Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
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TRIAGEM, ISOLAMENTO DE SUBSTÂNCIAS ANTIFÚNGICAS A PARTIR DO EXTRATO DO FUNGO ASPERGILLUS FELIS E AVALIAÇÃO DA AÇÃO ANTIFÚNGICA EM *PARACOCCIDIOIDES BRASILIENSIS*

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como um dos requisitos para a obtenção do título de Doutor em Ciências Biológicas com ênfase em Microbiologia.

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2016

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Aos meus pais Bernardino e Márcia,
às minhas irmãs Gisele e Daniele,
pelo apoio e compreensão.

Dedico este trabalho

Agradecimentos

A Deus, agradeço por ter me dado forças e por ter me mostrado o caminho correto a seguir;

A minha orientadora, Susana Johann, pela a oportunidade de trabalhar no laboratório, pela orientação, ensinamentos, compromisso e incentivo;

A minha co-orientadora, Betania Barros Cota, pela enorme dedicação, parceria, compromisso, afeto, incentivo e preocupação com o trabalho e comigo;

Ao professor Carlos Rosa e Luiz Rosa pelo apoio acadêmico;

A todos os professores, alunos e funcionários do Departamento de Microbiologia da UFMG agradeço pela convivência, ajuda, apoio, pelo conhecimento proporcionado e pelas amizades conquistadas;

Aos colegas do Laboratório de Produtos Naturais da Fiocruz, Dani, Micheli, Fátima, Markus, Ezequias, Carlos e Tânia, agradeço a ótima convivência e os bons momentos vividos;

Aos colegas do Laboratório de Taxonomia, Biodiversidade e Biotecnologia de Fungos da UFMG, agradeço pelos anos de boa convivência e amizades construídas! Gostaria principalmente de agradecer às amigas Thelma, Lana, Graci, Jordana, Ana Raquel, Bia, Camila e Mariana pela força e amizade;

À banca examindora que gentilmente aceitou o convite para avaliar este trabalho;

Ao meu namorado Raphael, pelo amor, companheirismo, carinho, apoio e paciência;

À minha família, meus pais, Bernardino e Márcia, minhas irmãs Daniele e Gisele, pela força, dedicação e por estarem sempre ao meu lado;

Agradeço à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, pela bolsa de estudos e apoio finceiro.

Sumário

Resumo

A paracoccidioidomicose (PCM), causada pelos fungos do gênero *Paracoccidioides,* é uma micose endêmica na América Latina. A PCM é a primeira causa de morte entre as micoses sistêmicas e a décima entre as doenças infecciosas e parasitárias. O tratamento medicamentoso desta micose é prolongado e, pode causar severos efeitos adversos e reações de hipersensibilidade. Deste modo é importante a busca de novos agentes antifúngicos que sejam mais eficazes e com efeitos adversos menos graves. Com o objetivo de se identificar fontes de substâncias ativas contra o fungo patogênico *P. brasiliensis* (Pb18), setenta e oito extratos etanólicos de fungos, isolados do Atacama, foram testados em ensaios de microdiluição nas concentrações de 500 a 0,9 μg/mL. Cerca de 18% dos extratos apresentaram valores de concentração inibitória mínima (CIM) menor ou igual a 125 μg/mL, com destaque para o isolado UFMGCB 8030 (CIM de 15,6 μg/mL). Este isolado foi identificado como *Aspergillus felis* por dados morfológicos e análises do sequenciamento das regiões transcrita interna, β-tubulina e gene ribossomal da polimerase II. A fim de se potencializar a atividade antifúngica deste extrato, o fungo foi cultivado em diferentes meios de cultura e a extração dos metabólitos secundários foi realizada com diferentes solventes. O meio batata dextrose ágar (BDA) e a extração com o solvente diclorometano (DCM) forneceram o extrato com o melhor valor de CIM (1,9 μg/mL). A identificação dos possíveis agentes antifúngicos que poderiam ser produzidos pelo fungo *A. felis* UFMGCB8030 foi realizada utilizando-se métodos analíticos de desreplicação, na qual a cromatografia líquida de alta eficiência acoplada à espectrometria de massas de alta resolução e comparação com os dados da literatura científica, indicaram a presença de substâncias do grupo das citocalasinas. Após este estudo químico prévio, o extrato do fungo foi feito em maior escala (BDA/DCM) para a obtenção das substâncias responsáveis pela atividade antifúngica. A partir dos processos cromatográficos de análise e purificação foram obtidas sete substâncias, das quais duas foram classificadas como inéditas e denominadas como 5-*N*-acetil-8-β-isopropil ardeemina e citocalasina Z15E. Além destas substâncias foram obtidos outros cinco produtos naturais já descritos pela literatura, roselicalasina, citocalasina E, gancidina, pseurotina A1 e 2,4-diidroxiacetofenona. Seis das substâncias apresentaram atividade contra o isolado Pb 18 de *P. brasiliensis* nas concentrações testadas (0,9-500,0 μg/mL), com destaque para a citosalasina E (CIM de 1,9

μg/mL; 3,6 μM). O tratamento das leveduras com esta substância resultou no impedimento da liberação dos brotos, produção de espécies reativas de oxigênio e danos na membrana plasmática e parede celular. Os resultados obtidos neste trabalho mostraram que fungos isolados de regiões extremas são fontes de metabólitos com propriedades antifúngicas contra o *P. brasiliensis* Pb 18 e representam fonte de novas substâncias. Para o nosso conhecimento, este é o primeiro relato sobre o isolamento de substâncias a partir do fungo *A. felis* e também é o primeiro relato de atividade antifúngica contra *P. brasiliensis* das substâncias isoladas neste trabalho.

Palavras chave: *Paracoccidioides brasiliensis*, atividade antifúngica, citocalasina E, 5-*N*-acetil-8-β-isopropil ardeemina, citocalasina Z15E e espécies reativas de oxigênio.

Abstract

The paracoccidioidomycosis (PCM) caused by fungi of the genus *Paracoccidioides* is an endemic mycosis in Latin America. The PCM is the leading cause of death among the systemic mycoses and the tenth among infectious and parasitic diseases. Drug treatment of mycosis is prolonged and can cause severe side effects and hypersensitivity reactions. Thus it is important to search for new antifungal agents that are more effective and have less serious side effects. In order to identify sources of bioactive substances against pathogenic fungus *P. brasiliensis* (Pb18), seventy-eight ethanolic extracts of fungi isolated from Atacama were tested in microdilution assays at concentrations from 500 to 0.9 μg/ml. About 18% had lower MICs than or equal to 125 μg/ml, especially isolated UFMGCB 8030 (MIC 15.6 μg/ml). This isolate was identified as *Aspergillus felis* from morphological data and analysis of the sequencing of the internal transcribed regions, β-tubulin and polymerase II rRNA gene. In order to potentiate the antifungal activity of this extract, the fungus was grown in different culture media and extraction of secondary metabolites was carried out with different solvents. The medium potato dextrose agar and extraction with dichloromethane solvent provided the extract with the best value of MIC (1.9 μg/ml, 3.6 μM). The identification of potential antifungal agents that could be produced by the fungus *A. felis* UFMGCB8030 was performed using analytical methods for dereplication, wherein the high-performance liquid chromatography coupled to spectrometry, high resolution mass and comparison with data from literature indicated the presence of the group of substances cytochalasins. After this chemical prior study, the fungus was grown on a larger scale to obtain the substances responsible for the antifungal activity. Chromatographic analysis and purification process resulted seven substances, of which two were classified as novel and named 5-*N*-acetyl-8-β-isopropyl ardeemin and cytochalasin Z15E. Besides these substances were obtained five other natural products already described in the literature, rosellichalasin, cytochalasin E, gancidin, pseurotin A1 and 2,4 dihydroxiacetophenone. Six of the substances showed activity against isolated Pb 18 *P. brasiliensis* in the tested concentrations (0.9 to 500 μg/ml), highlighting the citosalasina E (MIC 1.9 μg/ml). Treatment of yeast with this compound resulted in preventing the release of buds, generation of reactive oxygen species and damage to the plasma membrane and the cell wall. The results of this study showed that fungi isolated from extreme regions are sources of metabolites with antifungal properties against *P. brasiliensis* Pb 18 and

represent a source of new active componds. To our knowledge, this is the first report on the isolation of substances from the fungus *A. felis* and is also the first report of antifungal activity against *P. brasiliensis* compounds isolated in this work.

Key-words: *Paracoccidioides brasiliensis,* antifungal activity, cytochalasin E, 5-*N*-acetil-8-*β*-isopropil ardeemina, citocalasina Z15E and reactive oxigen species.

1- Relevância e justificativa

O *Paracoccidioides* é o agente etiológico da paracoccidioidomicose, uma importante micose sistêmica humana endêmica na America Latina. Esta micose é importante na área da saúde visto que é classificada como a primeira causa de morte entre as micoses sistêmicas sem doença de base associada e a décima entre as doenças infecciosas e parasitárias.

O tratamento da PCM é prolongado e começa com uma agressiva dose de agentes antifúngicos que pode durar de meses até anos. Apesar de apresentarem eficiência significativa, esses fármacos exercem severos efeitos adversos e, muitas vezes podem ocorrer recidivas. A anfotericina B é nefrotóxica e desencadeia diversos efeitos colaterais. Já as sulfas são eficazes no tratamento da PCM, mas devem ser administradas várias vezes ao dia e, a terapia pode ser mais prolongada quando comparada a outros fármacos. Além disto, efeitos colaterais, tais como, reação de hipersensibilidade, podem ocorrer nos pacientes. Os azólicos também detêm a progressão da doença, mas a sequela fibrótica pode persistir, o que pode causar uma reincidência da doença após o término do tratamento.

O desenvolvimento ideal de novos agentes antifúngicos deve levar em conta o índice de seletividade em relação às células fúngicas e não as do hospedeiro. Isso poderia resultar em medicamentos menos tóxicos e mais efetivos para o tratamento de micoses.

Os fungos representam uma importante fonte de moléculas ativas que podem servir de protótipos para novos fármacos. Em particular, os fungos encontrados em ambientes extremos, tais como, o Atacama, possuem adaptações que os possibilitam resistir a estes ambientes. Essas adaptações podem possibilitar a descoberta de novas vias metabólicas e, consequentemente, de novos metabólitos de interesse biotecnológico.

Os fungos apresentam grande vantagem frente às demais fontes de substâncias antimicrobianas, uma vez que, podem ser cultivados em larga escala, não havendo prejuízo ao ecossistema, como no caso de plantas e, não promovem problemas éticos como no caso do uso de animais. Já os produtos resultantes do seu metabolismo apresentam características biotecnologicamente importantes, tais como, grande diversidade química ainda inexplorada e potencial farmacológico em relação às atividades antimicrobiana, antitumoral, imunossupressora e antiparasitária. Além disso, muitos dos metabólitos de fungos com potencial antimicrobiano já são usados na terapêutica e indústria farmacêutica no tratamento de doenças infecciosas.

Neste trabalho, o objetivo principal foi avaliar a atividade antifúngica, contra o isolado Pb 18 de *P. brasiliensis,* de extratos de fungos de ambiente ainda pouco explorado, como o Deserto do Atacama e isolar metabólitos secundários do extrato mais ativo. Sabe-se da necessidade de se descobrir substâncias mais eficazes e com menos efeitos adversos contra infecções fúngicas para se ampliar as opções de tratamento para essas infecções, visto a emergência de isolados resistentes às drogas utilizadas atualmente.

2- Introdução

2.1- Paracoccidioidomicose

2.1.1- Histórico e epidemiologia

A paracoccidioidomicose (PCM) foi descrita pela primeira vez, em 1908, por Adolfo Lutz. Quatro anos depois, Splendore descreveu novos casos em pacientes da Santa Casa de Misericórdia de São Paulo e estudou minuciosamente a morfologia do fungo, denominando-o de *Zymonema brasiliensis*. Em 1930, Floriano Paulo de Almeida instituiu a denominação *Paracoccidioides brasiliensis*. A paracoccidioidomicose é, também, denominada doença, micose ou moléstia de Lutz, blastomicose sul-americana, blastomicose brasileira e Splendore-Almeida. O termo paracoccidioidomicose foi instituído em 1971 na reunião de micologistas das Américas em Medellin e persiste, até hoje, como nomenclatura oficial (Palmeiro et al., 2005; Lupi et al., 2005).

A PCM é uma importante micose sistêmica humana endêmica na América Latina (Tavares et al., 2005), em que cerca de 85% dos casos ocorrem no Brasil (Andrade et al., 2005) e atinge, principalmente, as regiões Sul, Sudeste, Centro-Oeste e Amazônia Ocidental e Oriental (Martinez 2015). No Brasil, a PCM tem o maior índice de mortalidade entre as micoses (Coutinho et al., 2002). Mais de 15.000 casos foram relatados entre 1930 e 2012, número este que reflete parcialmente a prevalência da doença na América Latina. A abordagem da epidemiologia desta doença encontra algumas dificuldades devido a vários fatores, dentre os quais, não obrigatoriedade de notificação dos casos, dificuldade no reconhecimento da infecção recentemente adquirida, ausência de surtos epidêmicos além de deficiente diagnóstico laboratorial em algumas áreas endêmicas (Martinez, 2015).

Ao longo das últimas décadas têm sido observadas notáveis alterações na frequência, nas características demográficas da população atingida e na distribuição geográfica da PCM. Dependendo da região, a incidência se alterou sem que possam justificar totalmente as suas causas. É possível que o aumento da urbanização e a melhoria do diagnóstico expliquem em parte estas alterações. Além disto, fatores ambientais decorrentes da abertura de novas fronteiras agrícolas, com a derrubada de florestas, sobretudo nas regiões Centro-Oeste e Norte, atingindo, principalmente a Amazônia, também contribuíram para o atual panorama da micose (Shikanai-Yasuda et al., 2006).

Esta micose é considerada um problema de saúde pública devido ao seu alto potencial incapacitante e a quantidade de mortes prematuras que provoca. Isto ocorre principalmente em segmentos sociais específicos, tais como os trabalhadores rurais (Shikanai-Yasuda et al., 2006).

Os humanos, em particular os trabalhadores rurais, possivelmente adquirem o fungo pelo contato com o solo de habitats de tatus. Isso foi possível ser observado pela alta similaridade apresentada pelas sequências genéticas de isolados clínicos e isolados obtidos de tatus (Sano et al., 1999). Além dos tatus, que apresentam alta frequência de infecções com *Paracoccidioides,* o fungo também já foi encontrado em solos, pinguim, fezes de morcegos (Restrepo et al., 2001), cães (Corte et al., 2012), cavalos (Albano et al., 2015), vacas (Silveira et al., 2008), etc.

A infecção geralmente ocorre na infância ou na adolescência e, após longo período de latência do fungo, as manifestações clínicas da PCM, em geral, são encontradas em pacientes adultos (Palmeiro et al., 2005) com cerca de 30 e 50 anos de idade. A doença afeta, principalmente, indivíduos imunodeprimidos (Shikanai-Yasuda et al., 2006).

Em indivíduos do sexo masculino, a incidência da PCM é maior e chega a uma taxa homem: mulher de 10-30:1 casos (Tavares et al., 2015). Esta diferença é decorrente de variações no perfil hormonal homem/mulher (Loose et al., 1983) pois na puberdade a doença se desenvolve da mesma forma em ambos os sexos. Neste contexto, Restrepo et al. (1984) demostraram que o hormônio feminino β-estradiol inibe a transição de micélio para levedura *in vitro*, sugerindo um papel protetor desse hormônio na mulher. Aristizábal e colaboradores (2002) avaliaram a influência do estado hormonal do hospedeiro nas respostas teciduais em modelo experimental de infecção em camundongos e sugeriram o papel do hormônio β-estradiol na resistência inata de fêmeas.

2.1.2- Agente etiológico: *Paracoccidioides*

Análises morfológicas e moleculares mostraram que *P. brasiliensis* pertence ao filo Ascomycota, ordem Onygenales (Bagagli et al., 2006). A sua classificação segue descrita abaixo:

Domínio – Eukaryota
Reino – Fungi
Filo – Ascomycota
Classe - Plectomycetes

Ordem – Onygenales

Família – Onygenaceae

Gênero – *Paracoccidioides*

Espécie – *P. brasiliensis e P. lutzii*

O gênero *Paracoccidioides* engloba três espécies filogenéticas, S1 (38 isolados), PS2 (6 isolados) e PS3 (21 isolados) e uma nova espécie, o *P. lutzii* (“Pb01 like”) (Tavares et al., 2015; Matute et al, 2006).

Os fungos desse gênero são dimórficos, apresentam-se na forma micelial não patogênica à temperatura ambiente (23°a 28°C) e transformam-se para a forma patogênica de levedura com múltiplos brotamentos a 37°C no hospedeiro (Tavares et al., 2005). As células leveduriformes são altamente polimórficas e multinucleadas (Almeida et al., 2006), enquanto que os conídios são estruturas uninucleadas (McEwen et al., 1987).

O processo de dimorfismo é uma adaptação às condições ambientais. No *P. brasiliensis* a conversão para a fase de levedura é um requisito para a progressão da infecção (San-Blas & Nino-Vega, 2008). A capacidade do fungo de se submeter a mudança de micélio para levedura é de particular interesse uma vez que representa uma parte da estratégia global de virulência do patógeno (Rooney & Klein., 2002). A transição dimórfica está correlacionada às mudanças na composição, organização e estrutura da parede celular reguladas durante o ciclo celular, em resposta às condições ambientais e ao stress (San-Blas & Ninõ-Veja, 2008). Isso confere às duas formas possíveis do fungo composições bioquímicas diferentes nas suas paredes celulares (Paris et al., 1986).

A parede celular do *P. brasiliensis* na forma de levedura apresenta maior quantidade no conteúdo de quitina do que a fase micelial. Além disso, apresenta alterações nas suas moléculas de glucano, um polissacarídeo de monómeros de D-glicose. A fase de micélio apresenta β-1,3 e β-1,6 glucanos enquanto que na fase leveduriforme 95% das glucanas são α-1,3-glucano e apenas 5% são de β-1,3-glucano (Kanetsuna et al., 1969).

A síntese de α-1,3-glucano é interrompida favorecendo a ligação β-glucano quando ocorre a mudança de temperatura de incubação de 37ºC para 23ºC (Paris et al., 1986). Assim, a α-glucano é proposta como um fator de virulência em *P. brasiliensis*, além de estar relacionada com o processo de dimorfismo apresentado por este fungo (San-Blas & Ninõ-Veja, 2008; Hogan & Klein, 1994).

A parede celular de ambas as fases ainda é formada por outro polissacarídeo que está presente como uma fina camada externa de fibras, as galactomananas (constituída por um esqueleto de manose com grupos laterais de galactose) (San-Blas & Niño-Veja, 2008;

Reddy et al., 2016). Além desses carboidratos que representam 81% dos constituintes, a parede celular da forma de levedura é composta também por aminoácidos (10%) e lipídeos (11%) (Puccia et al., 2011).

Os polissacarídeos constituintes da parede celular dos fungos são exclusivos deles e consequentemente possíveis inibidores das vias de biossíntese desses açúcares não devem ter efeitos secundários tóxicos em humanos (Latgé et al., 2005). Esses compostos são alvos interessantes para o desenvolvimento de drogas antifúngicas, pois são essenciais para integridade celular do fungo (San-Blas & Ninõ-Veja, 2008).

A constiuição da membrana celular do *P. brasiliensis* na forma de levedura é composta dos esteróis; brassicasterol (69.1%), ergosterol (26.8%) e lanosterol (4.1%) (Visbal et al., 2003). Esses esteróis são estruturalmente diferentes do colesterol encontrado na membrana das células dos animais (Visbal et al 2011). Deste modo, os esteróis específicos da membrana fúngica são susceptíveis aos inibidores que atuam na síntese do ergosterol, representando alvos potenciais para a descoberta de novas substâncias mais seletivas.

2.1.3- Caracterização da Doença

A PCM é adquirida pela inalação de propágulos aéreos derivados da forma micelial de *Paracocidioides* (Tavares et al., 2005) o qual nos pulmões se adere ao epitélio alveolar e se transforma na forma patogênica de levedura (Torres et al., 2010). Embora a maioria das formas clínicas da doença seja assintomática, infecções graves e progressivas podem ocorrer envolvendo tecidos pulmonares e extrapulmonares, tais como pele, linfonodos, mucosa orofaríngea, glândula suprarrenal e sistema nervoso central (Pigosso et al., 2013). A progressão da PCM vai depender da virulência de cada isolado fúngico e da resposta imune induzida no hospedeiro (Fortes et al., 2011).

A infecção geralmente ocorre na infância ou na adolescência e sua evolução depende da virulência do fungo, da quantidade de conídios inalados e da integridade do hospedeiro. Após longo período de latência do fungo, um desequilíbrio na relação entre hospedeiro e parasita pode ocorrer, e o paciente, então, evolui para os sinais e sintomas da doença. Sendo assim, apesar da infecção ocorrer, muitas vezes, na infância, as manifestações clínicas da PCM, em geral, são vistas em pacientes adultos (Palmeiro et al., 2005).

A PCM apresenta duas formas de manifestações clínicas: a forma cutânea ou subcutânea (tipo juvenil) e a forma crônica (tipo adulto). Ambas as formas são associadas com sequelas extensas, mediadas por infecções sistêmicas, incluindo lesões e uma resposta imune anormal mediada por células (Borges-Walmsley et al., 2002). A prevalência da forma clínica cutânea/subcutânea varia de acordo com a origem dos pacientes em relação aos estados e regiões e é menos frequente no Rio Grande do Sul e mais frequente no Maranhão, Pará, Minas Gerais, São Paulo, Mato Grosso do Sul e Rio de Janeiro (Fabris et al., 2014).

No adulto, a forma clínica predominante é a crônica, e nas crianças ou adolescentes apresenta-se na forma aguda ou subaguda. Quando não diagnosticada e tratada oportunamente, pode levar a formas disseminadas graves e letais, com envolvimento rápido e progressivo dos pulmões, tegumento, gânglios, baço, fígado e órgãos linfóides do tubo digestivo (Shikanai-Yasuda et al., 2006).

2.1.4- Tratamento

A paracoccidioidomicose é sensível à maioria das drogas antifúngicas, inclusive sulfamídicos. Consequentemente, vários antifúngicos podem ser utilizados para o tratamento dos pacientes, tais como anfotericina B, sulfamídicos (sulfadiazina, associação sulfametoxazol/trimetoprima) e azólicos (cetocanazol, fluconazol, itraconazol) (Shikanai-Yasuda et al., 2006). O tratamento é usualmente prolongado, de 1 a 2 anos. Na ausência de tratamento, a doença é frequentemnte fatal (Borges-Walmsley et al., 2002).

As sulfas foram as primeiras a serem empregadas no tratamento e continuam ser muito usadas (Ferreira, 2009). Porém, esses medicamentos apresentam a desvantagem de terem que ser administrados por via oral várias vezes ao dia, além de a duração da terapia ser mais prolongada (de até 2 anos) quando comparada a outras drogas. Para este tratamento pode haver efeitos adversos que incluem reação de hipersensibilidade (Palmeiro et al., 2005). Estas drogas interferem na síntese do ácido fólico pela competição com o substrato da enzima sintetase de diidroperoato, o ácido *p*-aminobenzóico (Visbal et al., 2011). A combinação sulfametoxazol/trimetoprima é amplamente usada na terapia ambulatorial devido ao baixo custo (Ramos E Silva & Saraiva, 2008).

Os azólicos (imidazol e derivados triazólicos) são drogas orais que são facilmente administradas e com excelente atividade contra o *Paracoccidioides.* Em pacientes com lesões no sistema nervoso central é utilizado o fluconazol e, naqueles com doença

disseminada e infecções pulmonares progressivas faz-se uso do itraconazol (Lupi et al., 2005). O período de tratamento do itraconazol em pacientes com formas graves é de 12 a 18 meses (Shikanai-Yasuda et al., 2006). Embora as drogas azólicas possam controlar a progressão da PCM, a sequela fibrótica pode persistir em alguns casos, provavelmente, constituindo uma fonte de leveduras que poderia conduzir a uma recidiva após o término do tratamento da doença (Borges-Walmsley et al., 2002).

A anfotericina B é outra opção de tratamento usada para as formas mais graves da doença (perda de peso > 10%, associada à dificuldade de deglutição e comprometimento do estado geral, insuficiência respiratória, sinais ou sintomas neurológicos ou evidências de comprometimento de adrenais) em crianças, adolescentes e adultos (Shikanai-Yasuda et al., 2006).

2.2- Antifúngicos

O desenvolvimento de um agente antifúngico é um desafio porque os fungos e o homem, por se tratarem de organismos eucariotos, compartilham alguns alvos enzimáticos relativos à toxicidade. Apesar disso muitos alvos podem ser explorados visando à inibição da síntese de constituintes da membrana fúngica (ergosterol) e a inibição de constituintes que são exclusivos das paredes celulares fúngicas (glucano) (Srinivasan et al., 2014; Odds et al., 2003).

A primeira geração de antifúngicos foi caracterizada pelos poliênios anfotericina B e nistatina na década de 50. O primeiro poliênio foi isolado de *Streptomyces noursei* e o segundo isolado de *S. nodosum*. Estas substâncias são altamente nefrotóxicas e hepatotóxicas, por isso comprometem à saúde (Fanning & Mitchell, 2012; Kathiravan et al., 2012). Anfotericina B age ligando-se ao ergosterol presente na membrana celular do fungo, quanto ao colesterol da membrana do paciente. Esta ligação forma um complexo que perturba a membrana plasmática, o que resulta no aumento de sua permeabilidade, extravasamento do conteúdo plasmático e por fim a morte do fungo (Visbal et al., 2011; Fanning & Mitchell, 2012; Kathiravan et al., 2012).

Duas décadas depois, os quimioterápicos azólicos foram desenvolvidos (Srinivasan et al., 2014). Os principais efeitos adversos dessas drogas relacionam-se a intolerância gastrintestinal, hepatotoxicidade e hipersensibilidade, além disso, são drogas teratogênicas e não devem ser administradas a gestantes (Martinez, 2006). Sua atuação é na fase inicial da síntese do ergosterol, o maior componente da membrana celular fúngica. Essa droga inibe a enzima lanosterol 14α-demetilase dependente do citocromo P450, modifica a

síntese de lipídeos e inativa enzimas do processo oxidativo dos fungos. Isto resulta em acumulação do esterol intermediário que coloca a célula fúngica num estresse tóxico, interfere na arquitetura da membrana, altera suas funções normais, como sua permeabilidade e fluidez, além de inibir o crescimento celular (Domenico, 1999; Ferreira, 2009; Srinivasan et al., 2014; Martinez, 2006; Visbal et al., 2011).

As equinocadinas, isoladas pela primeira vez em 2001 de fungos filamentosos, representam uma nova classe de antifúngicos. As equinocandinas são moléculas lipopeptídicas que atuam inibindo a síntese de β-1,3-glucano, um polissacarídeo essencial na formação da parede celular de muitos fungos patogênicos. Essas drogas agem diferentemente de outros antifúngicos, na parede celular e não na membrana celular do fungo. Assim, os seus efeitos não ocorrem nas membranas celulares do hospedeiro, e por isso essas substâncias são menos tóxicas para o homem (Kartsonis et al., 2003). A caspofungina representa a primeira substância dessa nova classe de agentes antifúngicos (Letscher-Bru & Herbrecht, 2003). Ela é um derivado semi-sintético da pneumocandina B, obtida pela fermentação do fungo *Glarea loroyensis* (Chen et al., 2015). Além destas substâncias, há a anidulafungina, derivado semi-sintético de um produto natural resultante da fermentação de *Aspergillus nidulans* (Hof & Dietz, 2009) e a micafungina, derivado semi-sintético de um peptídeo, produto da fermentação de *Coleophoma empetri* (Hashimoto, 2009).

Apesar das equinocadinas serem efetivas para o tratamento de infecções fúngicas causadas pelos isolados resistentes aos azólicos, o aumento da emergência de linhagens resistentes às equinocadinas pode ser considerado como um sinal claro para a urgência na busca de uma nova geração de substâncias antifúngicas (Srinivasan et al., 2014).

2.3- Produtos naturais como fonte de novos fármacos

Os produtos naturais representam uma das mais importantes fontes de descoberta de novos fármacos. Eles são substâncias que apresentam elevada diversidade estrutural química e muitas são ativas contra uma variedade de alvos com potencial para o tratamento de diversas doenças. Essas subtâncias também podem ser utilizadas como protótipos para o desenvolvimento de fármacos menos tóxicos e/ou mais ativos e/ou mais específicos em relação ao alvo terapêutico (Arivudainambi et al., 2011).

Embora os produtos naturais tenham sido usados popularmente de forma empírica, apenas no século 20 as substâncias isoladas a partir dos mesmos começaram a ser identificadas e caracterizadas sistematicamente. O interesse maior nesses produtos é devido a sua aplicabilidade para tratamento de várias doenças infecciosas, câncer e como fonte de novos agentes terapêuticos (Brakhage & Schroeckh, 2011).

Animais, plantas, algas, liquens, e micro-organismos são fontes de metabólitos secundários (Newman & Cragg, 2012). Com relação aos micro-organismos, dos 1500 metabólitos fúngicos que foram isolados e caracterizados entre 1993 e 2001, mais da metade tem atividade antibacteriana, antifúngica e antitumoral (Keller *et al.,* 2005*).*

Sabe-se que somente uma pequena quantidade de micro-organismos que vivem na biosfera já foi descrita e estudada. Por isto, há uma enorme fonte de substâncias naturais de grande diversidade estrutural ainda a ser explorada para o desenvolvimento de novos antifúngicos (Newman & Cragg, 2012).

2.4- Metabólitos secundários de fungos

Os metabólitos secundários são produtos do metabolismo não essenciais para o crescimento normal, desenvolvimento ou reprodução de um organismo (Vaishnav & Demain, 2010). Estas substâncias servem para atender às necessidades secundárias do organismo produtor, tais como: 1) viver em competição, 2) prover mecanismos de defesa, 3) inibir o crescimento do competidor e 4) facilitar processos reprodutivos. Sabe-se que os micro-organismos vivem em ecossistemas complexos onde competem e se comunicam com outros organismos e necessitam desenvolver estratégias que garantam sua sobrevivência, por isso, a produção dos metabólitos está relacionada ao meio no qual o fungo está inserido (Brakage & Schroeckh, 2011).

Os metabólitos secundários fúngicos são usualmente separados nos seguintes grupos: alcalóides; peptídeos não-ribossomais (sirodesmina, peramina e sideróforos tais como ferricrocina); policetídeos (aflatoxinas e fumonisinas), terpenos (toxina T-2, desoxinivalenol-DON) e compostos fenólicos (Kempken & Rohlfs, 2010; Fox & Howlett, 2008, Ansari et al., 2013).

Diferentemente do metabolismo secundário, o primário está relacionado à síntese de macromoléculas (lipídeos, proteínas e ácidos nucléicos) (Malik,1980) que desempenham funções vitais básicas, como por exemplo, divisão e crescimento celular, respiração e reprodução (Fumagali et al., 2008). Por outro lado, os metabólitos secundários

usualmente possuem baixo peso molecular e tem sua distribuição taxonômica restrita. Eles não são requeridos para a sobrevivência do micro-organismo em meio laboratorial mas podem fornecer vantagens de sobrevivência no meio ambiente (Katz & Baltz; 2016). Sua produção pode estar relacionada a partes específicas do ciclo de vida e apesar de muitas vezes poderem ser bioativos, não possuem suas propriedades totalmente elucidadas (Keller et al., 2005).

Muitos metabólitos podem ser prejudiciais ao homem, como as micotoxinas, enquanto outros são benéficos, como os antibióticos. Apesar de conferirem benefícios não totalmente conhecidos aos organismos produtores, os metabólitos secundários podem inibir o crescimento de bactérias, fungos, protozoários, parasitas, insetos, vírus e tumores celulares humanos e apresentarem, deste modo, importância médica, industrial e agrícola (Keller et al., 2005).

Os fungos são bem conhecidos como produtores de metabólitos secundários bioativos com variedade de atividades biológicas, tais como, antifúngica, antibacteriana, antiviral, citotóxica e imunossupressora (Brakage & Schroeckh, 2011). Dentre os fungos já relatados na literatura como produtores de substâncias antifúngicas, pode-se citar: *Pestalotiopsis foedan*, obtido da planta *Bruguiera sexangula*, produtor de lactonas monoterpênicas com atividade contra *Candida albicans* (Xu et al., 2016); *Keissleriella* sp. produtor de keisslona contra *Candida albicans*, *Tricophyton rubrum* e *Aspergillus niger* (Liu et al., 2002); *Myrothecium* sp. que vive associado ao peixe *Argyrosomus argentatus* é produtor de tricotecenos com atividade contra *A. niger*, *T. rubrum* e *C. albicans* (Liu et al., 2006); *Trichoderma brevicompactum*, fungo endofítico do alho, produtor do tricodermina com atividade contra *Rhizoctonia solani*, *Botrytis cinerea* e *Colletotrichum lindemuthianum* (Shentu et al., 2014) e *Cladosporium cladosporioides* produtor da cladosporina e isocladosporina com atividade contra patógenos de plantas *Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides* e *Phomopsis viticola* (Wang et al., 2013). Apesar de haver muitos trabalhos sobre isolamentos de substâncias antifúngicas de fungos, ainda existem poucos fungos bem caracterizados e muitos desses podem produzir metabólitos desconhecidos, o que os leva a ser uma fonte promissora para a descoberta de novas substâncias ativas (Moldes-Anaya et al., 2011).

2.5- Substâncias antifúngicas derivadas de fungos contra *Paracoccidioides*

Os dados da literatura mostram que há poucos relatos de isolamento de substâncias com atividade antifúngica contra *P. brasiliensis* a partir de fungos. Rodríguez-Brito e colaboradores (2010) relataram a atividade de caspofungina, obtida de *Glarea loroyensis*, contra as formas leveduriforme e micelial de isolados de *P. brasiliensis*. Apesar de inibir as duas formas, a caspofungina inibe mais o crescimento da forma micelial porque esta possui maior quantidade *β*-1-3-glucano na parede celular.

Johann e colaboradores (2012) testaram a altenusina, um derivado bifenólico, isolado do fungo endofítico *Alternaria* sp.. Esta substância apresentou atividade antifúngica contra os 11 diferentes isolados de *Paracoccidioides*. Os autores sugerem que a altenusina possa atuar na parede celular do fungo.

A partir de outro fungo endofítico, *Fusarium* sp., três micotoxinas do grupo tricoteceno foram isoladas: a toxina T2 e uma mistura de 8-*n*-isobutirilsolaniol e 8-n-butirilneosolaniol. Essas substâncias também apresentaram atividade antifúngica contra 11 isolados de *Paracoccidioides* (Campos et al., 2011).

2.6- Fungos de regiões extremas: Atacama

Sabe-se que apenas uma pequena fração de micro-organismos foi sistematicamente avaliada quanto ao seu potencial biotecnológico, assim, ainda existe uma fonte rica e inexplorada de substâncias bioativas a serem estudadas. Este fato renovou o interesse na exploração de táxons microbianos, nichos específicos e/ou habitats pouco exploradas (Monciardini et al., 2014), tais como, o deserto do Atacama. Os fatores extremos que atuam sobre os micro-organismos podem possibilitar a descoberta de novas vias metabólicas e consequentemente de novos metabólitos de interesse biotecnológico, uma vez que, estes organismos possuem um alto nível de adaptação e são capazes de resistir a condições extremas (Oarga, 2009; Santiago et al., 2012).

O Deserto do Atacama, localizado no Norte do Chile, representa outro ambiente extremo. Este local é considerado o deserto mais seco da Terra com condições que dificultam a sobrevivência da maioria dos organismos, tais como; baixa disponibilidade de água, alta concentração de sal e intensa radiação UV. Apesar disso, muitos micro-organismos conseguem sobreviver nessas condições e podem ser encontrados mesmo em áreas mais secas do deserto (Azua-Bustos et al., 2012).

Os períodos de longa aridez no Deserto do Atacama agrega valor ao estudo das adaptações biológicas porque os organismos foram expostos a condições ambientais extremas por tempo suficientemente longo para expressar características adquiridas em processos de seleção natural (Wierzchos et al., 2013). Acredita-se que as espécies adaptadas a tais ambientes constituem potenciais fontes de enzimas com características especiais e novos produtos gênicos com possíveis aplicações industriais (Dalmaso et al., 2015).

Os fungos que vivem associados a rochas estão entre os organismos mais tolerantes ao estresse pois são capazes de lidar com uma variedade de estressores associados a rochas nuas em ambientes extremos de quente e frio (Tesei et al., 2012). Estas superfícies são habitats únicos os quais mudanças rápidas na radiação, temperatura, água e disponibilidade de nutrientes representam um desafio para a sobrevivência microbiana em diferentes ambientes (Gueidan et al., 2008).

Gonçalves e colaboradores (2016) relataram pela primeira vez que as rochas podem ser habitats para fungos no deserto do Atacama, representando microambiente favorável à colonização fúngica, sobrevivência e dispersão em condições extremas. Este estudo indicou a presença de comunidade fúngica que inclui espécies sapróbias, patogênicas e micotoxigênicas. *Penicillium chrysogenum* produziu os compostos ácido α-linolênico e ergosterol endoperóxido, que foram ativos contra o *Cryptococcus neoformans* e *Staphylococcus aureus* resistance ao antibiótico meticilina respectivamente.

2.7- Gênero *Aspergillus*

Aspergillus é um gênero de fungos diversificado com mais de 300 espécies relatadas na literatura (Lee et al., 2016). Ele é subdividido em 7 subgêneros, que por sua vez são divididos em seções. O gênero é facilmente identificado pelo seu conidióforo característico, mas a identificação e diferenciação das espécies são mais complexas (Rodriguez et al., 2007). As espécies deste gênero são muito importantes porque algumas são alérgenos conhecidos, patógenos humanos oportunistas (Lee et al., 2016), causadores de aspergilose (Barrs et al., 2013; Kim et al., 2016; Bhaskaran et al., 2016) e possuem potencial toxigênico (Kumeda & Asao, 2001). Apesar disso, algumas espécies apresentam grande potencial industrial como produtores de enzimas (Shi et al., 2009; Cortesini et al., 2010) e farmacêutico na produção de drogas ou substâncias ativas que podem ser utilizadas como protótipo de drogas (Patil et al., 2015).

O estudo químico de espécies do gênero *Aspergillus* forneceu muitos metabólitos que foram descritos na literatura com diversas atividades biológicas. Dentre esses, as estatinas (lovastatina, mevastatina, pravastatina e monacolina J) isoladas de *Aspergillus terreus* atuam na inibição da enzima 3-hidroxi-3-metilglutaril-coenzima A redutase na biossíntese do colesterol, assim podem ser utilizadas na terapia medicamentosa para a diminuição do colesterol (Manzoni et al., 1998). Além disso, as estatinas podem ser utilizadas como antifúngico (Galgoczy et al., 2011; Bellanger et al., 2016; Javed et al., 2016) e em outras aplicações biomédicas, tais como, no tratamento de doenças cardíacas e renais, mal de Alzheimer e lesão óssea (Barrios-González & Miranda, 2010).

Outros metabólitos isolados de espécies de *Aspergillus* que podem ser citados são: dicetopiprazina obtida de *A. fumigatus* com atividade antibacteriana contra *Staphylococcus aureus e Micrococcus luteus* (Busi et al., 2009); 3,3-diidroxiterfenilina, 3-hidroxiterfenilina e candidusina B com propriedade antioxidante a partir de *Aspergillus candidus* (Yen et al., 2001); austalideos, metabólito meroterpenóide de *A. aureolatus*, sendo que os austalideo T e B apresentaram atividade antiviral contra o vírus influenza (H1N1), com valores de IC_{50} de 90 e 99 μM, respectivamente (Peng et al., 2016); flavonas aspergivona A e B obtidas de *A. candidus*, sendo que a B mostrou-se ser um inibidor fraco da alfa-glucosidase (Ma et al., 2016), a inibição desta enzima pode ser usada para tratar o diabetes mellitus tipo 2 (Sun et al., 2016) e butenolídeos obtidos de *A. terreus* apresentam moderada atividade contra células tumorais (Guo et al., 2016).

3- Objetivos

3.1-Objetivo Geral

Isolar substâncias com atividade antifúngica contra o fungo patogênico *P. brasiliensis* a partir de extrato de fungo do deserto do Atacama e propor seus possíveis alvos de ação.

3.2- Objetivos Específicos

- Avaliar os extratos brutos de fungos isolados do Atacama contra o isolado Pb18 do *P. brasiliensis* em ensaio de microdiluição*;*
- Identificar os extratos mais ativos por meio do ensaio de Concentração Inibitória Mínima (CIM);
- Recultivar os fungos que apresentaram melhor atividade antifúngica nos ensaios de triagem e obter os valores de CIMs contra o isolado Pb18 do *P. brasiliensis*;
- Otimizar as condições de cultivo e de extração dos metabólitos secundários do fungo que apresentou melhor valor de CIM;
- Produzir extrato do fungo mais ativo em larga escala;
- Realizar o fracionamento biomonitorado para atividade antifúngica do extrato mais promissor;
- Obter os valores de CIMs das substâncias isoladas;
- Testar a toxicidade dos extratos selecionados e das substâncias em macrófagos murinos.
- Elucidar os possíveis alvos de ação das substâncias obtidas.

4- Capítulos

4.1- Capítulo 1- **"**Antifungal activity of extracts from Atacama Desert fungi against *Paracoccidioides brasiliensis* and identification of *Aspergillus felis* as a promising source of natural bioactive compounds"

Graziele Mendes, Vívian N Gonçalves, Elaine M Souza-Fagundes, Markus Kohlhoff, Carlos A Rosa, Carlos L Zani, Betania B Cota, Luiz H Rosa, Susana Johann. **Memórias do Instituto Oswaldo Cruz**, Rio de Janeiro, Vol. 111(3): 209-217, 2016. doi: 10.1590/0074-02760150451.

A atividade antifúngica de extratos de fungos obtidos do Deserto do Atacama contra o *P. brasiliensis* foi avaliada neste trabalho. Dentre os setenta e oito extratos, cinco deles (UFMGCB8015, UFMGCB21, UFMGCB24, UFMGCB26 e UFMGCB30) apresentaram valores de concentração inibitória mínima mais interessantes (valor de CIM de 62,5 μg/mL) com destaque para o extrato do fungo UFMGCB8030 (CIM 15,6 μg/mL). Este isolado foi identificado como *Aspergillus felis* a partir de dados morfológicos (macroscópicos e microscópicos) e de análises do sequenciamento da região transcrita interna, *β*-tubulina e gene ribossomal da polimerase II. Com o objetivo de se potencializar a atividade antifúngica deste extrato, o fungo foi cultivado em diferentes meios de cultura e a extração dos metabólitos secundários foi realizada com diferentes solventes. O meio batata dextrose e a extração usando o solvente diclorometano forneceram o extrato com o melhor valor de CIM (1,9 μg/mL) contra o isolado Pb18 de *P. brasiliensis.* Além disso, este extrato não apresentou citotoxicidade nas concentrações testadas em células normais de mamífero (Vero). Com base nestes resultados, um estudo químico deste extrato foi realizado na tentativa de se identificar os possíveis agentes antifúngicos que poderiam ser produzidos pelo *A. felis* UFMGCB8030 cultivado nas condições que resultaram no extrato mais ativo. O fracionamento biomonitorado foi realizado utilizando cromatografia líquida de alta eficiência (CLAE) em escala analítica e posteriormente as frações foram submetidas ao ensaio antifúngico contra o isolado Pb 18 de *P. brasiliensis*. As frações ativas foram analisadas em CLAE acoplada à espectrômetria de massas de alta resolução o que indicou a presença de substâncias do grupo das citocalasinas. As substâncias presentes nas outras frações ativas não foram identificadas porque suas massas moleculares não

coincidiram com nenhuma substância presente nos bancos de dados. Essa análise sugeriu a existência de substâncias novas neste extrato. Os resultados deste trabalho revelaram o potencial de *A. felis* como produtor de substâncias bioativas com atividade antifúngica.

Os objetivos deste trabalho foram:

1. Avaliar a atividade antifúngica de extratos brutos de uma coleção de fungos obtidos de rochas do deserto do Atacama contra o fungo patogênico *P. brasiliensis* (Pb 18) utilizando o ensaio de CIM;
2. Identificar a espécie do fungo que forneceu o extrato mais ativo por técnicas morfológicas e moleculares;
3. Determinar as melhores condições de cultivo e extração dos metabólitos secundários;
4. Identificar as substâncias responsáveis pela atividade antifúngica nas frações ativas do extrato bruto utilizando a técnica da desreplicação.

Antifungal activity of extracts from Atacama Desert fungi against *Paracoccidioides brasiliensis* and identification of *Aspergillus felis* as a promising source of natural bioactive compounds

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Fungi of the genus Paracoccidioides *are responsible for paracoccidioidomycosis. The occurrence of drug toxicity and relapse in this disease justify the development of new antifungal agents. Compounds extracted from fungal extract have showing antifungal activity. Extracts of 78 fungi isolated from rocks of the Atacama Desert were tested in a microdilution assay against* Paracoccidioides brasiliensis Pb*18. Approximately 18% (5) of the extracts showed minimum inhibitory concentration (MIC) values ≤ 125.0 µg/mL. Among these, extract from the fungus UFMGCB 8030 demonstrated the best results, with an MIC of 15.6 µg/mL. This isolate was identified as* Aspergillus felis *(by macro and micromorphologies, and internal transcribed spacer, β-tubulin, and ribosomal polymerase II gene analyses) and was grown in five different culture media and extracted with various solvents to optimise its antifungal activity. Potato dextrose agar culture and dichloromethane extraction resulted in an MIC of 1.9 µg/mL against* P. brasiliensis *and did not show cytotoxicity at the concentrations tested in normal mammalian cell (Vero). This extract was subjected to bioassay-guided fractionation using analytical C18RP-high-performance liquid chromatography (HPLC) and an antifungal assay using* P. brasiliensis. *Analysis of the active fractions by HPLC-high resolution mass spectrometry allowed us to identify the antifungal agents present in the* A. felis *extracts cytochalasins. These results reveal the potential of* A. felis *as a producer of bioactive compounds with antifungal activity.*

Key words: rock-inhabiting fungi - Atacama Desert - *Paracoccidioides brasiliensis* - antifungal - *Aspergillus felis*

Paracoccidioidomycosis (PCM) is a human systemic mycosis endemic in Latin America (Tavares et al. 2005). Approximately 10 million people in this region are infected (Stürme et al. 2011), with 85% of cases occurring in Brazil (Andrade et al. 2005), and specific social groups, such as rural workers, being particularly affected (Shikanai-Yasuda et al. 2006). PCM is acquired by inhaling airborne propagules derived from the mycelial form of *Paracoccidioides brasiliensis* (Tavares et al. 2005) and *Paracoccidioides lutzii* (Teixeira et al. 2009). They adhere to the alveolar epithelium, where they transform into pathogenic yeasts (Torres et al. 2010).

Despite the effectiveness of treatments with currently available drugs (amphotericin B, azoles, and sulfonamides), they require long term administration protocols capable of causing toxic effects (Borges-Walmsley et al. 2002, Palmeiro et al. 2005, Shikanai-Yasuda et al. 2006, Visbal et al. 2011).

In addition, antifungal chemotherapy does not ensure the complete elimination of the fungus from the patient (Travassos & Taborda 2012). The discovery of new antifungal agents with higher efficacies and fewer side effects is needed in order to increase treatment options for this infection.

Abadio et al. (2015) using the rational combination of molecular modelling simulations and virtual screening identified compounds against thioredoxin reductase of *P. lutzii*, which is a promising target for drugs. Transcriptome is another potential experimental strategy to elucidate the mechanism of action of bioactive compounds using the change in gene expression. Argentilactone, for example, appears to be capable of modulating cellular targets by inducing oxidative stress and interfere with cell wall biosynthesis in *P. lutzii* (Araújo et al. 2016). Proteomic profile of this fungus indicated a global metabolic adaptation in the presence of argentilactone. Enzymes of important pathways were repressed in *P. lutzii*, while proteins involved in cell rescue, defense, and stress response were induced in the presence of argentilactone (Prado et al. 2015).

Rock-inhabiting fungi are among the most stress-tolerant organisms on Earth, able to cope with the variety of stressors associated with bare rocks in environments of hot and cold extremes (Tesei et al. 2012). These surfaces are unique habitats where rapid changes in radiation, temperature, water and nutrient availability represent a challenge to microbial survival in different environments across the world (Gueidan et al. 2008).

The Atacama Desert may be the oldest desert on Earth (Azua-Bustos et al. 2012). Atacama's long-stand-

doi: 10.1590/0074-02760150451
Financial support: CNPq, FAPEMIG (CBB-APQ-02587-14), CAPES, UFMG
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Received 11 December 2015
Accepted 15 February 2016

ing aridity adds value to the study of biological adaptations, since that, organisms have been exposed to challenging environmental conditions for sufficiently long to bear witness to evolution and natural selection processes (Wierzchos et al. 2013). It is believed that species adapted to live in such environments constitute potential sources of enzymes with special characteristics and novel genes with possible industrial applications (Dalmaso et al. 2015).

The present study aimed to evaluate the activity of crude extracts from a collection of fungi isolated from the Atacama Desert against the human pathogenic fungus *P. brasiliensis*. Extract of the strain UFMGCB 8030 showed outstanding antifungal activity against this fungus of medical importance, and thus it was selected for further investigation.

MATERIALS AND METHODS

Fungal material - The 78 fungal isolates used in this study were obtained from rocks collected in the Atacama Desert (Gonçalves et al. 2015). These fungi have been deposited in the Collection of Microorganisms and Cells of the Federal University of Minas Gerais (UFMG), Brazil, under codes UFMGCB 8010-8090 (Table I).

Fungal cultivation and preparation of extracts for biological assays - All fungal isolates were cultivated and extracts prepared according to protocols established by Rosa et al. (2013). A stock solution of each extract was prepared in dimethyl sulfoxide (DMSO) (Merck, USA) at a concentration of 100 mg/mL and stored at -20°C. Extract of sterile yeast mold medium (YM) (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2% glucose, and 2% agar), generated using the same extraction protocol, was used as a control in the screening procedure.

Antifungal assay - Fungal isolate and inoculum - Antifungal activity of the extracts was evaluated using *P. brasiliensis Pb*18 (Fungi Collection of the Faculty of Medicine of São Paulo University, Brazil). Isolate *Pb*18 belongs to the cryptic phylogenetic species S1 (Matute et al. 2006) and was maintained at the Microbiology Department of the UFMG by weekly transfer onto solid yeast peptone dextrose medium (1% yeast extract, 0.1% peptone, 1% dextrose, and 2% agar) at 37°C. Isolated *Pb*18 cells were suspended in sterile saline and the transmittance of the resulting suspension at a wavelength of 530 nm was adjusted to 70% (1-5 × 10^6 cells/mL) using a spectrophotometer (SP-22; Biospectro, Brazil). The yeast-cell stock suspension was diluted in a 1:10 solution of RPMI-1640 medium (Sigma-Aldrich, USA) plus 3-(*N*-morpholino)-propanesulfonic acid broth (Sigma-Aldrich) for a final inoculum of 1-5 × 10^5 cells/mL (Cruz et al. 2012).

Antifungal activity screen - Extracts were diluted in RPMI medium for final concentrations of 500 µg/mL with DMSO at 0.5% v/v. RPMI medium with inoculum was used as a growth control, while the former was used on its own as a sterility control. DMSO (0.5% v/v) was used as a control for toxicity and itraconazole (0.05-0.0005 µg/mL) (Sigma-Aldrich) as a susceptibility control. The 96-well plates were prepared in duplicate and incubated at 37°C for 10 days. After this period, the plates were visually assessed and 10 µL of 5 mg/mL thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich) was added to each well prior to 4-h incubation. Following MTT metabolism, 100 µl of 5% v/v sodium dodecyl sulfate/isopropanol was added per well. The absorbance of test wells was measured at 530 nm using a microtitre plate spectrophotometer (VersaMax; Molecular Devices, USA) and compared with that of the growth control well. The inhibition of yeast growth (% inhib.) was calculated as a percentage according to the following equation where OD signifies optical density:

$$\%\ \text{inhib.} = \frac{(\text{OD of negative control well} - \text{OD of sample tested}) \times 100}{\text{OD of negative control well}}$$

Extracts demonstrating 70% inhibition of isolate *Pb*18 growth were considered active and subjected to a minimum inhibitory concentration (MIC) assay.

Determination of MIC - Microdilution assays were performed using the same conditions as those described for the antifungal activity screen (CLSI 2008, Johann et al. 2010). By dilution in RPMI-1640 broth, 10 two-fold serial dilutions of the selected extracts, ranging from 500.0-0.9 µg/mL, were tested. DMSO (0.5% v/v) was used as a control for toxicity and itraconazole (0.05-0.0005 µg/mL) as a susceptibility control. The MIC was considered to be the lowest concentration completely inhibiting *Pb*18 growth compared to the growth control, expressed in µg/mL. All tests were performed in duplicate in three independent experiments.

Molecular identification - The DNA extraction protocol and amplification of the internal transcribed spacer (ITS) region, achieved using the universal primers ITS1 and ITS4 (White et al. 1990), have been described by Rosa et al. (2009). Amplification of β-tubulin (Glass & Donaldson 1995) and ribosomal polymerase II genes (RPB2) (Houbraken et al. 2012) was performed with Bt2a/Bt2b and RPB2-5F-Pc/RPB2-7CR-Pc 7CR primers, respectively, according to protocols established by Godinho et al. (2013). To achieve species-rank identification based on ITS, β-tubulin, and RPB2 data, consensus sequences were aligned using all sequences of related species retrieved from the National Center for Biotechnology Information GenBank database using the Basic Local Alignment Search Tool (Altschul et al. 1997). The sequences obtained were subjected to ITS, β-tubulin, and RPB2-based phylogenetic analyses using comparisons with sequences of type species deposited in GenBank, with estimations calculated by MEGA v.5.0 (Tamura et al. 2011). The maximum composite likelihood method was employed to estimate evolutionary distances, with bootstrap values calculated from 1,000 replicate runs. Information concerning fungal classification generally follows Kirk et al. (2008) and the MycoBank (mycobank.org) and Index Fungorum (indexfungorum.org) databases.

Morphological identification - Macroscopic fungal parameters (colony colour and texture, border type, and radial growth rate) and colony diameters were observed on Czapek yeast autolysate (CYA) (0.5% w/v yeast extract, 3.5% w/v Czapeck, 2% w/v agar) and malt extract agar (MEA) [2% w/v malt extract, 0.1% w/v peptone, 2% w/v glucose (HiMedia, India)]. Three-point inoculations

TABLE I

Minimum inhibitory concentrations (MIC) of extracts of fungi isolated from Atacama Desert rocks against *Paracoccidioides brasiliensis Pb*18

Fungal species	UFMGCB[a]	MIC (µg/mL)
Alternaria cf. *arborescens*	8010	500.0
Aspergillus felis	8011	**250.0**
Alternaria sp. 1	8012	500.0
Alternaria sp. 2	8013	500.0
Cladosporium halotolerans	8014	-
Neosartorya cf. *udagawae*	8015	**125.0**
Cladosporium cf. *cladosporioides*	8017	500.0
A. cf. *arborescens*	8018	-
A. felis	8019	500.0
Hypoxylon cf. *trugodes*	8020	500.0
N. cf. *udagawae*	8021	**62.5**
Fusarium oxysporum	8023	-
A. felis	8024	**31.2**
A. felis	8025	-
A. felis	8026	**31.2**
Eupenicillium javanicum	8027	500.0
Cladosporium cf. *oxysporum*	8028	-
Aspergillus sp.	8029	-
A. felis	8030	**15.6**
Aspergillus lentulus	8031	500.0
Neosartorya sp. 2	8032	500.0
F. oxysporum	8033	500.0
E. javanicum	8034	500.0
A. lentulus	8035	-
Penicillium cf. *puvillorum*	8036	-
Neosartorya sp. 2	8037	500.0
E. javanicum	8038	-
Neosartorya sp. 2	8039	500.0
A. felis	8040	-
C. halotolerans	8041	-
C. halotolerans	8042	-
Penicillium crysogenum	8043	500.0
Didymellaceae sp.	8044	-
P. crysogenum	8045	500.0
Aspergillus persii	8046	-
Aspergillus westerdijkiae	8047	500.0
Cladosporium cf. *gossypiicola*	8048	-
P. crysogenum	8049	500.0
Macroventuria cf. *anomachaeta*	8050	-
Penicillium cf. *citrinum*	8051	-
P. cf. *citrinum*	8052	-
P. crysogenum	8053	-
P. crysogenum	8054	-
P. crysogenum	8055	500.0
C. halotolerans	8056	-
P. crysogenum	8057	500.0
Aspergillus sydowii	8058	-
P. cf. *citrinum*	8059	-
P. cf. *citrinum*	8060	-
Devriesia sp.	8061	-
P. cf. *citrinum*	8062	-
Neosartorya sp. 2	8063	-
Neosartorya sp. 2	8064	-
Neosartorya sp. 2	8065	-
Neosartorya sp. 1	8066	-
Neosartorya sp. 1	8067	-
Neosartorya sp. 2	8068	-
Neosartorya sp. 2	8069	-
C. halotolerans	8070	-
Neosartorya sp. 2	8071	-
Neosartorya sp. 2	8072	-
P. cf. *citrinum*	8073	-
P. crysogenum	8074	500.0
C. halotolerans	8075	-
C. cf. *gossypiicola*	8076	-
C. halotolerans	8077	-
P. cf. *citrinum*	8078	-
Neosartorya cf. *udagave*	8079	-
P. cf. *citrinum*	8080	-
P. crysogenum	8081	-
Pseudogymnoascus cf.	8082	-
Cladosporium cf.	8083	-
Cladosporium cf.	8084	-
Cladosporium cf.	8085	-
A. cf. *arborescens*	8086	-
C. halotolerans	8087	-
P. cf. *citrinum*	8089	500.0
P. cf. *citrinum*	8090	-

a: Collection of Microorganisms and Cells of the Federal University of Minas Gerais, Brazil; -: no antifungal activity. Bold values: good antifungal activity.

of the fungus UFMGCB 8030 were incubated for seven days in the dark at 25ºC. Fungal reproductive structures were produced by microculture technique, stained with lactophenol cotton blue (0.05% w/v), and evaluated under an optical microscope (DM750; Leica, Germany) at 40X magnification (Klich 2002).

Cultivation and extraction of UFMGCB 8030 using different culture media - The fungus UFMGCB 8030 was grown on the following five culture media in order to evaluate the antifungal activity of its extracts: potato dextrose (PDA) [2% w/v glucose, 30% w/v potato infusion (HiMedia)], YM, MEA, corn meal (HiMedia), and minimal medium containing 6.98 g/L K_2HPO_4, 5.44 g/L KH_2PO, and 4.1 g/L $(NH_4)_2SO_4$, and supplemented with 5, 10, 15, 20, and 30 g/L glucose. The cultures were incubated at 25 ± 2ºC for 15 days and extracted with ethanol (Vetec, Brazil) for 24 h at ambient temperature. After filtration, the organic phase was concentrated on a rotary evaporator. Residual solvent was removed with a SpeedVac system (Savant SPD 121P; Thermo Scientific, USA) at 40ºC to yield crude extracts.

Production of UFMGCB 8030 extracts using different solvents - UFMGCB 8030 was grown on PDA medium at 25 ± 2ºC for 15 days, with cultures being subjected to extraction three times at 48 h intervals using 20 mL of hexane, dichloromethane (DCM), ethyl acetate or ethanol (all Vetec). The extracts were obtained by the procedure described above.

Cytotoxicity assay - The VERO (African green monkey kidney cells) lineage was used as a model of normal cells. This lineage was maintained in the logarithmic phase of growth in Dulbecco's modified Eagle's medium supplemented with 100 IU/mL penicillin and 100.0 µg/mL streptomycin enriched with 5% foetal bovine serum. VERO cells were maintained at 37ºC in a humidified incubator with 5% CO_2 and 95% air. The medium was changed twice weekly and the cells were regularly examined and used until 20 passages. Vero cells were seeded at a density of 1×10^4 cells before being pre-incubated for 24 h at 37ºC to allow for their adaptation prior to addition of the test sample. The extract was dissolved in DMSO (0.5% v/v) before dilution and tested over a range of concentrations (8 nonserial dilutions from 100-1.5 µg/mL). All cell cultures were incubated in a humidified 5% CO_2/95% air atmosphere at 37ºC for 48 h. The negative control comprised treatment with 0.5% v/v DMSO. Controls included drug-containing medium (background) and drug-free complete medium. Drug-free complete medium was used as a control (blank) and was treated in the same way as the drug-containing media. Results were expressed as a percentage of inhibition of cell viability compared to the 0.5% DMSO control and were calculated as follows: % inhibition of cell viability (%) = 100 - (mean OD treated - mean OD background)/(mean OD untreated culture, i.e., 0.5% DMSO - mean OD blank wells) x 100. Interactions between compounds and media were estimated on the basis of variations between drug-containing media and drug-free media to avoid false-positives or false-negatives (Monks et al. 1991). All samples were tested in triplicate in two independent experiments.

Chromatographic separation of UFMGCB 8030 DCM extract and identification of active compounds - Analytical chromatography was performed on a reversed-phase high-performance liquid chromatography (RP-HPLC) system (Shimadzu, Japan) equipped with a manual injector, two pumps (LC-10A), and a diode array detector (SPD-M10A). DCM extract (500 µg) was injected into an analytical HPLC column [Shim-pack ODS, 4 µm, 3.9 × 150 mm (Shimadzu)] and eluted at a flow rate of 1 mL/min using a gradient of 15-100% acetonitrile (ACN) in water for 16 min, followed by 100% ACN for 8 min. The effluent was collected in a 96-well plate (300 µL per well in 80 wells) using a fraction collector (SF2120; Advantec MFS, USA). The experiment was repeated four times and the plates obtained were dried in a SpeedVac vacuum centrifuge at 40ºC. Fractions from two plates were dissolved in 100 µL RPMI medium containing 0.5% v/v DMSO before being transferred to fresh plates for the *P. brasiliensis Pb*18 bioassay. Fractions showing 70% inhibition of isolate *Pb*18 growth were considered active.

Active compounds were dissolved by addition of ACN to the appropriate wells prior to liquid chromatography-mass spectrometry (LC-MS) [tandem MS (MS/MS)] analysis. This was performed on a Nexera UHPLC system (Shimadzu) coupled to a maXis ETD high-resolution ESI-QTOF mass spectrometer (Bruker, USA) and controlled by the Compass 1.5 software package (Bruker). Fractions (20 µL) were injected into a Shim-Pack XR-ODS III column [C18, 2.2 µm, 2.2 × 200 mm (Shimadzu)] at 40ºC using a flow rate of 200 µL/min. The components of the mobile phase, A and B (0.1% formic acid in water and ACN, respectively), formed an eluent gradient as follows: 5% B for the initial 0.5 min, then a linear gradient to 100% B over 12.5 min, and a final hold for 1 min of 100% B. Ultraviolet chromatograms were recorded at wavelengths of 214 and 254 nm. The mass spectra were acquired in positive mode at a spectra rate of 2 Hz. Ion-source parameters were set to 500 V end plate offset, 4,500 V capillary voltage, 2.0 bar nebuliser pressure, and 8.0 L/min and 200ºC dry gas flow and temperature, respectively. Data-dependent of precursor fragmentation was performed at collision energies of 40 eV. Ion cooler settings were optimised within an *m/z* range of 40-1,000 using a solution of 10 mM sodium formate in 50% 2-propanol for calibration. Mass calibration was achieved by an initial ion-source infusion of 20 µL calibration solution and post-acquisition recalibration of the raw data.

Compound detection was performed by chromatographic peak analysis with subsequent formula determination according to exact mass and isotope pattern (MS1) and database comparison of compound fragment spectra (MS2). An in-house database of standard compounds and the public spectra database MassBank (Horai et al. 2010) served as sources of reference ESI fragment spectra.

LC-MS mass data files were used to identify the active compounds from KNApSAcK and SciFinder/Chemical Abstracts Service databases. Manual interpretation of MS/MS spectra was also performed using the MassBank database.

RESULTS

When the 78 extracts of fungi obtained from rocks in the Atacama Desert were tested at a single concentration (500.0 µg/mL) against *P. brasiliensis Pb*18 35% were found to inhibit 70% of growth. These were considered to be active and their MICs were determined. Approximately 18% (5) of these active extracts exhibited MICs ≤ 125.0 µg/mL (Table I). UFMGCB 8030 extract demonstrated the lowest MIC with a value of 15.6 µg/mL followed by those of isolates UFMGCB 8024 and UFMGCB 8026, with values of 31.2 µg/mL, and UFMGCB 8021, with an MIC of 62.5µg/mL. The fungal isolates have been identified based on ITS sequence analysis by Gonçalves et al. (2015), being grouped into 30 species belonging to 13 genera. The fungi providing the most active extracts in the present work were *Neosartorya* cf. *udagawae* (UFMGCB 8015 and 8021, with MICs of 125.0 and 62.5 µg/mL, respectively) and *Aspergillus felis* (UFMGCB 8024, 8026, and 8030, with MICs of 31.2-15.6 µg/mL) (Table I). Among the most active extracts, *A. felis* UFMGCB8030 was of particular interest, showing promising activity against *P. brasiliensis Pb*18 (MIC = 15.6 µg/mL). Thus, a more detailed study to identify both this fungus and the active compounds in its extract was performed.

Although the ITS-based identification of *A. felis* UFMGCB 8030 gave satisfactory results (Gonçalves et al. 2015), we also sequenced its β-tubulin and RPB2 (Fig. 1). A combination of phylogenetic evaluation (Fig. 1) and analysis of micro and macro-morphological features (Fig. 2) increased the degree of confidence in this identification.

The ITS nucleotide sequence showed 100% query coverage and 100% similarity with that of *A. felis* (GenBank accession KF558318). In addition, the β-tubulin and RPB2 sequences of this isolate shared 84% and 100% query coverage and 99% and 98% of similarity, respectively, with the corresponding *A. felis* sequences (GenBank accessions KJ914694 and KJ914735, respectively). ITS, β-tubulin, and RPB2 references or type species sequences were retrieved from GenBank and used in a neighbour-joining phylogenetic analysis with 1,000 bootstrap replicates (Fig. 1). This approach revealed distinct clustering of the organism of interest in this study with *A. felis*, confirming it to be the species most genetically similar to isolate UFMGCB 8030.

The following characteristics of the *Aspergillus* isolate were observed, as shown in Fig. 2: colony diameters of 5.0 and 5.5 cm after seven days at 25ºC on CYA and MEA media, respectively, and sporulation on MEA at 25ºC on the 14th day of culture. On CYA medium, colony texture is mostly floccose; colonies are usually white, with a cream-to-light-brown reverse, and often sporulate poorly. Furthermore, yellow soluble pigments are diffused into the agar. On MEA, colonies are somewhat velvety with greenish sporulation occurring after seven days. Colonies have a cream reverse. Conidiophores are uniseriate with greenish stipes (12 × 5.0 µm) and green globose conidia 1.5-2.5 µm in length. Phialides are 6.0 × 2.0 µm and vesicles are pyriform with a diameter of

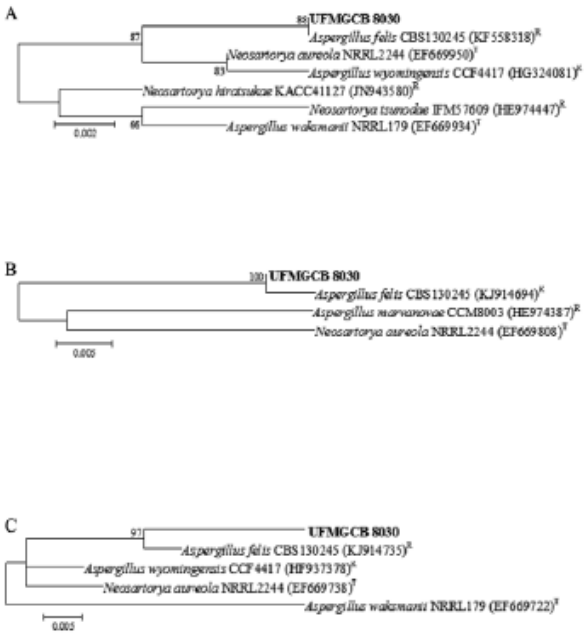


Fig. 1: phylogenetic analysis of nucleotide sequences obtained from fungus UFMGCB 8030 (in bold) associated with rocks from the Atacama Desert in comparison with type (T) and reference (R) sequences deposited in GenBank. Trees were constructed based on ITS1-5.8S-ITS2 (A), β-tubulin (B), and ribosomal polymerase II gene (C) sequences using the maximum composite likelihood model.

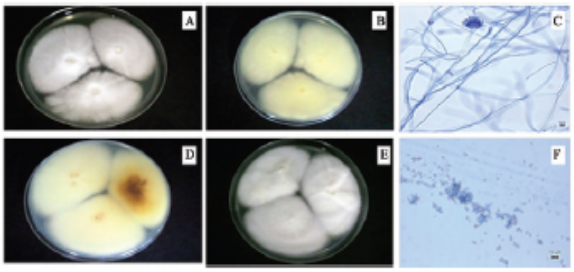


Fig. 2: *Aspergillus felis* colonies after seven days at 25°C on malt extract agar (A, B) and Czapeck yeast autolysate agar (D, E). Conidiophores and conidia (C, F) at 40X magnification (10 µm). Top-down (A, E) and reverse (B-D) aspects of cultures.

13 mm. After taxonomic analysis using molecular and morphological methods, fungus UFMGCB 8030 was confirmed to be *A. felis* (Barrs et al. 2013).

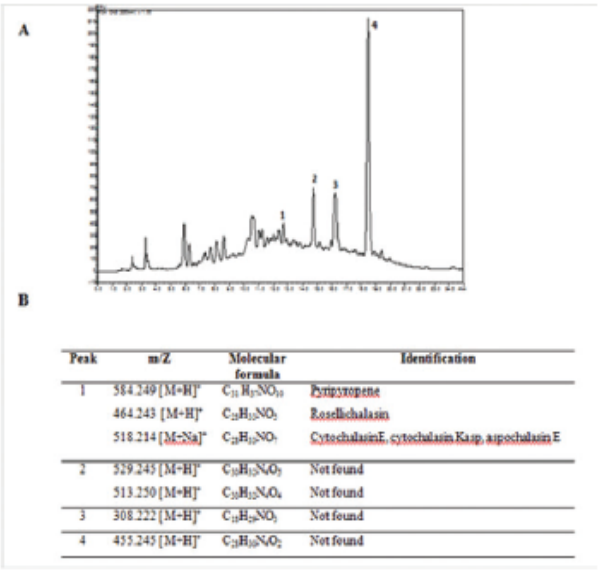
In the present study, the production of bioactive compounds was assessed by varying certain culture conditions of *A. felis* UFMGCB 8030 and testing the resulting extracts with a *P. brasiliensis* bioassay. In regard to culture media,

TABLE II

Minimum inhibitory concentrations (MIC) against *Paracoccidioides brasiliensis Pb*18 of ethanol extracts from *Aspergillus felis* (UFMGCB 8030) grown on different culture media

Culture medium (g/L)	MIC (µg/mL)
MM (5)	-
MM (10)	-
MM (15)	-
MM (20)	-
MM (30)	-
PDA	7.8
YM	15.6
MEA	62.5
Corn meal	15.6
Itraconazole	0.001

MEA: malt extract agar; MM: minimal medium supplemented with 5-30 g/L glucose; PDA: potato dextrose agar; YM: yeast mold; -: no activity.



Peak	m/Z	Molecular formula	Identification
1	584.249 [M+H]+	$C_{31}H_{37}NO_{10}$	Pyripyropene
	464.243 [M+H]+	$C_{28}H_{33}NO_5$	Rosellichalasin
	518.214 [M+Na]+	$C_{28}H_{33}NO_7$	CytochalasinE, cytochalasin Kasp, aspochalasin E
2	529.245 [M+H]+	$C_{30}H_{32}N_4O_3$	Not found
	513.250 [M+H]+	$C_{30}H_{32}N_4O_4$	Not found
3	308.222 [M+H]+	$C_{18}H_{29}NO_3$	Not found
4	455.245 [M+H]+	$C_{28}H_{30}N_4O_2$	Not found

Fig. 3: identification of secondary metabolites in dichloromethane (DCM) extract of *Aspergillus felis* UFMGCB 8030 grown for 15 days on potato dextrose agar medium. A: high-performance liquid chromatography chromatogram of *A. felis* DCM extract (ultraviolet detection at 220 nm) showing active fractions 1-4 (≥ 70% inhibition of isolate *Pb*18 growth); B: table showing the base-peak values of active fractions 1-4 with their molecular formulae and manual verification of high resolution mass spectrometry results using SciFinder and KNApSAcK data.

TABLE III

Minimum inhibitory concentrations (MIC) against *Paracoccidioides brasiliensis Pb*18 of various solvent extracts from *Aspergillus felis* (UFMGCB 8030) cultures grown on potato dextrose agar

Solvent	MIC (µg/mL)
Hexane	250.0
Dichloromethane	1.9
Ethyl acetate	500.0
Ethanol	7.8

the most striking results were obtained with extracts from fungi cultivated on PDA (MIC = 7.8 µg/mL) followed by those from YM and corn meal cultures (MIC = 15.6 µg/mL). On MEA medium, the MIC of the ethanol extract was 62.5 µg/mL (Table II). Extracts obtained after cultivation of this isolate on minimal medium supplemented with glucose showed no antifungal activity against *Pb*18.

As the ethanolic extract of *A. felis* UFMGCB 8030 grown on PDA demonstrated the lowest MIC, this medium was used to identify the optimal solvent for the production of extracts with the highest antifungal activity. The extract obtained using DCM was found to be the most active against *P. brasiliensis Pb*18 (MIC = 1.9 µg/mL), followed by that produced with ethanol (MIC = 7.8 µg/mL). Extracts prepared with ethyl acetate and hexane were only minimally active (with MICs of 500.0 and 250.0 µg/mL, respectively) (Table III). The PDA/DCM *A. felis* UFMGCB 8030 extract did not show cytotoxicity at the concentrations tested when assayed with Vero cells, demonstrating that this extract exhibits some selectivity towards fungal cells compared to mammalian cells.

The PDA/DCM extract was then subjected to bioassay-guided fractionation using RP-HPLC and a *P. brasiliensis* assay (Fig. 3). The active fractions were analysed by HPLC-high resolution mass spectrometry (HRMS) with electrospray ionisation in positive-ion mode to obtain accurate mass measurements. A tentative identification based on the resulting mass spectra was achieved by manual verification using SciFinder and KNApSAcK data. The HRMS data corresponding to active fraction 1 consisted of *m/z* signals at 584.249 $[M+H]^+$, 464.243 $[M+H]^+$, and 518.214 $[M+Na]^+$ that were tentatively identified as known compounds pyripyropene A (Omura et al. 1993), rosellichalasin (Kimura et al. 1989), and cytochalasin E (Aldridge et al. 1972), cytochalasin Kasp (Kimura et al. 1989), or aspochalasin E (Steyn et al. 1982), respectively. Active fraction 1 comprised multiple compounds, but the effective identification of these based on patterns of substitution was not possible due to a lack of information in the literature. The resulting formulas obtained from the fractions 2-4 did not match against SciFinder and KNApSAcK database to search for known metabolites. It could be hypothesised that these fractions can contain metabolites that were not previously isolated from *Aspergillus* species.

DISCUSSION

In the present work, the DCM extract of *A. felis* UFMGCB 8030 displayed promising activity against *P. brasiliensis Pb*18, although in a previous screen with *Candida albicans*, *Candida krusei*, and *Cladosporium sphaerospermum* it was shown to be inactive (Gonçalves et al. 2015). Although this fungus has previously been identified using ITS sequences (Gonçalves et al. 2015), in this work the identity of isolate UFMGCB 8030 was confirmed using molecular, morphological, and phylogenetic methodologies. According to Barrs et al. (2013), species belonging to the *Aspergillus*, section *Fumigati*, cannot be identified only on the basis of morphological aspects only, therefore the use of other approaches for the identification of such organisms is key. Barrs et al. (2013) recently described the identification of *A. felis* in human and animal hosts (dogs and cats) with invasive aspergillosis. The isolation of this fungus from environmental samples was first reported by our group, as a result of an investigation of Atacama Desert's rock samples (Gonçalves et al. 2015).

In fungi, the biosynthesis of secondary metabolites is regulated in response to nutrient availability or as a result of changes in the environment or developmental phase (Sanchez & Demain 2002, Zain et al. 2011). Altering the media used to culture microorganisms can enhance the production of bioactive compounds (Abdel-Fattah & Olama 2002). A good understanding of the role of culture conditions in the biosynthesis of metabolites may lead to improved exploitation of microorganisms-derived compounds (Miao et al. 2006). The ethanol extract obtained by cultivation of *A. felis* UFMGCB 8030 on PDA resulted in the strongest effect, i.e., the lowest MIC, in an evaluation of culture media, while minimal medium failed to provide conditions suitable for the production of antifungal compounds against *Pb*18. Bhattacharyya and Jha (2011) showed that in salt-rich media such as Czapek-Dox, the growth and antimicrobial activity of an *Aspergillus* strain was lower than that observed using a complex medium such as PDA. In addition, Mathan et al. (2013) demonstrated that low-nutrient medium has a detrimental effect on mycelial growth and metabolite profile in *Aspergillus terreus*. This suggests that in salt-rich or nutrient-poor media, mycelial growth interferes with the production of antifungal metabolites by *Aspergillus* spp.

As *A. felis* was described only very recently, we were unable to find any records in the literature concerning investigation of its secondary metabolites, a fact that encouraged us to determine the compounds in the UFMGCB 8030 DCM extract responsible for its antifungal activity.

Concerning the compounds identified in this extract, no reports of antifungal activity exist for pyripyropene, rosellichalasin, cytochalasin Kasp, or aspochalasin E. However, cytochalasin E has been tested against *Fusarium solani* (MIC > 100 µM), *Gibberella saubinetti* (MIC = 100 µM), *Botrytis cinerea* (MIC = 100 µM), and *Alternaria solani* (MIC = 50 µM), showing weak antifungal activity with MIC values generally greater than 50 µM (Zhang et al. 2014). Although antifungal activity against organisms of agricultural importance has thus been documented, no investigations into the effect of the compounds identified in this work against fungi of medical interest have been carried out.

The fast tentative identification of natural products using the dereplication process can be very efficient to detect promising source of new bioactive compounds (Kildgaard et al. 2014, Petersen et al. 2014, Boruta & Bizukojc 2015). In one of the fractions displaying antifungal activity, cytochalasins were identified as the active metabolites. Cytochalasins are a group of fungal secondary metabolites with a 10-phenylperhydroisoindol-1-one skeleton and a macrocyclic ring and are capable of various biological activities (Qiao et al. 2011). They have been described not only in the genus *Aspergillus* (Demain et al. 1976, Udagawa et al. 2000, Lin et al. 2009, Zheng et al. 2013), but also in *Xylaria* (Silva et al. 2010), *Cladosporium* (Cafêu et al. 2005), *Arthrinium* (Wang 2015), and *Phomopsis* (Shen et al. 2014). According to Guerra et al. (2014), cytochalasins inhibit actin polimerisation and act preventing actin interaction with host cells in the fungal pathogen *Cryptococcus neoformans*. *C. neoformans* is internalised by receptor-mediated or "triggered" phagocytosis, dependent on actin recruitment. Additionally, they can act as microfilament-disrupting agents, alter cell motility, adherence, secretion, drug efflux, deformability, morphology, and size, among many other cell properties critical to neoplastic cell pathology (Van Goietsenoven et al. 2011). Rosellichalasin and cytochalasin E isolated from *Aspergillus* sp. exhibit potent cytotoxic activity against human tumour cell lines (Xiao et al. 2013). Besides these compounds, aspochalasin E shows potent activity against murine melanoma B16-F10 and human colon carcinoma HCT-116 cells (Naruse et al. 1993). Pyripyropene A acts in decrease of intestinal cholesterol absorption and cholesteryl oleate levels, resulting in protection of atherosclerosis development (Ohshiro et al. 2011).

The literature contains few reports on the isolation of compounds from fungi exhibiting activity against *P. brasiliensis*. However, among these are altenusin, isolated from an *Alternaria* sp. (Johann et al. 2012), and trichothecene mycotoxins (T-2 toxin and a mixture of 8-*n*-isobutyrylsolaniol and 8-*n*-butyryl*neo*solaniol (Campos et al. 2011).

This study indicated that fungi isolated from Atacama Desert rocks may constitute potential sources of novel bioactive compounds. *A. felis* UFMGCB 8030 produced the most active extract among those studied and its antifungal activity was enhanced by changes in culture conditions. The DCM extract of this fungus showed low cytotoxicity in preliminary tests and outstanding activity against one of the fungi responsible for PCM. Our results demonstrate the importance of further studies into the fungus *A. felis*, since the analyses presented here suggest that previously unknown bioactive compounds can be produced by this species.

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4.2- *Capítulo 2- "*Alkaloidal metabolites from *Aspergillus felis* and their activities against *Paracoccidioides brasiliensis*".

Graziele Mendes; Djalma M Oliveira; Markus Kolloff; Carlos A Rosa; Tânia Alves; Carlos L Zani; Luiz H Rosa; Susana Johann; Betania Barros Cota.

Neste trabalho foi realizado o isolamento e a caracterização estrutural de substâncias ativas contra *P. brasiliensis* Pb 18 a partir do extrato orgânico do fungo *A. felis* UFMGCB8030. Este extrato foi feito com base nos resultados do trabalho apresentado no capítulo1 e nas condições que este fungo apresentou os melhores valores de CIM, ou seja, no cultivo com o meio batata dextrose ágar (BDA) e diclorometano (DCM) para a extração dos metabólitos ativos. A partir deste extrato foram obtidas sete substâncias, dentre as quais, duas foram classificadas como inéditas. Uma delas pertence ao grupo das citocalasinas e foi denominada como citocalasina Z15E. A outra é classificada como um derivado de ardeemina e foi denominada 5-*N*-acetil-8-β-isopropil ardeemina. Estas susbtâncias apresentaram atividade antifúngica contra o Pb 18, com destaque para a 5-*N*-acetil-8-β-isopropil ardeemina (valor de CIM de 62,5 μg/mL; 125,0 μM). Além dessas substâncias, cinco metabólitos conhecidos também foram isolados: roselicalasina, citocalasina E, gancidina, pseurotina A1 e 2,4-diidroxiacetofenona. Dentre estas susbtâncias, quatro apresentaram atividade antifúngica nas concentrações testadas, com destaque para a citocalasina E (valor de CIM de 1,9 μg/mL; 3,60 μM). Para o nosso conhecimento, este é o primeiro relato sobre o isolamento de substâncias a partir do fungo *A. felis* e também é o primeiro relato de atividade antifúngica contra *P.brasiliensis* das substâncias isoladas neste trabalho.

Os objetivos deste trabalho foram:

- Produzir extrato do fungo *A. felis* UFMGCB8030 em maior escala;
- Realizar o fracionamento biomonitorado do extrato BDA/DCM do fungo *A. felis* UFMGCB8030 para atividade antifúngica contra *P. brasiliensis*;
- Determinar os valores de CIM das frações e substâncias isoladas;
- Realizar a caracterização estrutural das substâncias através de métodos espectrométricos.

Phytochemistry Letters 17 (2016) 28–35

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Alkaloidal metabolites from *Aspergillus felis* and their activities against *Paracoccidioides brasiliensis*

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ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form 15 May 2016
Accepted 9 June 2016
Available online xxx

Keywords:
Aspergillus felis
Alkaloidal metabolites
Perhydroisoindol
Paracoccidioides brasiliensis
Antifungal activity

ABSTRACT

In this study, the fungus *Aspergillus felis* (strain UFMGCB 8030) was isolated from rocks in the Atacama Desert, Chile. Its CH_2Cl_2 extract exhibited antifungal activity (MIC of 1.9 μg/mL) against *Paracoccidioides brasiliensis* (Pb18), which is the etiological agent for paracoccidioidomycosis. The crude extract was purified, and a new cytochalasin derivative, cytochalasin Z_{15E} (**1**), and one ardeemin derivative, 5-*N*-acetyl-8-β-isopropyl-ardeemin (**4**), in addition to five known secondary metabolites, rosellichalasin (**2**), cytochalasin E (**3**), gancidin (**5**), pseurotin A1 (**6**), and 2,4-dihydroxyacetophenone (**7**), were isolated. The structures of these compounds were confirmed by 1D and 2D NMR spectroscopy and HR-ESI–MS. Cytochalasin E was the most active compound with MIC an value of 3.6 μM, while the other isolated compounds exhibited weak antifungal activities (MIC values >100.0 μM).

1. Introduction

The driest region in the world is the hyperarid Atacama Desert, Chile, where non-symbiotic fungi are widely distributed (Sterflinger et al., 2012). During our ongoing investigation toward the isolation of bioactive metabolites from fungi found in underexplored environments, we focused our attention on the *Aspergillus felis* strain UFMGCB 8030 isolated from the rocks of the Atacama Desert in northern Chile. The organic extract from this strain exhibited outstanding antifungal activity (MIC of 1.9 μg/mL) against the pathogenic fungus *Paracoccidioides brasiliensis*. *P. brasiliensis* causes paracoccidioidomycosis in humans, one of the most prevalent systemic mycoses in Latin America; it is a persistent infection that often develops after a long period of latency and cannot be easily eradicated with antifungal drugs (Bocca et al., 2013). Few research groups have explored natural products for finding drug leads aimed at finding alternative treatments or overcoming the issues that the drugs currently in clinical use cannot address. In this study, the isolation and structural determination of two new compounds, cytochalasin Z_{15E} (**1**) and 5-*N*-acetyl-8-β-isopropyl-ardeemin (**4**), along with the known compounds (**2–3**, **5–7**) was reported. The isolated compounds were tested against the pathogenic fungus *P. brasiliensis*, and the results showed that cytochalasin E exhibits antifungal activity. In addition, secondary metabolites isolated from *A. felis* have been reported for the first time.

2. Results and discussion

The CH_2Cl_2 extract of *A. felis* was subjected to liquid–solid chromatography on a silica gel column to afford 12 fractions (F1–F12). Then, fractions F6 and F9 were subjected to RP18 semi-prep. HPLC to afford four (**1**, **4–6**) and two alkaloidal metabolites (**2–3**) (Fig. 1), respectively. All known compounds were identified by the comparison of their spectral data (MS, and ^1H and ^{13}C NMR) with those published in literature.

Compound **1** was isolated as colorless oil with a specific rotation $[\alpha]_D^{25}$ of +30.34° (*c* 0.78 in MeOH). The molecular formula was assigned as $C_{25}H_{33}NO_5$, as determined by HR-ESI–MS (*m/z* 428.2433, $[M+H]^+$; calc. for $C_{25}H_{34}NO_5$: 428.2431). Based on

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http://dx.doi.org/10.1016/j.phytol.2016.06.006
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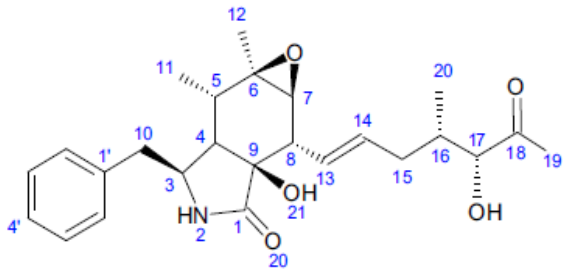

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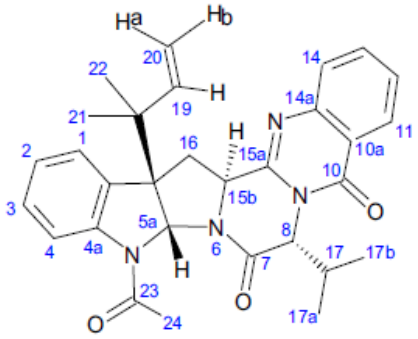

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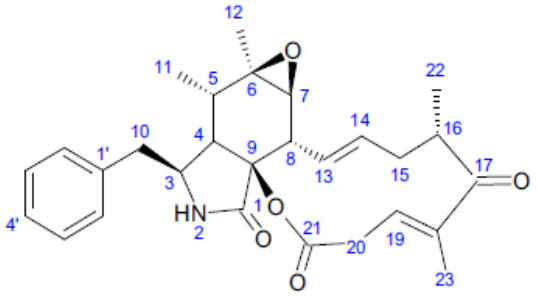

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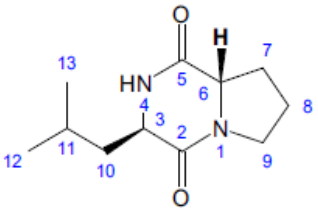

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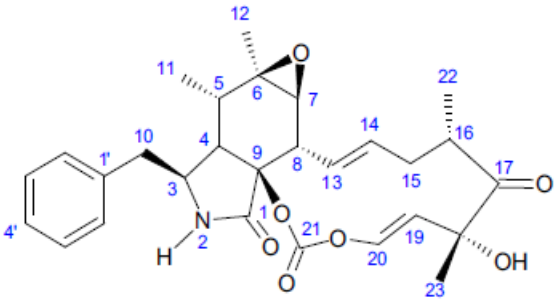

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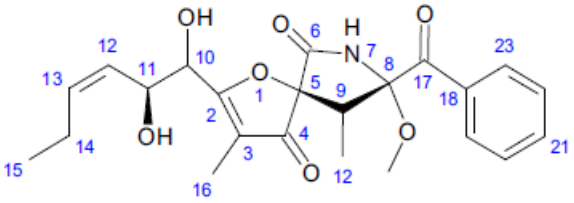

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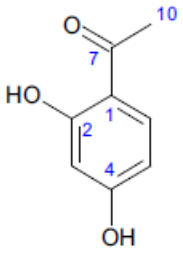

7

Fig. 1. Structures of compounds **1–7** isolated from *Aspergillus felis*.

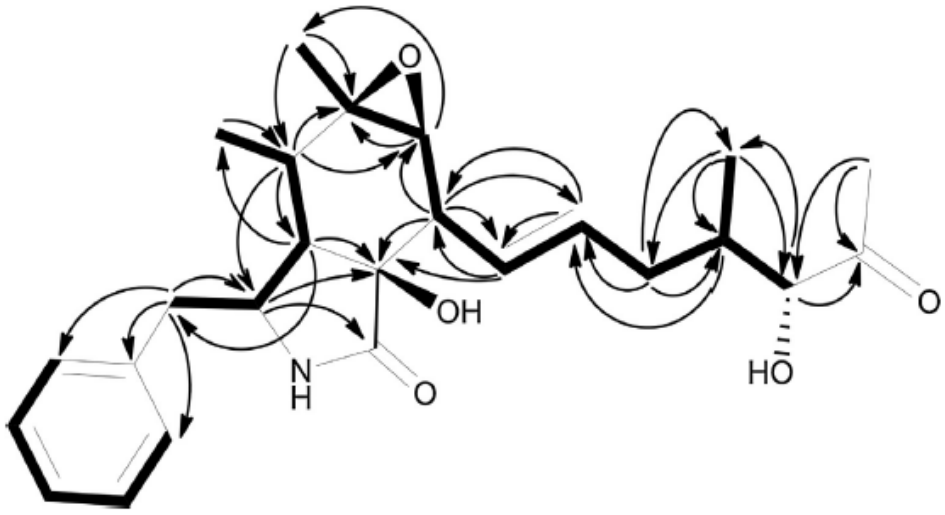

Fig. 2. Structure, key HMBC (→) and ^{1}H–^{1}H COSY (—) correlations of the cytochalasin Z_{15E} (**1**).

the molecular formula, it was deduced that **1** has 10° of unsaturation. Its UV spectrum exhibited λ_{max} at 204 and 256 nm. The ^{1}H, ^{13}C NMR, and HSQC data of **1** revealed two carbonyl and three non-hydrogenated carbons, as well as fourteen methine, two methylene, and four methyl groups. In the ^{1}H NMR spectrum of **1**, aromatic signals were observed at a δ_H of 7.33 (2H, dt, J = 7.5, 1.8 Hz, H-3′/5′), δ_H 7.30–7.27 (1H, m, H-4′), and δ_H 7.19–7.16 (2H, m, H-2′/6′), indicative of a monosubstituted phenyl ring. The COSY spectrum showed correlations between aromatic protons, attributed to the presence of cross peaks between H-2′/6′ and H-3′/5′ and H-3′/5′ and H-4′. The correlations between H-2′/6′ and C-10 (δ_C 45.10, CH_2) indicated the presence of a benzyl moiety, which was attached at C-3 (δ_C 54.45, CH), attributed to the cross peak between H_2-10 (δ_H 2.98 and δ_H 2.69) and C-3 in the HMBC spectrum (Fig. 2). In the ^{1}H NMR spectrum of **1** (Table 1), a signal corresponding to amide NH (δ_H 6.19) was observed, and in the ^{13}C NMR spectrum, two carbonyl carbon signals were observed at δ_C of 210.5 and 175.5, attributed to ketone and amide carbons, respectively. The 1D and 2D NMR spectra of **1** also showed peaks corresponding to a 10-phenylsubstituted perhydroisoindol-1-one skeleton with a 6,7-epoxy moiety, as well as spectral data very similar to the known series of cytochalasins, mainly with the cytochalasins Z_{20} and Z_{21} (Wang et al., 2011; Lin et al., 2009; Liu et al., 2006, 2008). The chemical shifts of C-6 and C-7 in **1** observed at δ_C 59.9 (C) and 61.5 (CH), respectively, were very similar to those recorded for cytochalasin Z_{20} (C-6: δ_C 60.5, C and C-7: δ_C 61.2, CH) (Lin et al., 2009) and Z_{21} (C-6: δ_C 59.3, C and C-7: δ_C 61.2, CH) (Wang et al., 2011). COSY correlations confirmed the perhydroisoindol-1-one skeleton with a 6,7-epoxy moiety by the presence of cross peaks between H-3 and H-4, H-4 and H-5, H-5 and H-11, and H-7 and H-8/H-12 (Fig. 2). The HMBC spectrum helped to confirm this individual fragment by correlations between H-10 and C-1′/C-3/C-

Table 1
NMR spectroscopic data for cytochalasin Z_{15E} (**1**) (at 400 MHz for ^{1}H and 100 MHz for ^{13}C).

Position	**1** in $CDCl_3$			
	δ_C, type	δ_H (J in Hz)	COSY	HMBC[a]
1	175.5, C			
2	N—H	6.19, br. s.		
3	54.5, CH	3.63, dt (9.0, 4.5)	4, 10a, 10b	1, 4, 5, 9, 1′
4	50.5, CH	2.40, dd (8.0, 4.5)	3, 5	1, 3, 5, 6, 8, 9, 10, 11
5	33.9, CH	2.34, dd (8.0, 7.0)	4, 11	3, 4, 6, 7, 9, 11,12
6	59.9, C			
7	61.5, CH	2.97, br. d (5.0)	8, 12	6, 8, 9, 12, 13
8	48.8, CH	2.75, br. dd (8.0, 5.0)	7, 13, 14	1, 4, 6, 7, 9, 13, 14
9	77.9, C			
10	45.1, CH_2	a: 2.69, dd (13.0, 9.5)	3, 10b	3, 4, 1′, 2′/6′
		b: 3.04–2.93, m	3, 10a	3, 4, 1′, 2′/6′
11	14.2, CH_3	1.11, d (7.0)	5	4, 5, 6
12	21.1, CH_3	1.33, br. s	7	5, 6, 7
13	127.2, CH	5.75–5.69, m	8, 14, 15a, 15b	7, 8, 9, 14, 15
14	135.1, CH	5.74 ddd (15.5, 9.0, 6.8)	8, 13, 15a, 15b	8, 13
15	37.4, CH_2	a: 2.24–2.16, m	8, 14, 15b, 16	13, 14, 16, 17, 20
		b: 2.25, dddd (14.1, 7.0, 6.8, 1.7)	13, 14, 15a, 16	13, 14, 17, 20
16	36.0, CH	2.07, dtd (8.6, 6.8, 6.8, 2.0)	15a, 15b, 17, 20	14, 15, 20
17	78.6, CH	4.17, d (2.0)	16	15, 16, 18, 20
18	210.5, C			
19	25.7, CH_3	2.15, s		17, 18
20	13.0, CH_3	0.71, d (6.8)	16	15, 16, 17
1′	137.4, C			
2′/6′	129.2, CH	7.18–7.16, m	3′,5′	10, 3′/5′, 4′
3′/5′	129.2, CH	7.33, dt (7.5, 1.8)	2′, 6′	1′, 2′/6′
4′	127.4, CH	7.30–7.28, m	3′,5′	2′/6′

[a] HMBC correlations are from proton (s) stated to the indicated carbon.

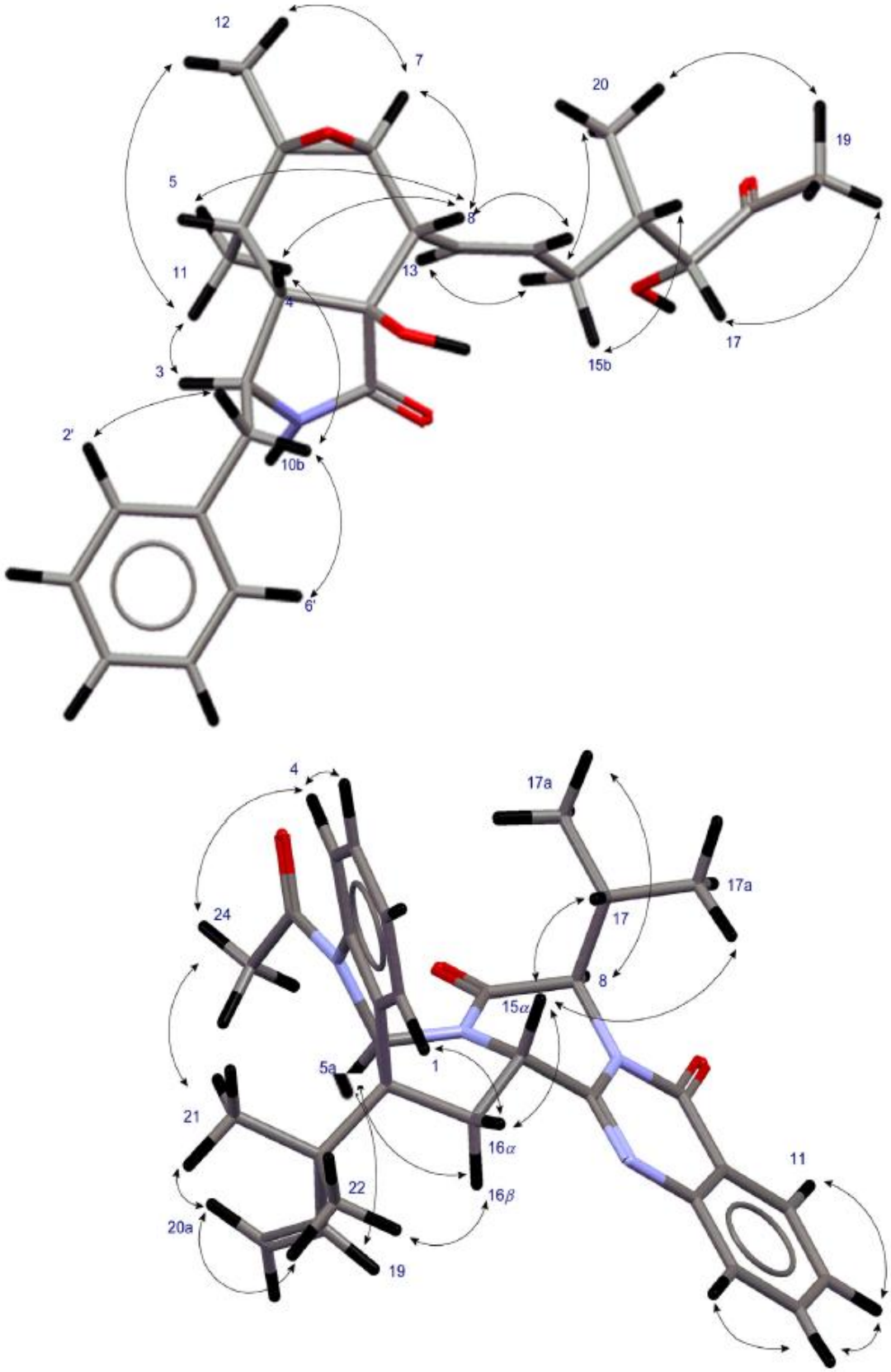

Fig. 3. Main NOESY correlations of the cytochalasin Z_{15E} (**1**) and 5-*N*-acetyl-8-β-isopropyl-ardeemin (**4**).

4, H-3 and C-1/C-4/C-5/C-9/C-1′, H-5 and C-3/C-4/C-6/C-11/C-12, H-7 and C-6/C-8/C-9/C-12, and H-8 and C-9/C-1 (Fig. 2). Comparison between the NMR data of compound **1** and those reported to Z_{21} showed R^2 values ($\alpha = 0.05$) very significant for both δ_C (0.999) and δ_H (0.998).

The ^{1}H NMR spectrum of **1** also showed signals at δ_H 5.74 and 5.73–5.69, attributed to a double bond. The double bond was confirmed by HSQC analysis, which showed two corresponding methine signals at δ_C 135.1 and 127.2, respectively. The ^{1}H NMR spectrum of **1** was analyzed using TopSpin 3.2 and PERCH NMR software, which enabled the recovery of information with respect to the spin coupling constants related to H-14, the signal of which was partially overlapped by that of H-13. The coupling constants measured between H-14 and H-13 ($J = 15.5$ Hz), H-15a ($J = 9.0$ Hz), H-15b ($J = 6.8$ Hz) allowed for the assignment of the signal at δ_H 5.74 (1H, ddd, $J = 15.5, 9.0, 6.8$ Hz, H-14). Thus, the geometry of the double bond between C-13 and C-14 is likely *trans*, attributed to the coupling constant value ($J_{13-14} = 15.5$ Hz) (Fig. 2). The NOESY correlations corroborate this assignment because the H-14 signal is correlated with H-7 and H-8 (Fig. 3), while the H-13/H-14 cross peak was not recorded.

Besides the 10-phenylsubstituted perhydroisoindol-1-one skeleton, the 1D and 2D NMR indicated that **1** has an open 8-carbon chain system rather than a macrocyclic ring (Fig. 1, Table 1). In the COSY spectrum, cross peaks were observed between H-8 and H-13/H-14, H-14 and H-15a, H-15a and H-14/H-15b, H-15b and H-13/H-14/H-16, and H-16 and H-17/H-20. This third spin system was also deduced by the HMBC spectrum (Fig. 2), confirmed by the correlation between H-13 and C-14, as well as the correlations of both H-15a and H-15b with C-13/C-14/C-17/C-20 and H-16 with C-14/C-15/C-20. In addition, in the HMBC contour map, correlations were observed between H-17 and C-15/C-16/C-18/C-20; finally, H-19 exhibited cross peaks with C-17 and C-18. On the other hand, the comparison of the 1D NMR spectra of **1** with that of cytochalasin Z_{20} (Lin et al., 2009) indicated that these compounds are very similar, except for the position of the carbonyl and hydroxyl in the 8-carbon chain system. The main differences in the ^{13}C NMR of **1** were observed at C-16 to C-20 (C-16: δ_C 36.0; C-17: δ_C 78.6; C-18: δ_C 210.5; C-19: δ_C 25.7; and C-20: δ_C 13.0) (Table 1). The chemical shifts of C-17 (δ_C 78.6) and CH_3-20 (δ_C 13.0) in **1** were downfield and upfield, respectively, considering the same carbons at cytochalasin Z_{20} (C-17:δ_C 73.2 and CH_3-20: δ_C 17.2) (Lin et al., 2009).

The magnitude of the coupling constant corresponding to the signal at δ_H 2.75 ($J_{8,7} = 5.0$ Hz, H-8) reveal that H-8 and H-7 have dihedral angles of approximately 53°, indicating α-equatorial and β-axial location of H-7 and H-8, respectively (Smith and Barfield, 1993). The relative configuration of the stereocenters in the isoindolone moiety of **1** was deduced from the NOESY correlations of H-5 and H-8 and between H-4 and H-8, which indicated that H-4, H-5, and H-8 are on the same face of the molecule and assigned to have β-orientation. In addition, all cytochalasins isolated thus far have the same configuration of cyclohexane and isoindole moieties (Lin et al., 2009). Therefore, the 1D and 2D NMR data comparison, the results of NOESY analysis and the biogenetic origin allowed to assign the same relative configuration previously found in Z_{20} and Z_{21} to the epoxyperhydroisoindol 6,7-1-1-one moiety of **1**.

The relative configurations of the stereogenic centers at C-16 and C-17 in the open 8-carbon chain in **1** were proposed on the basis of the correlations between H-17 (δ_H 4.17) and H-16 (δ_H 2.07) and H-15b (δ_H 2.25) in the NOESY spectrum, indicative of an *anti* relationship between H-17 and H-20. This assumption was confirmed by the HMBC correlation of a δ_H of 2.07 and a δ_C of 13.0 ($^3J_{C\text{-}20,\ H\text{-}17}$), as well as the non-detection of cross peak of the coupling constant $^2J_{C\text{-}17,\ H\text{-}16}$, suggesting that H-16 and the hydroxyl at C-17 are in *anti* arrangement. Furthermore, the absence of correlation between H-17 (δ_H 4.17) and H-20 (δ_H 0.71) confirmed this relative orientation, and the peak between CH_3-20 (δ_H 0.71) and H-15a (δ_H 2.16–2.24) in the NOESY spectrum indicated that these two protons are on the same side.

In addition, the relative stereochemistry of the fragment between C-15 and C-18 of **1** was assigned on the basis of the homonuclear and heteronuclear coupling constant values obtained by HMBC and ^{1}H NMR spectral analysis. The molecular modeling system (MMS) of PERCH was utilized for the molecular modeling of **1**, and the ^{13}C and ^{1}H NMR spectra were predicted using PERCH-NMR software. Table 2 shows the experimental and estimated values of the coupling constants $^{2,3}J_{C,H}$ and $^3J_{H,H}$ for the 1-methyl-2-hydroxy system relative to C-16/C-17, which were calculated using the measurement and estimation from ^{1}H NMR spectra and phase-sensitive HMBC. The data indicated that the fragment exhibits a majority of the *threo*-1,2-methyl-hydroxyl configuration (Matsumori et al., 1999; Bifulco et al., 2007). Thus, the configuration of the stereogenic centers at C-16 and C-17 is presumed to be *S* and *R*, respectively, according to open-chain cytochalasins Z_{15} (Liu et al., 2008), and on the basis of biogenetic origin, all cytochalasins isolated thus far have the same absolute configuration in the 10-phenylsubstituted perhydroisoindol-1-one skeleton and at C-16 of the macrocyclic ring moieties (Lin et al., 2009; Liu et al., 2008). Thus, the structure of **1** is tentatively established as (13*E*)-(3*S*,4*S*,5*S*,6*R*,7*S*,16*R*,17*R*)-6,7-epoxy-16-methyl-17-hydroxy-10-phenyl-[14]cytochalasa-13-ene-1,18-dione, namely cytochalasin Z_{15E}.

Compound **4** was isolated as a colorless amorphous solid. Its UV spectrum exhibited λ_{max} at 206, 228, 270, and 305 nm. The molecular formula was $C_{30}H_{32}N_4O_3$, as determined by HR-ESI–MS (m/z 497.2553, $[M+H]^+$; calc. for $C_{30}H_{33}N_4O_3$: 497.2547). The COSY experiment indicated the presence of two aromatic spin systems with coupled protons at δ_H of 7.92 (H-4), 7.54 (H-1), 7.37 (H-3), and 7.26 (H-2), and another one at a δ_H of 8.20 (H-11), 7.83 (H-13), 7.71 (H-14), and 7.52 (H-12). 1D and 2D NMR analysis of **4** indicated that it has a structure similar to that of ardeemin derivatives (Hochlowski et al., 1993). The molecular formula was assigned as $C_{30}H_{32}N_4O_3$, implying17° of unsaturation. The unsaturations were accommodated such as 5-*N*-acetylardeemin: eight in the two substituted benzene rings, two in carbonyl functions, one in the olefin of the isoprenoid unit, one in amidine function, and four in the other four rings (Hochlowski et al., 1993). The only difference was the presence of an isopropyl moiety (δ_C 32.6/δ_H 1.97, C-17/CH;

Table 2
Estimated and calculated $^{2,3}J_{C,H}$ e $^3J_{H,H}$ values for the C16-C17 segment in **1**.

$^{2,3}J_{XH}$	Estimated (Hz)[b]	Calculated (Hz)[c]	Magnitude[d]
$^3J_{H\text{-}16,H\text{-}17}$	1.8	1.3	Small
$^3J_{C\text{-}15,H\text{-}17}$	3.9	1.4	Medium
$^3J_{C\text{-}18,H\text{-}16}$	1.0	1.2	Small
$^3J_{C\text{-}20,H\text{-}17}$	6.4	7.2	Large
$^2J_{C\text{-}17,H\text{-}16}$	<−0.5	−0.5[a]	Small

[a] Empirical value based on the non-detected correlation $^2J_{C17,H16}$ in the HMBC spectrum. Experimentally, this value ranges from 0 to −2 Hz.

[b] $^3J_{H,H}$ values were extracted from the ^{1}H NMR spectrum. The $^{2,3}J_{C,H}$ values were estimated by the PS-HMBC spectrum through the rationalization of the relative intensities of the cross peaks by the equation $I_{Ca,H}/I_{Cb,H} = \sin^2(\Delta\pi J_{Ca,H})/\sin^2(\Delta\pi J_{Cb,H})$, where $I_{Ca,H}$ and $I_{Cb,H}$ are the cross peak intensity, attributed to H-Ca and H-Cb couplings, respectively, whereas Δ represents the delay of long-range proton-carbon coupling evolution of the HMBC experiment.

[c] Values calculated by Karplus-type equations. The dihedral angles of **1** were calculated by a molecular modeling system (MMS) with 3D molecular models having energetic optimization by Merck molecular force field (MMFF94).

[d] Systems 1-methyl-2-hydroxy, the magnitude of coupling constant values, in Hz, are estimated as follows: Small = [$(1 < {}^3J_{H,H} < 4)$, $(1 < {}^3J_{C,H} < 3)$, $(0 < {}^2J_{C,H} < -2)$]; large = [$(8 < {}^3J_{H,H} < 11)$, $(6 < {}^3J_{C,H} < 8)$, $(-5 < {}^2J_{C,H} < -7)$].

δ_C 20.3/δ_H 0.88; C-17b/CH_3; and δ_C 18.9/δ_H 0.85, C-17a/CH_3) in **4** instead of a methyl group in position 8, as previously reported for 5-*N*-acetylardeemin (Zhang et al., 2014; Zhang et al., 2010; Hochlowski et al., 1993). Furthermore, the HMBC experiment indicated cross peaks between δ_H 5.10 (H-8) and δ_C 32.6 (C-17), δ_C 18.9 (C-17a), and δ_C 20.3 (C-17b), which permitted the confirmation of this connectivity. Based on NOESY correlations (δ_H 5.10, H-8 and δ_H 1.97, H-17) (Fig. 3, Table 3) and the coupling constant of vicinal protons J_{8-17} (8.4 Hz), **4** was elucidated as (7*R*,9a*S*,14b*R*,15a*R*)-10-acetyl-10,14b,15,15a-tetrahydro-7-isopropyl-14b-(2-methylbut-3-en-2 yl)indolo[3″,2″:4′,5′]pyrrolo [2′,1′:3,4]pyrazino[2,1-*b*]quinazoline-5,8(7H,9aH)-dione, namely 5-*N*-acetyl-8-β-isopropyl-ardeemin.

The crude CH_2Cl_2 extract from *A. felis* exhibited an MIC value of 1.9 μg/mL against *P. brasiliensis*. All isolated compounds were tested by the same assay for evaluating their antifungal activities. Cytochalasin E exhibited the highest activity with an MIC of 1.8 μg/mL (3.6 μM), followed by **4** (MIC = 62.5 μg/mL = 125.9 μM), **2** (MIC = 62.5 μg/mL = 134.8 μM), and **6** (MIC = 62.5 μg/mL = 144.9 μM) (Table 4). Cytochalasin E and rosellichalasin have been both isolated from *Rosellinia necatrix* (Kimura et al., 1989), *Aspergillus flavipes* (Lin et al., 2009), *Aspergillus terreus* (Ge et al., 2010; Zhang et al., 2010), *Aspergillus* sp. nov. F1 (Xiao et al., 2013), and *Xylaria* sp. (Zhang et al., 2015); their structures primarily differ in the side chains at carbons 18–21. According to Xiao et al. (2013), both cytochalasin E and rosellichalasin exhibit cytotoxic activity against A549, Hela, BEL7402, and RKO cells with IC_{50} values varying from 37.3–78.5 μM. However, only cytochalasin E exhibits activity against HepG2 and CaSki cancer cells with IC_{50} values of 25 μM and 29 μM, respectively (Zhang et al., 2015).

In our assays, only cytochalasin E exhibited outstanding antifungal activity against *P. brasiliensis*. Cytochalasin E has been previously reported to be the antifungal component in *Xylaria sp.* XC-16, exhibiting activity against *Alternaria solani* (MIC of 50 μM), *Botrytis cinerea*, and *Gibberella saubinetti* (MICs of 100 μM) (Zhang et al., 2014).

3. Experimental

3.1. General

UV spectra were measured on a SpectraMax® M5 multi-mode microplate reader (Molecular Devices, California, USA). Optical rotation was recorded using an Anton Paar MCP 300 polarimeter. 1D and 2D NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer using TMS as the internal standard. High-resolution mass spectra were recorded on a Bruker ETD-maXis quadrupole TOF (Bruker Daltonics, Bremen, Germany) coupled to a Thermo Surveyor Plus HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Finnigan PDA Surveyor Plus diode-array detector. Analyses were conducted on a reverse-phase column (Atlantis C_{18}, Waters, USA, particle diameter of 3 μm, 150 mm × 2.1 mm i.d.) using a linear gradient of ACN:H_2O from 10 to 100% in 12.5 min. The following conditions were utilized: end plate offset, −500 V; capillary voltage, 4500 V; nebulizer pressure, 0.4 bar; dry gas (nitrogen) flow rate, 4.0 L/min; dry temperature, 180 °C; ISCID energy, 25 eV; collision energy, 7 eV; ion cooler RF, 25 Vpp; transfer time, 45–49 μs; and mass range, 50–1500 Da. Analytical HPLC was performed on a Shim-pack® C18 column (5 μm, 4.6 mm × 250 mm, i.d.) using a system (Shimadzu, Kyoto, Japan) equipped with an LC10AD pump and an SPD M-10A VP diode array detector. Semi-prep. purification was conducted using an HPLC system (Shimadzu, Kyoto, Japan) equipped with two LC6AD pumps and an SPD-10A-UV detector. TLC analyses were

Table 3
NMR spectroscopic data for 5-*N*-Acetyl-8-β-isopropyl-ardeemin (**4**) (at 400 MHz for ^1H and 100 MHz for ^{13}C).

	4 in CD_3OH			
Position	δ_C, type	δ_H (*J* in Hz)	COSY	HMBC[a]
1	126.4, CH	7.54, dd (8.0, 1.5)	2	3, 4, 4a, 16a
2	126.2, CH	7.26, td (7.5, 1.5)	1, 3	4, 4a
3	130.1, CH	7.37, td (7.5, 1.5)	2, 4	2, 4, 4a
4	120.3, CH	7.92, br. d (7.5)	3	
4a	144.0, C			
5a	80.9, N—H	6.13, br. s.		
7	166.8, C			
8	63.9, CH	5.10, d (8.4)	17	7, 10, 15a, 17, 17a, 17b
10	162.2, C			
10a	121.5, C			
11	127.8, CH	8.20, dd (8.5, 1.5)	12, 13	10, 12, 13, 14a
12	128.5, CH	7.52, dd (7.5, 1.5)	11, 13	10, 14a
13	136.1, CH	7.83, ddd (8.5, 7.0, 1.5)	12, 14	10a, 11, 14a
14	128.3, CH	7.71, br. d (8.5)	12, 13	10, 10a, 12, 14a
14a	148.6, C			
15a	153.0, C			
15b	60.2, CH	4.53, dd (10.5, 6.0)	16α, 16β	14a, 15a, 16
16	37.4, CH_2	β: 2.77, dd (13.0, 10.5)	15b, 16α	15a, 15a, 18
		α: 3.08, dd (13.0, 6.0)	15b, 16β	5a, 15b, 16b
16a	62.4, C			
16b	136.1, C			
17	32.6, CH	1.97, dquin (8.4, 6.8 × 4)	8, 17a	7, 8, 17a, 17b
17a	18.9, CH_3	0.85, d (6.8)	17	8, 17, 17b
17b	20.3, CH_3	0.88, d (6.8)		8, 17a
18	41.4, C			
19	144.9, CH	5.87, dd (17.4, 10.8)	20a	16a, 18, 22
20	114.7, CH_2	a: 5.14, br. d (17.4)	19, 20b	18, 19
		b: 5.08, d (10.8, 0.9)	20a	18, 19
21	22.8, CH_3	1.22, s	22	16a, 18, 19, 20, 22
22	23.8, CH_3	1.05, s	21	16a, 18, 19, 20, 21
23	172.5, C			
24	23.9, N—H	2.66, br. s.		23

[a] HMBC correlations are from proton (s) stated to the indicated carbon.

Table 4
MIC values of isolated compounds from *Aspergillus felis* against *Paracoccidioides brasiliensis* (Pb18) using microdiluition broth assay.

Sample	Pb 18 μg/ml
Crude extract	1.9
Cytochalasin Z_{15E} (**1**)	250.0
Rosellichalasin (**2**)	62.5
Cytochalasin E (**3**)	1.8
5-*N*-Acetyl-8-*β*-isopropyl-ardeemin (**4**)	62.5
Gancidin (**5**)	>250.0
Pseurotin A1 (**6**)	62.5
2,4-Dihydroxyacetophenone (**7**)	250.0
Itraconazol	0.001
Amphotericin	0.06

conducted on pre-coated silica gel G-60/F254 plates (0.25 mm, Merck, Darmstadt, Germany).

3.2. Isolation of fungal material

The fungus *A. felis* (UFMGCB 8030) was isolated from rocks collected from the Atacama Desert, Chile. It was preserved in sterile distilled water (Castellani, 1967) in the Collection of Microorganisms and Cells at the "Universidade Federal de Minas Gerais," Brazil.

3.3. Identification of fungus

The fungus UFMGCB 8030 was identified on the basis of the analysis of ITS, β-tubulin, and RPB2 gene regions. The nucleotide sequence of ITS of UFMGCB 8030 indicated 100% of query coverage and 100% similarity with a sequence of *A. felis* (GenBank code KF558318). In addition, β-tubulin sequences indicated 84% of query coverage and 99% similarity with a sequence of *A. felis* (GenBank code KJ914694), while RPB2 nucleotide sequences indicated 100% of query coverage and 98% similarity with the sequence of *A. felis* (GenBank code KJ914735). The fungus UFMGCB 8030 was identified as *A. felis* (Gonçalves et al., 2016; Mendes et al., 2016). The sequences were deposited in GenBank under accession codes KX098586 (ITS) and KX229737 (β-tubulin).

3.4. Fermentation and extraction

The fungus *A. felis* was grown under static conditions at 25 °C for 15 days in 600 Petri dishes (90 mm diameter) containing 20 mL of potato dextrose agar composed of dextrose (2% w/v), potato infusion (30% w/v), and agar (2% w/v). The culture was extracted two times with CH_2Cl_2 for 48 h. The suspension was filtered using filter paper, and the organic phases were concentrated on a rotary evaporator. The residual solvent was dried by vacuum centrifugation at 40 °C overnight, affording the CH_2Cl_2 extract (5.8 g).

3.5. Purification and isolation

Next, the CH_2Cl_2 extract (5.8 g) was subjected to column chromatography (CC) using a silica-gel (230–400 mesh) open column using a gradient of solvents with increasing polarity: Hex, Hex/CH_2Cl_2 (1:1, v/v), CH_2Cl_2, CH_2Cl_2:EtOAc (1:1–7:3, v/v), EtOAc, EtOAc/MeOH (1:1, v/v), and finally with MeOH, affording 12 fractions (F1–12). Fraction 6 (350 mg) was purified by semi-prep. RP-HPLC (220 and 254 nm, Shim-pack® C18, 5 μm, 20 × 250 mm, i.d., 7 mL/min) using a gradient of ACN in H_2O (10–100% over 50 min) followed by 100% ACN for 20 min, affording **1** (20 mg), **4** (6 mg), **5** (15 mg), and **6** (4 mg). Fraction 9 (162 mg) was subjected to semi-prep. RP-HPLC (220 and 254 nm, Shim-pack® C18, 5 μm, 20 × 250 mm, i.d., 7 mL/min) using a gradient of ACN in H_2O, 10–80% over 60 min, followed by 80–100% of ACN for 4 min, and 100% of ACN for 10 min, affording **2** (9 mg), **3**, (11 mg), and **7** (8 mg).

3.5.1. Compound *1*

Cytochalasin Z_{15E}, (13*E*)-(3*S*,4*S*,5*S*, 6*R*,7*S*,16*R*,17*R*)-6,7-epoxy-16-methyl-17-hydroxy-10-phenyl-[14]cytochalasa-13-ene-1,18-dione: 20 mg; colorless oil; $[\alpha]_D^{25}$ +30.34° (*c* 0.78, MeOH); UV (MeOH) λ_{max} (log ε) 204 (3.65), 256 (2.66); for ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRMS *m/z* 428.2433, $[M+H]^+$ (calc. for $C_{25}H_{34}NO_5$: 428.2431).

3.5.2. Compound *4*

5-N-acetyl-8-β-isopropyl-ardeemin, (7*R*,9a*S*,14b*R*,15a*R*)-10-acetyl-10,14b,15,15a-tetrahydro-7-isopropyl-14b-(2-methylbut-3-en-2 yl)indolo[3″,2″:4′,5′]pyrrolo[2′,1′:3,4]pyrazino[2,1-*b*]quinazoline-5,8(7H,9aH)-dione: 6 mg; colorless amorphous solid; $[\alpha]_D^{25}$ −31.67° (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.41), 228 (4.24), 270 (3.79), and 305 (3.42) nm; for ^1H and ^{13}C NMR spectroscopic data, see Table 3; HRMS *m/z* 497.2553, $[M+H]^+$ (calc. for $C_{30}H_{32}N_4O_3$: 497.2547).

3.6. Antifungal assay

The antifungal activity of the samples was evaluated against a *P. brasiliensis* (Pb18) isolate belonging to the phylogenetic species S1 by the broth microdilution susceptibility test. The Pb18 isolate was maintained by weekly transfer in solid YPD medium (peptone, yeast extract, and dextrose) at 37 °C. The isolated Pb18 cells were suspended in sterile saline, and the transmittance of the supernatant at 530 nm was adjusted to 70% ($1 \times 10^6 - 5 \times 10^6$ cells/mL). The cell suspension was diluted in 1:10 RPMI 1640 broth plus 3-(*N*-morpholino)propanesulfonic acid buffer to the final inoculum of $1 \times 10^5 - 5 \times 10^5$ cells/mL (Cruz et al., 2012; Clinical Laboratory Standards Institute, 2008). Samples were diluted serially using RPMI 1640 broth at concentrations ranging from 500.0 to 0.9 μg/mL for obtaining minimal inhibitory concentrations (MICs). Itraconazol (0.5–0.0005 μg/mL) and amphotericin (0.5–0.0005 μg/mL) were used as positive controls. MIC, expressed in μg/mL, was defined as the lowest concentration at which 100% inhibition of the growth of isolated Pb 18 could be achieved. Results were obtained in three independent experiments performed in duplicate.

3.7. Computational simulations

The intensities of the long-distance correlations $^{2,3}J_{H,C}$ and $^3J_{HH}$ were measured by a spectrum obtained by a standard experiment, obtained from a program hmbcetgpl3nd pulses (Cicero et al., 2001), using a BRUKER spectrometer of 400 MHz. $CDCl_3$ was used as the solvent. Phase-sensitive HMBC using an echo/antiecho gradient selection was used with a three-fold low-pass *J*-filter for suppressing one-bond correlations as well as decoupling during acquisition. This experiment was processed to obtain a phase-sensitive ^1H–^{13}C long-range correlation spectrum (PS-HMBC) using TOPSPIN software (version 3.2). This pulse program was chosen because the echo-anti-echo detection mode results in high sensitivity, in addition to the 3rd order low-pass filter, which is more efficient at eliminating ^1J artifacts. The *J* values in this parameter set were selected for optimizing ^2J-^3J correlations. HMBC experiments were recorded at frequencies of 400.15 MHz (^1H) and 100.62 (^{13}C) with a delay Δ set at 62.5 ms with a size date of 2048 (F2) × 256 (F1) points, total number of scans set at 16, spectral widths of 3422.99 Hz (^1H) and 22299.63 Hz (^{13}C), and the spectral resolution of 1.672196 (F2) and 21.798269 (F1). The data were processed using sine (t_2) and a sine-bell squared (t_1) window functions.

Acknowledgments

We acknowledge the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) for financial support and scholarship. This study was also supported by the Program of Technological Development in Tools for Health-PDTIS-FIOCRUZ analytical platform (RPT13A) for NMR experiments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.06.006.

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4.3- Capítulo 3- Antifungal activity of cytochalasin E and its action in different cell structures of *Paracoccidioides brasiliensis*

Graziele Mendes, Ludmila de Matos Baltazar, Nívea Pereira de Sá, Luiz Henrique Rosa, Carlos Augusto Rosa, Elaine M Souza-Fagundes, Betania Barros Cota, Susana Johann.

Neste trabalho foram avaliados o tratamento da citocalasina E, isolada do fungo *Aspergilus fellis*, nas células leveduriformes de *P. brasiliensis* Pb 18. Os efeitos na morfologia e nas estruturas celulares (membrana e parede celulares) foram avaliados por análise da microscopia: óptica e de fluorescência, e também da eletrônica de varredura e de transmissão e pelo ensaio de perda de componentes celulares. Além disso foram avaliados a capacidade da citocalasina E em induzir a produção de espécies reativas de oxigênio (EROs) e de interferir na síntese do ergosterol da membrana fúngica. Os nossos resultados mostraram que a citocalasina E não apresentou citotoxicidade nas células de mamíferos estudadas (IC50 > 500 μg/mL) e mostrou seletividade para as células fúngicas. Além disso mostrou-se fungicida em concentração maior (7,2 μM) que a concentração inibitória mínima (3,6 μM). As células submetidas à concentração sub-inibitória (1,8 μM) induziram a produção de espécies de oxigênio reativas (EROs), bem como, provocou danos de membrana celular e de parede fúngica, além de aumento do número e tamanho dos vacúolos. As leveduras expostas a concentrações abaixo da sub-inibitória (0,9 μM) apresentaram impedimento da liberação dos brotos a partir das células mães. Sugerimos que a ação na membrana plasmática do fungo possivelmente ocorreu pela indução de EROs. Já os danos na parede celular podem ser resultado do aumento de pressão intracelular ou por efeito direto das EROs nos polissacarídeos que constituem a camada mais externa da parede celular.

O objetivo geral deste trabalho foi estudar os possíveis efeitos do tratamento das células leveduriformes do isolado Pb 18 de *P. brasiliensis* com citocalasina E através dos seguintes objetivos específicos:

- Determinar a atividade fungicida e o índice de seletividade;
- Avaliar a citotoxicidade em macrófagos murinos;
- Caracterizar as possíveis mudanças morfológicas ocorridas pelo tratamento com a citocalasina E através de microscopia ótica, eletrônica de varredura e de transmissão;
- Avaliar se a substância é capaz de atuar em algum componente da parede celular por microscopia de fluorecência;
- Estudar possíveis efeitos na membrana plasmática através do ensaio de perda de material celular;
- Quantificar o ergosterol presente na membrana celular de *P. brasiliensis* após o tratamento com a citocalasina E a fim de se determinar o efeito desta substsância sobre a síntese do ergosterol;
- Verificar se a citocalasina E é capaz de induzir a produção de espécies reativas de oxigênio.

Antifungal activity of cytochalasin E and its action in different cell structures of *Paracoccidioides brasiliensis*

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ABSTRACT

Paracoccidioides spp. is an etiologic agent of paracoccidioidomycosis (PCM), a systemic endemic mycotic disease in Latin America. Usually, the treatment is long term to allow the control of the clinical manifestations of the mycosis and prevent relapse that are common in PCM. The discovery of new antifungal agents with higher efficacies and fewer side effects is needed in order to increase treatment options for this infection. In this work, the action of cytochalasin E, isolated from the extremophile fungus *Aspergillus felis*, on cells isolated *Paracoccidioides brasiliensis* Pb 18 was acessed. The efects in yeasts caused by treatment with cytochalasin E were determined by microscopy: optical, scanning and transmission electron and fluorescence, assay release of cellular material and production of reactive oxigen species (ROS). Our results showed that this compound did not show cytotoxicity to mammalian cells studied (IC50 > 500/mL) and showed selectivity for

fungal cells. Besides that, the treatment of *P. brasiliensis* cells with cytochalasin E induced production of ROS, acted on fungal cell and wall membrane and, moreover, it caused at low concentrations the preventing the release of buds.

Key words: Cytochalasin E, antifungal activity, cell wall, cell membrane, reactives oxygen species.

INTRODUCTION

Paracoccidioides spp. is an etiologic agent of paracoccidioidomycosis (PCM), a systemic endemic mycotic disease that affects Latin America (Franco et al., 1993). The disease is characterized by a chronic granulomatous inflammation, and patients may present a spectrum of clinical manifestations, ranging from benign and localized to severe and disseminated forms (Franco et al., 1989).

Treatment of patients with PCM is long to allow control of the clinical manifestations of mycosis and to prevent recurrence. It can be made with various antifungal agents such as amphotericin B, sulfamides (sulfadiazine, combination sulfamethoxazole/ trimethoprim) and azoles (cetoconazol, fluconazole, itraconazole) (Shikanai-Yasuda et al., 2006). In mild and moderate forms of the disease, the therapeutic option is itraconazole that can be combined with sulfamethoxazole/ trimethoprim. Patients with severe form of the disease the treatment is made with amphotericin B (Ramos-e-Silva and Scott, 2008).

The discovery of new antifungal agents with higher efficacies and fewer side effects is needed in order to increase treatment options for this infection. In a previous work, our group isolated cytochalasin E as antifungal metabolite against *P. brasiliensis* Pb 18 from *Aspergillus felis* UFMGCB 8030, a fungus isolated from rocks of Atacama desert (Mendes et al, 2016). This compound has activity against fungi of agricultural importance such as *Gibberella saubinetti, Botrytis cinerea* and *Alternaria solani* (Zhang et al., 2014). Although the antifungal activity has been described for citochalasin E, there are no reports about the effects of this compound against fungi medical interest, including *P. brasiliensis*. So, in present study, we intended to characterize the antifungal effects of this compound on *P. brasiliensis* Pb 18 cells.

MATERIALS AND METHODS

Isolation of cytochalasin E

The cytochalasin E (Figure 1) was isolated from dichloromethane extract from *Aspergillus fellis* UFMGCB 8030, a fungus isolated from the rocks collected in the Desert of Atacama (Mendes et al., 2016).

Fungal isolate and inoculum

Antifungal activity of the compounds was evaluated using *P. brasiliensis* Pb18 (Fungi Collection of the Faculty of Medicine of São Paulo University, Brazil). Isolate Pb18 belongs to the cryptic phylogenetic species S1 (Matute et al. 2006) was maintained at the by weekly transfer in solid YPD medium (0.1% peptone, 1% yeast extract, 1% dextrose and 2% agar) at 37ºC. The isolated Pb18 cells were suspended in sterile saline and the transmittance and the transmittance of the suspension at 530 nm was adjusted to 70% (1-5 $\times 10^6$ cells/ml) using a spectrophotometer (SP-22; Biospectro, Brazil). The cell suspension was diluted in a 1:10 RPMI-1640 broth plus 3-(*N*-morpholino)- propanesulfonic acid buffer (Sigma-Aldrich-USA) to the final inoculum of 1-5 $\times 10^5$ cells/ml (Cruz et al. 2012).

Antifungal assay

The determination of Minimal Inhibitory Concentration (MIC) was performed using the microdilution assays (CLSI 2008, Johann et al. 2010). Samples were diluted using RPMI-1640 broth at concentrations ranging from 500.0 to 0.9 μg/ml. DMSO (0.5% v/v) was used as control for toxicity and itracozanole (0.05 - 0.0005 μg/ml), amphotericin B (0.5 – 0.0005 μg/ml) (Sigma-Aldrich) and sulfamethoxazole/trimethoprim (500.0 – 0,9 μg/ml) as susceptibility controls. The 96-well plates were prepared in duplicate and incubated at 37ºC. The MIC value, expressed in μg/ml, was defined as the lowest concentration which completely inhibited the Pb18 growth in 10 days compared to the growth control.

Minimum fungicidal concentration

The minimum fungicidal concentration (MFC) of the cytochalasin E was determined by transferring the entire contents of each well that presents 100% inhibition in the MIC test on Petri plates containing YPD medium. These plates were incubated at 37 ° C for 10 days. MFC is the lowest concentration of the compound at which there is no growth of colonies (REGASINI et al., 2009).

Cytotoxicity in assays with murine macrophages

Peritoneal macrophages were obtained from three female Balb/c mice of 6 weeks old. To this, 2 ml of the 3% (w/v) sodium thioglycolate (3%) were injected intraperitoneally. After 72 hours, the mice were euthanized and dipped in alcohol 70 ° GL for disinfection. In laminar flow, the peritoneal membranes were exposed and 5 ml of RPMI 1640 culture medium without serum (Sigma) were injected into the peritoneal cavity to collect macrophages. This volume was aspirated with a syringe, transferred to 15 ml tube and then centrifuged (10 min, 150 *g*, 4 °C). The cell pellet was suspended in RPMI with 10% fetal bovine serum (FBS) and cell density determined by counting in a Neubauer chamber.

The macrophages (1 x 10^5 cells/well) were pre incubated for 12 hours at 37 °C in incubator with 5% CO_2. After 12 hours of incubation, the culture medium was removed and 100 µL were added of RPMI medium + 10% FBS (fetal bovine serum) containing differents concentrations of cytochalasin E. In each well identified as "blank" are added 100 µL of RPMI + 10% FCS culture medium. After treatment, the cells were incubated for 24 hours at 37 °C in incubator with 5% CO_2. The medium was removed and 100 μl of RPMI medium + 10% FBS containing MTT (0.5 mg/ml) were added. After 4 hours incubation, the supernatant was removed and the formazan crystals were dissolved with isopropanol/HCl solution 0.04M. The absorbance (Abs) of the sample at 570 nm and 670 nm were determined on the SpectraMax M5 microplate reader (Molecular Devices). For the determination of cell death the following calculations was made: 1) Abs570 - Abs670 = Abs; 2) Abs – Abs blank = Abs sample; 3) Average Abs control cells (Abs CC); 4) Viability = 100 x (Abs sample / Abs control cells) and 5) Cell death = 100 – viabilility.

Selectivity index

IC50 data and MIC values were used to calculate the selectivity index (SI) for citochalasin E, as previously proposed by Protopopova et al (2005). The SI was determined by the ratio between the IC50 value obtained in the assays using murine macrophages and the value of MIC of the compound. The values of SI larger than 10 are considered indicative of lack of toxicity and the values below 10 are considered toxic.

Morphological alterations

The cells *P. brasiliensis* Pb18 were treated with ¼ MIC (0.4 μg/ml) and ½ MIC (0.9 μg/ml) concentration of the cytochalasin E in RPMI-1640 medium at ±36ºC for 7 days.

The cells were collected and examined through light microscopy for morphological alterations (Leica DM 750).

Scanning and Transmission Electron Microscopy

P. brasiliensis Pb18 was grown (7 days at ±36°C) in YPD medium containing ½ MIC (0.9 μg/ml). After, the cells were fixed by immersion in a solution containing 2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in phosphate buffer saline (PBS), pH 7.2, for 12h at room temperature. The material was washed with PBS and the cells post-fixed in 1% (w/v) osmium tetroxide (OsO_4) and 0.8% (w/v) potassium ferricianide in sodium cacodylate buffer. The cells were washed to remove excess OsO_4 and dehydrated in a graded acetone series from 30% to 100% (v/v). The samples were mounted on stubs, dried by critical point method, coated with gold-palladium alloy and visualized using a DSM 950 (Zeiss, West Germany) scaning electron microscope. For transmission electron microscopy, followed with post fixation step was carried out in OsO_4 1% (w/v) was followed by the dehydration of the material in a graded acetone series from 30% to 100% (v/v) and embedded in Spurr resin. Semi-thin sections were stained with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed using a EM10 electron microscope (Zeiss, Oberkochen, Germanny) (Santos et al 2007).

Fluorescence microscopy

The yeasts were cultured for 7 days at ±37 °C in YPD containing ½ MIC concentration of cytochalasin E (0.9 μg/ml). The cells were stained with 5 µL of calcofluor white (10.0 mg/ml) and the images were obtained with the fluorescence Nikon Ti Elcipse microscope (Nikon, Melville, NY).

Release of cellular material

The methodology used to quantitate the release of cellular material was previously described by Bennis et al. (2004) with modifications. Aliquots of 5.0 ml of the yeast suspension (10^5 cells/ml) in saline 0.85% (p/v) were used in three treatments groups: 1) control cells suspended in saline; 2) cells treated for 2hours at concentrations equal to the ½ MIC, MIC, 2x MIC and 4x MIC of cytochalasin E or amphotericin B. The cells were centrifuged at 7000 rpm for 5 min, and the absorbance of the supernatant was measured at 260 nm in a UV spectrophotometer (Hitachi U-1100, Tokyo, Japan).

Measurement of ROS production

Endogenous amounts of ROS were measured by fluorometric assay with specific probes. Cells of *P. brasiliensis* Pb 18 (1.0×10^5 cells/ml) were treated with subinhibitory concentration of the cytochalasin E (0.9 μg/ml), itraconazole (5.0×10^{-4} μg/ml), amphotericin B (3.0×10^{-2} μg/ml), sulfamethoxazole/trimethoprim (62.5 μg/ml) or 0.88 mM of hydrogen peroxide in RPMI-1640 phenol red medium free (Sigma-Aldrich, EUA) and incubated with 50 μM of 2′,7′-dichlorofluorescein diacetate (Invitrogen, EUA) for ROS quantification.The fluorescence was measured with Synergy 2 multi-mode microplate reader (BioTek, USA) at a wavelengths of 485 nm (excitation) and 530 nm (emission). The kinetics of ROS production was performed at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 hours. All results were expressed as the means ± standard errors of the mean (SEM) of fluorescence over the control (Baltazar et al 2013).

Ergosterol quantification

Cellular ergosterol was quantified in fungal cell membranes of *P. brasiliensis* Pb 18 as described by Arthington - Skaggs et al (1999) with some modifications. Approximately 25 mg of fungal cell mass were treated with cytochalasin E, amphotericin B or itraconazole in subinhibitory concentrations and incubated in YPD medium for 7 days under agitation at ±36 °C. After incubation, the tubes were centrifuged (Jouan, BR4i model) at 7000 rpm for 10 minutes at 4°C and the supernatants were removed. The cells were washed with sterile distilled water. The cell weighs was calculated. For the extraction of lipids, 3000 ml of ethanolic solution of 25% potassium hydroxide was added to cell mass and agitated for 1 min. The tubes were incubated in a water bath at 85 °C for 1 h and then maintained at room temperature. A mixture of 1 ml of sterile water and 3 ml of n-heptane (Sigma-Aldrich) was added, followed by agitation in vortex for 3 minutes. The supernatant was removed and the reading was performed using a UV/VIS spectrophotometer (Shimadzu, Japan) at 282 and 230 nm. A calibration curve with standard ergosterol (Sigma-Aldrich) was constructed and used to quantification of the ergosterol. The results were expressed as the percentage of ergosterol in camparation with the growth control.

Statistical analysis

Statistical analyses were performed in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) using a One-Way Analysis of Variance

(ANOVA), Kruskal–Wallis and Newman–Keuls multiple comparison tests. The level of significance used was 0.05.

RESULTS

The MIC values of cytochalasin E (figure 1) was 3.6 μM (1.9 μg/ml) against *P. brasiliensis* Pb 18. This value was smaller than sulfamethoxazole/trimethoprim (MIC of 71.5/411.0 μM) and larger than itraconazole (MIC of 0.001 μM) and amphotericin B (MIC of 0.06 μM) (Table 1). The MFC value for cytochalasin E (7.2 μM; 3.8 μg/ml) was twice higher than it MIC value. The IC50 of cytochalasin showed value > 500 μg/ml (964.0 μM) and the SI was 263 to macrophages. These results showed that cytochalasin E was not cytotoxic to mammalian cells tested. In addition it was 263 times less toxic to mammalian cells than for yeast cells.

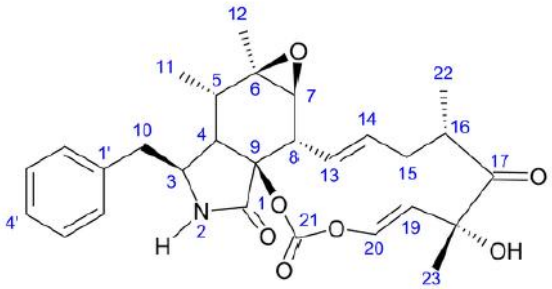

Figure 1. Structure of cytochalasin E from *Aspergillus fellis* UFMGCB 8030 (Mendes et al., 2016)

Table 1: MIC values for cytochalasin E, itraconazole, amphotericin, sulfamethoxazole/trimethoprim against *Paracoccidioides brasiliensis* (Pb18) using microdiluition broth assay.

Compounds	**µg/ml**	**µM**
Cytochalasin E	1.9	3.6
Itraconazole	0.001	0.001
Amphotericin B	0.06	0.06
Sulfamethoxazole/trimethoprim	125.0	71.66/411.29

The optical microscope images (Figure 2) showed that cells treated with cytochalasin E in ¼ MIC (0.4 μg/ml) exhibited difficulty in releasing the buds (Fig 2a-white arrow). The damages at *P. brasiliensis* Pb 18 cells treated with ½ MIC (0.9 μg/ml) were more apparent with release of cellular material (black arrow). The control whitout treatment (Fig 2c) showed that the cells had the morphology spherical appearance, without damages.

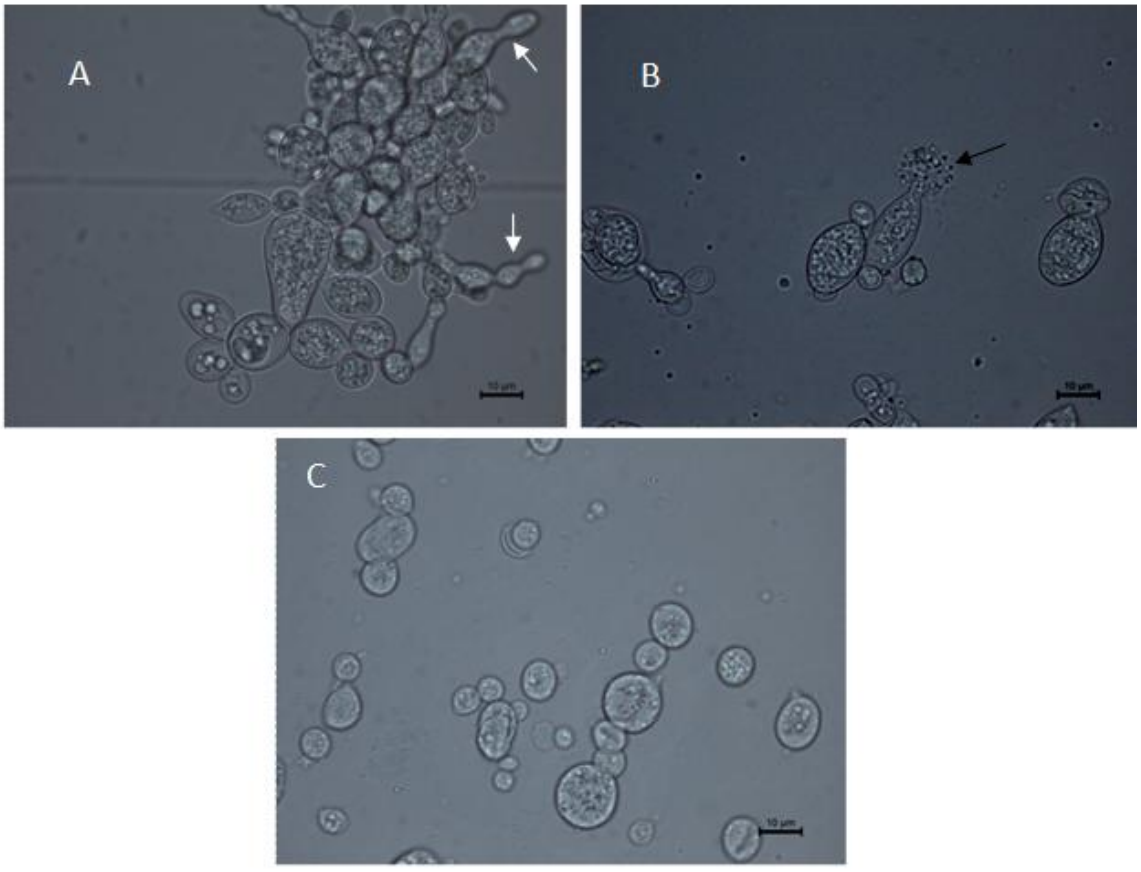

Figure 2: Analysis by optical microscopy (100x) of *Paracoccidiodes brasiliensis* Pb 18 after treatment with ¼ MIC (A) and ½ MIC (B) of cytochalasin E after incubation for 7 days in yeast peptone dextrose medium at 37ºC compared to control cells without treatment (C). Note the presence of cellular content leak (black arrow) and buds connection in parent cells (white arrow).

Ultrastructural analysis by scanning microscopy of *P. brasiliensis* Pb 18 yeast without treatment (figure 3a) was comparable to yeast treatment with ½ MIC of cytochalasin E. The treated cells with cytochalasin E showed crake of cell wall (3b), release cell material (3b), changes in cell shape, depression in their surface and withered cells (3c). Additionally, the treatment with amphotericin B, itraconazole and sulfamethoxazole/trimethoprim, resulted at release of cell material (3d and 3f), cell shape changes (3e), cell surface squashing (3e) and cells with malformation in buds (3e).

Analysis by electron microscopy transmission also showed morphological differences between untreated yeasts (4a and 4f) and treated yeasts with ½ MIC (0.9 μg/ml) of cytochalasin E (4b-4e). There was release of cell material (4b) and changes in cell morphology, such as, fusion plasma membrane with vacuoles, retraction of the cell membrane in some points (4d) and reduction of the outermost layer of the cell wall (4e).

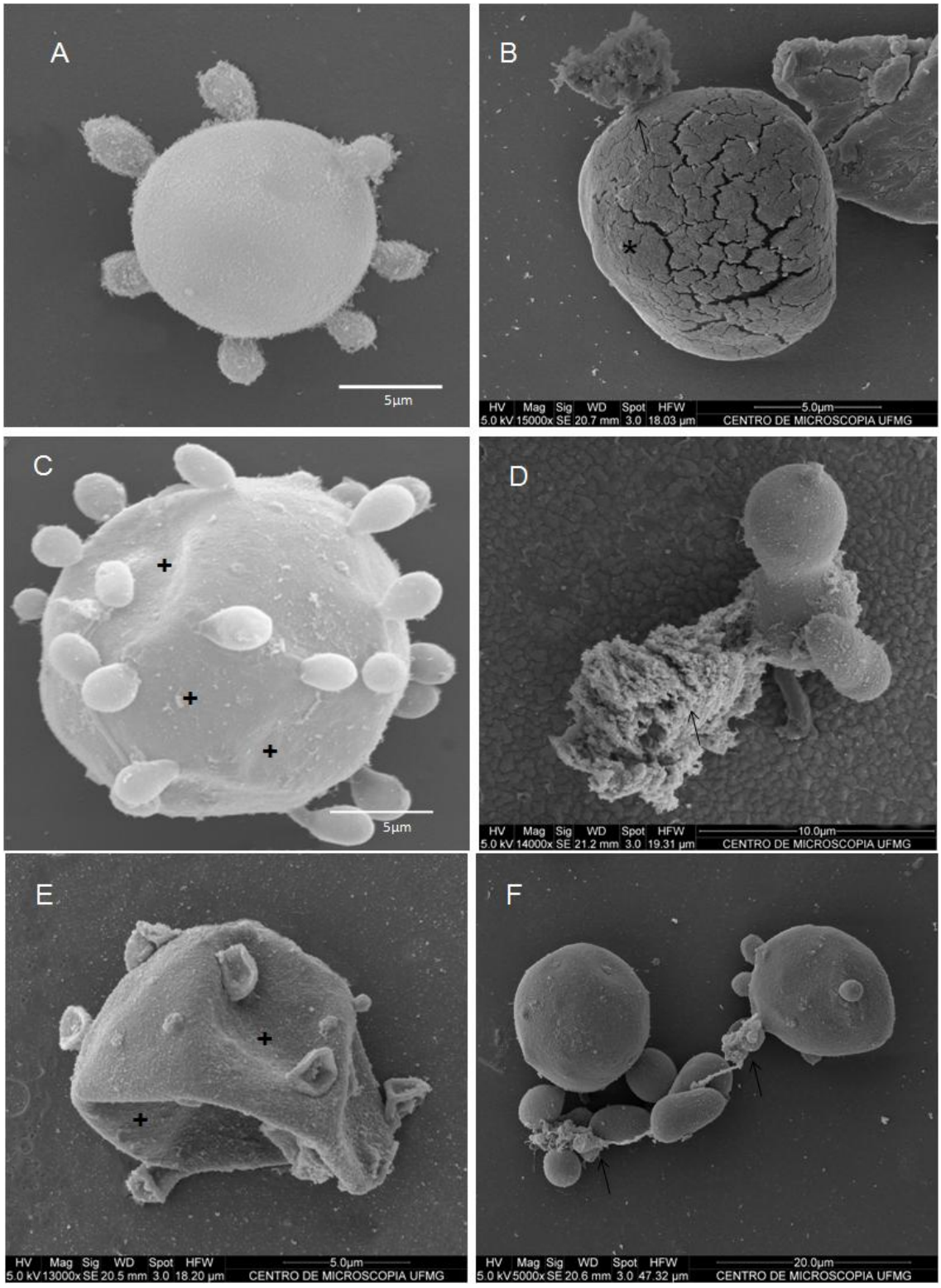

Figure 3: Scanning electron microscopy of *Paracoccidiodes brasiliensis* Pb 18 without treatment (A) and after treatment with ½ MIC of cytochalasin E (B and C), amphotericin B (D), itraconazole (E) and sulfamethoxazole/trimethoprim (F) for 7 days. Note the presence of cracks (*), release of cellular material (black arrow), cell surface squashing and depression (+).

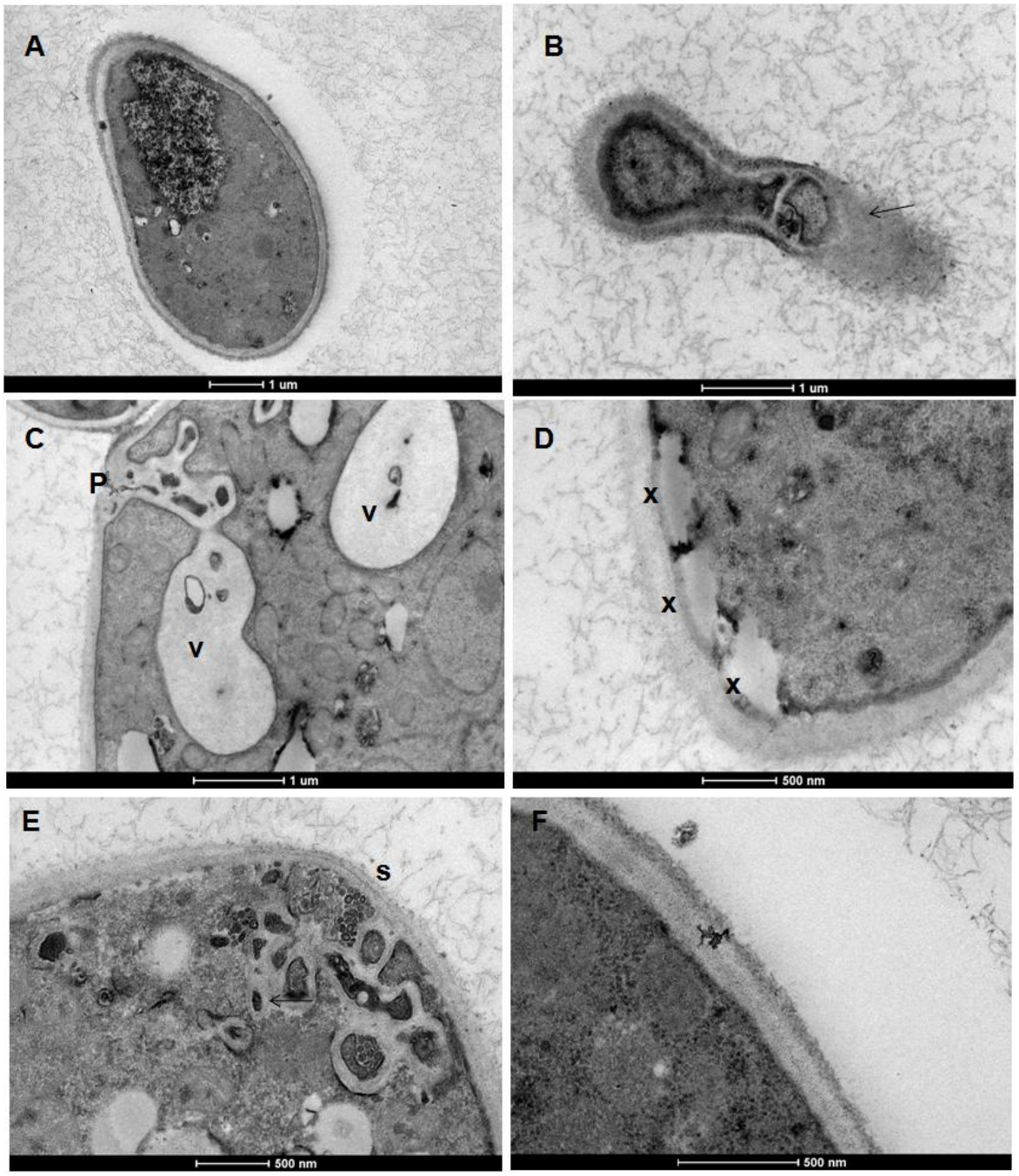

Figure 4: Transmission electron microscopy of *Paracoccidioides brasiliensis* Pb 18 without treatment (A and F) and after treatment with ½ MIC of cytochalasin E (B-E). Note the presence of release of cellular material (black arrow), fusion plasma membrane with vacuoles (p), formation of vacuoles (v), cytoplasm membrane recess (x) and changes in thickness (S).

Fluorescence microscopy of *P. brasiliensis* Pb 18 cells with calcofluor white fluorochrome showed that there was no difference in fluorescence microscopy analyzis of the untreated control (5a) and the treatment with ½ MIC of cytochalasin E (5b). Thus, the changes in the composition and organization of cell walls can be seen by the difference in dye markings.

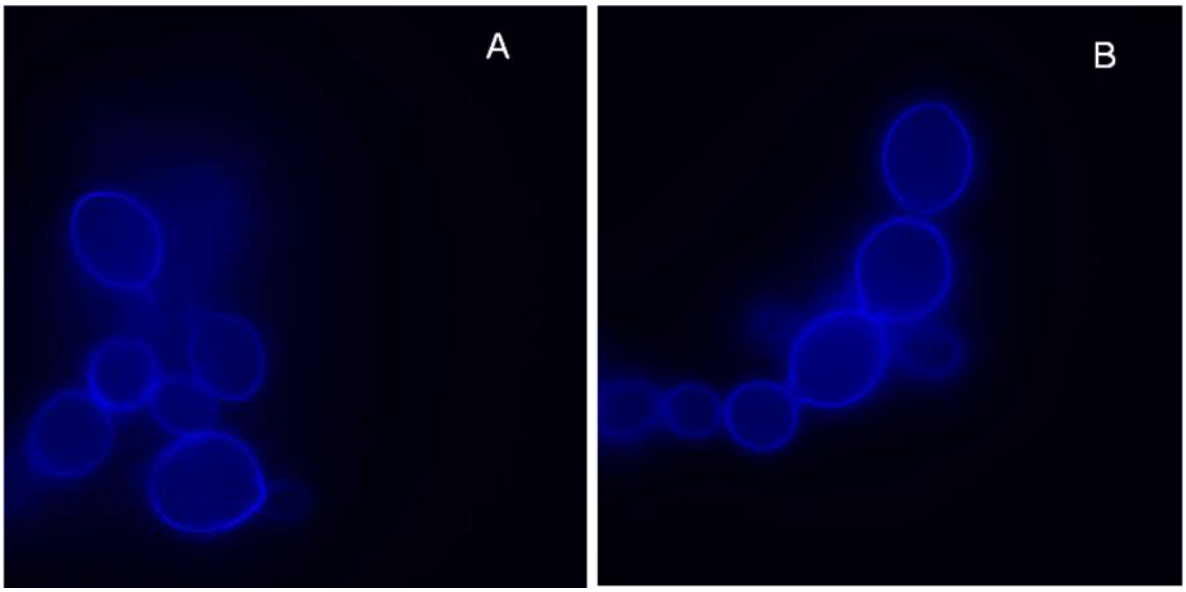

Figure 5: Fluorescence microscopy of *Paracoccidioides brasiliensis* Pb 18 stained by calcofluor white (CFW). Pb 18 control stained with CFW(A); Pb 18 after treatment with ½ MIC of cytochalasin E stained with CFW (B).

Effects of cytochalasin E in membrane cell were available by release of cellular material assay (figure 6). The cells of *P. brasiliensis* Pb 18 treated with ½ MIC of cytochalasin E showed loss of its intracellular constituents detected by absorbance. This effect was also maintained at higher concentrations of the compound (MIC, 2 x MIC and 4 x MIC) and also when the cells were exposed in all concentrations of amphotericin B (1/2 MIC to 4 x MIC). The two types of treatments (cytochalasin E and amphotericin B) at all concentrations tested showed a significant difference comparison to the untreated control ($p < 0.0001$). These results show that cytochalasin E acts on the plasma membrane of cells of *P. brasiliensis* Pb 18.

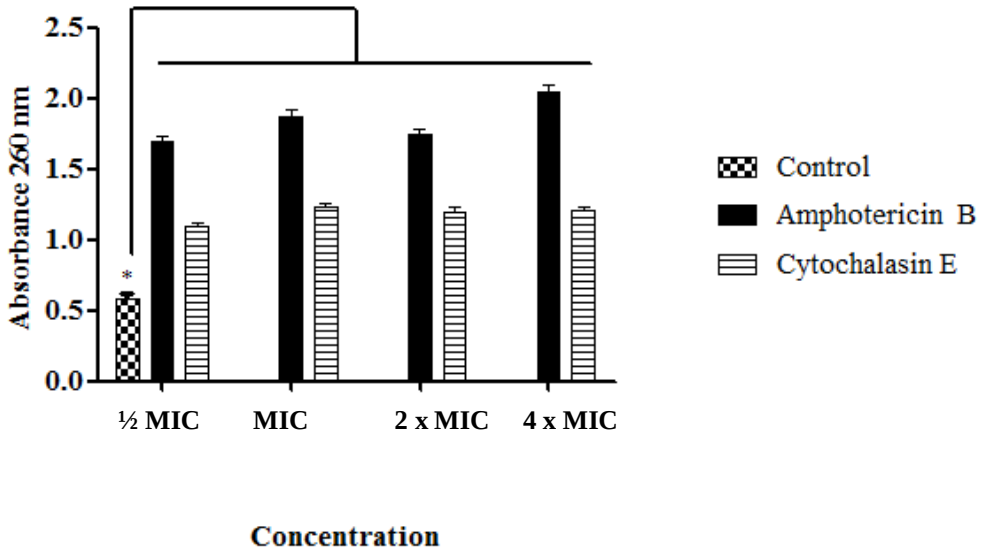

Fig 6: The release of cell material was measured at wavelength of 260 nm from *P. brasiliensis* Pb 18 cells by the action of ½ MIC, MIC, 2 x MIC and 4 x MIC of cytochalasin E.*Statistically significant difference (P < 0,0001). The error bars indicate SEM.

Our results showed that the amount of ergosterol extracted from the untreated cells and treated with ½ MIC of cytochalasin E and amphotericin B were statistically equal. The amount of extracted ergosterol of the cells treated with ½ MIC of itraconazole was statistically lower in comparasion to the other (data not shown).

The treatment of *P. brasiliesis* with cytochalasin E at ½ MIC induced ROS production in cells with significant differences ($p < 0.05$) after 2 hours of incubation when compared to the untreated control. This production had their levels increased up until 3 hours of incubation ($p < 0.05$) (Figure 7). The amphotericin B and itraconazole at ½ MIC showed no significant induction of ROS production compared to the untreated control at all times analised, whereas sulfamethoxazole/trimethoprim showed significantly lower values of ROS after 2 and 3 hours of treatment in comparison to untreated cells.

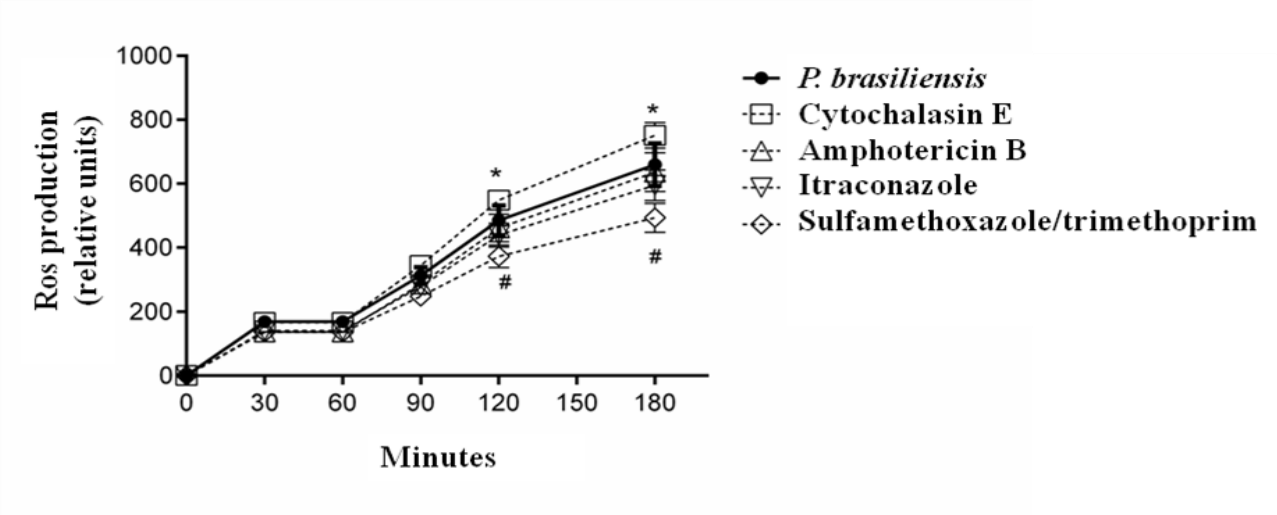

Figure 7: Analysis of the generation of reactive oxygen species (ROS) by *Paracoccidioides brasiliensis* Pb 18 yeast cells after of the treatment with ½ MIC of cytochalasin E, itraconazole, amphotericin B and sulfamethoxazole/trimethoprim.*statistically significant difference in comparison to the control for each drug treatment (P<0,05). The error bars indicate SEM.

DISCUSSION

This study showed the action of cytochalasin E, isolated from *Aspergillus felis*, in yeast of *P. brasiliensis* Pb 18. Our group has shown elsewhere (Mendes et al., 2016) that this compound has antifungal activity.

MIC values obtained for antifungal activity of cytochalasin E varies according to the fungal species tested. It was more active against *P. brasiliensis* Pb 18 (1.9 μM) compared to fungal pathogens *Alternaria solani* (50 μM), *Botrytis cinerea* and *Gibberella saubinetti* (100 μM) (Zhang et al., 2014). Regarding toxicity, cytochalasin E was not toxic to macrophages in tested concentrations. Nevertheless, it may be toxic against other targets such as *Artemia* microcrustacean (LC50 2.79 μM) (Zhang et al., 2014), cancer cell lines P388 (0.093 μM) and A-549 (0.0062 μM) (Liu et al., 2006).

Changes in the structural organization of the yeast cells treated with sub-inhibitory concentrations (½ MIC) of citochalasin E were evaluated with microscopy analysis. Optical microscopy analysis showed that the yeast exhibited leakage of intracellular content that can be explained by the action of the citochalasin E in fungal membrane. This

action was confirmed by the results obtained in release of cellular material assay that yeasts treated with cytochalasin E and amphotericin B showed the detection of their intracellular content in 260 nm. It is known that amphotericin B acts on the plasma membrane of fungi by binding to ergosterol forming a complex which disrupts the cell membrane, resulting in increased permeability, leakage of intracellular content and eventually death of the fungus (Visbal et al., 2011; Fanning & Mitchell, 2012; Kathiravan et al., 2012). The action of cytochalasin E in the cell membrane of *P. brasiliensis* Pb 18 can also be evidenced by the results obtained in analysis by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), as well as fungicidal activity.

The action on the membrane did not occur by interfering in the synthesis of ergosterol as showed by itraconazole, which is a drug that interferes in the synthesis this compound (Visbal et al., 2011). The action of cytochalasin E in the membrane cells can be related to the induction of reactive oxygen species (ROS) by this compound. The overproduction of ROS leads to oxidative degradation of lipids resulting in cellular damage (Ferreira et al., 2013). Other types of mechanism of action related to fungal membrane can be studied, such as binding of the fungal ergosterol present in the membrane (Gray et al., 2012) and inhibition of the synthesis of phospholipids (Sangamwar et al., 2008).

Yeast treated with ¼MIC cytochalasin E showed that the buds or daughter cells remained attached to stem cells. This treatment prevented the buds separated from the mother cell to form new cell. Cytochalasins, a class of fungal metabolites, inhibit actin polymerization by binding with high affinity to growing ends and preventing addition of monomers (Casella et al., 1981). Besides that it affects a wide variety of motile functions and contraction during cell division in eukaryotic (Burke et al., 2014) that resulted in no separation of the daughter cells.

The formation of large vacuoles (visualized in TEM) in intracellular spaces may be related to the increased of the pressure in the cells. These vacuoles suggest that they were formed by water influx from the extracellular environment that would probably lead to rupture of the cells. The vacuole formation was also reported by Santos et al 2007 in assay with *P. brasiliensis* Pb 01 in the presence of oenotein.

Analyzes in SEM and TEM showed changes in the cell wall. These may possibly have been caused by changes in the internal pressure of the cell which resulted in weakness, expansion and rupture of the wall. On the other hand, some changes in the cell wall may have been a result of ROS action. These species are capable of degrading macromolecules, such as, polysaccharides, and alter their function. Besides that, the

degradation result in smaller fragments that to influence the structural cellular organization (Duan & Kasper, 2011). TEM analysis showed a large decrease in layer more eletrodensa of the cell wall. This layer clearly fibrillar, in which there was damage is mainly composed of α-1,3-glucan polysaccharide (Filho et al. 1987) covered by fibrillar layers galactomannan (Puccia et al. 2011).

The results in fluorescence microscopy did not showed difference in the staining with fluorochrome calcofluor-white between cell control and cell treatment. This stain enters the cell and interacts with polymers having *β*-1,4 bonds as chitin. It also has weak affinity with bonds *β*-1,3 glucan (Zacchino & Gupta 2007). Thus, it can be inferred that the cytochalasin E does not act on the polysaccharide chitin and β-1,3-glucan present in the cell walls of *P. brasiliensis* Pb18.

CONCLUSION

Our results showed that cytochalasin E induced the production of ROS, acted both on the membrane and wall cell of the *P. brasiliensis*. Additionally, it caused, in low concentrations, the formation of pseudohyphaes. The action on the cell wall may have been caused as a result of the increase of intracellular pressure or a direct effect of ROS on cell wall polysaccharides. We suggest that the ROS can cause the damage in the cell membrane by oxidation of lipids.

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5- Discussão

Cerca de 100. 000 espécies de fungos são conhecidos, embora muito mais do que um milhão são esperadas. A variedade de espécies e a diversidade de seus habitats, alguns deles menos explorados, permitem concluir que os fungos continuam a ser uma fonte rica de novos metabólitos (Schueffler & Anke, 2014). O potencial de fungos extremófilos em produzir tais moléculas ainda é pouco estudado (Godinho et al., 2013; Azua-Bustos & González-Silva, 2014). Este trabalho explorou o potencial dos fungos que vivem associados a rochas no Deserto do Atacama em produzir substâncias ativas contra o *P. brasiliensis* e até mesmo substâncias inéditas. Além disso, investigou os efeitos do tratamento das leveduras com a citocalasina E.

Os fungos associados a rochas do Deserto do Atacama estão entre os organismos mais tolerantes às condições ambientais terrestres estressantes (Tesei et al., 2012). Acredita-se que possuam genes e vias de produção de metabólitos que os possibilitam viver em tais ambientes (Dalmaso et al., 2015). Deste modo, estes fungos representam fontes potenciais de novas substâncias bioativas. Dentre os fungos estudados no presente trabalho, aqueles que apresentaram atividade antifúngica promissora contra o *P. brasiliensis* foram os isolados das espécies *A. felis* e *Neosartorya* cf. *udagawae*, obtidos rochas do Deserto do Atacama com destaque para o isolado *A. felis* UFMGCB 8030. O extrato deste fungo exibiu o menor valor de CIM entre todos os extratos testados e por isso foi escolhido para o isolamento das substancias bioativas.

A identificação do fungo *A. felis* (UFMGCB 8030) foi feita utilizando metodologias morfológicas, moleculares e filogenéticas uma vez que espécies do gênero *Aspergillus*, seção Fumigati, não podem ser identificadas com base apenas em aspectos morfológicos (Barr et al., 2013). A utilização de outras abordagens, além da morfológica, para a identificação do isolado de UFMGCB 8030 foi fundamental para a confirmação da identificação. Este fungo foi descrito pela primeira vez a partir de amostras obtidas de hospedeiros humanos e animais (cães e gatos) acometidos com aspergilose invasiva. Mas o primeiro relato sobre o isolamento deste fungo no ambiente foi realizado por Gonçalves et al. (2016) como resultado da investigação de amostras de rocha do deserto do Atacama.

Com relação a obtenção de substâncias ativas a partir de fungos, sabe-se que uma boa compreensão do papel das condições de cultivo na biossíntese de metabólitos pode levar a uma melhor exploração desses metabólitos (Miao et al., 2006). A biossíntese de metabólitos secundários em fungos é regulada em resposta à disponibilidade de nutrientes

(Zain et al., 2011; Sanchez & Demain 2002). Assim, a alteração no meio de cultura no qual o micro-organismo está se desenvolvendo pode melhorar a sua produção de substâncias bioativas (Abdel-Fattah & Olama 2002). Com o objetivo de se utilizar o meio de cultura mais eficaz para que o fungo UFMGCB 8030 produzisse substâncias ativas, diferentes meios de cultura foram testados. Além disto, foram testados solventes de diferentes polaridades para que a extração destas susbtâncias fosse efetiva. Dada a heterogeneidade dos grupos funcionais das substâncias produzidas pelos fungos (Kempken & Rohlfs, 2010), o sucesso da extração depende do solvente extrator. Assim, as condições que possibilitaram a produção de extratos mais ativos pelo fungo *A. felis* UFMGCB8030 foi o meio de cultura ágar batata dextrose e a extração com o solvente diclorometano. O extrato obtido do cultivo do fungo em meio mínimo não apresentou atividade antifúngica nas condições testadas no presente trabalho. Alguns autores sugerem que o cultivo em meios ricos em sais, como por exemplo, o Czapek-Dox e o meio mínimo (utilizado no presente trabalho), influencia negativamente o crescimento de *Aspergillus* sp., a atividade antimicrobiana e o perfil dos metabólitos obtidos quando comparado às culturas obtidas em meio complexo, como ágar batata dextrose (Bhattacharyya & Jha, 2011; Mathan et al., 2013). Isto sugere que meios ricos em sais ou pobre em nutrientes interferem no o crescimento micelial e na produção de metabólitos antifúngicos por *Aspergillus* spp.

Até o momento não existem relatos na literatura sobre a investigação de metabólitos secundários produzidos por *A. felis*. Aliado a este fato, o extrato BDA/DCM de *A. felis* não apresentou citotoxicidade em células Vero e a atividade antifúngica foi promissora contra o isolado Pb 18 de *P. brasiliensis*. Portanto esse extrato foi selecionado para ser submetido ao fracionamento cromatográfico com o objetivo de se determinar as substâncias responsáveis pela atividade antifúngica.

A identificação prévia de substâncias ativas no extrato BDA/DCM do *A. felis* UFMGCB 8030 foi feita utilizando a técnica de desreplicação. Esta metodologia é empregada para a identificação de substâncias ou classe de substâncias ativas que já tenham sido descritas na literatura. Ela envolve uma rápida caracterização dos extratos através de bibliotecas e bancos de dados utilizando suas massas moleculares obtidas por espectrometria de massas de alta resolução (Kildgaard et al., 2014; Petersen et al., 2014; Boruta & Bizukojc, 2016). A técnica de desreplicação do extrato de *A. felis* UFMGCB 8030 identificou substâncias do grupo das citocalasinas (piripiropeno A, roselicalasina, citocalasina E, citocalasina Kasp e aspocalasina E) em uma das frações com atividade antifúngica. Algumas substâncias não puderam ser identificadas mostrando que havia a

possibilidade de se encontrar susbtâncias novas neste extrato. Dentre as substâncias identificadas no extato bruto, há relatos de atividade antifúngica descritos na literatura apenas para a citocalasina E. Esta substância apresenta atividade contra fungos de importância agrícola, tais como, *Gibberella saubinetti* (CIM de 100 µM), *Botrytis cinerea* (CIM de 100 µM) e *Alternaria solani* (CIM de 50 µM) (Zhang et al., 2014). Embora a atividade antifúngica tenha sido descrita, não há ainda investigações sobre o efeito desta substância contra fungos de interesse médico, inclusive *P. brasiliensis*.

Citocalasinas são um grupo de metabólitos secundários fúngicos que possuem um esqueleto 10-fenilperhidroisoindol-1-ona e um anel macrocíclico e várias atividades biológicas (Qiao et al., 2011). Elas têm sido descritas não só no gênero *Aspergillus* (Demain et al., 1976; Udagawa et al., 2000; Lin et al., 2009, Zheng et al., 2013), mas também em *Xylaria* (Silva et al., 2010), *Cladosporium* (Cafêu et al., 2005), *Arthrinium* (Wang 2015) e *Phomopsis* (Shen et al;. 2014). De acordo com Guerra et al. (2014), a citocalasina D pode inibir a polimerização da actina em *C. neoformans* e pode causar uma diminuição significativa na internalização desta levedura por macrófagos pois a internalização por fagocitose é dependente de recrutamento de actina. Esta proteína é um elemento do citoesqueleto que tem uma multiplicidade de papéis nos processos celulares, incluindo a adesão celular, motilidade, sinalização celular, transporte intracelular e citocinese. As citocalasinas podem, então, alterar a motilidade celular, adesão, secreção, efluxo de drogas, deformabilidade, morfologia e tamanho (Van Goietsenoven et al., 2011). Aspocalasina E mostra atividade contra melanoma murino (IC50 18,5 μg/mL) e carcinoma do cólon humano (IC50 6,3 μg/mL) (Naruse et al., 1993). Piripiropeno A atua na diminuição da absorção intestinal de colesterol o que resulta na proteção do desenvolvimento da aterosclerose (Ohshiro et al., 2011). Roselicalasina e citocalasina E, isoladas a partir de *Aspergillus* sp., exibiram potente atividade citotóxica contra linhagens celulares tumorais humanas de cólon (Xiao et al., 2013).

A literatura contém poucos relatos de substâncias isoladas de fungos que exibem atividade contra *P. brasiliensis*. Algumas das substâncias descritas até o momento foram: a altenusina isolada a partir do fungo endofítico *Alternaria* sp. (Johann et al,. 2012), a toxina T2 e a mistura 8-n-isobutirilsolaniol e 8-n-butirilneosolaniol de *Fusarium* sp (Campos et al., 2011). Além dessas substâncias, a caspofungina também mostra atividade contra as duas formas de *P. brasiliensis* (Rodríguez-Brito et al., 2010). Este fármaco é um derivado semi-sintético da pneumocandina B, obtida pela fermentação do fungo *Glarea loroyensis* (Chen et al., 2015).

Nossos resultados demonstraram a importância do prosseguimento dos estudos com o fungo *A. felis* UFMGCB8030, uma vez que as análises de desreplicação demostraram a existência de substâncias ativas conhecidas e sugeriram que substâncias novas poderiam ser produzidas por este isolado. Assim, após o estudo químico do extrato bruto de *A. felis* UFMGCB8030 e a identificação de metabólitos ativos por meio da desreplicação, foi realizado o extrato deste fungo em maior escala.

A purificação do extrato bruto resultou no isolamento de sete substâncias, duas delas foram descritas pela primeira vez e cinco já haviam sido descritas pela literatura. Os metabólitos secundários conhecidos foram identificados como: roselicalasina, citocalasina E, gancidina, pseurotina A1, e 2,4 -diidroxiacetofenona. Além dessas substâncias conhecidas foi isolada uma citocalasina inédita denominada como citocalasina Z_{15E} (fig. 8A). Esta substância difere da citocalasina E (fig. 8B) por apresentar uma cadeia alifática na porção da molécula onde na citocalasina E forma-se um anel macrocíclico. Substâncias do grupo das citocalasinas, denominadas 10-fenil-12 citocalasina Z11, Z13, Z16 e Z17 (fig. 8C-8F), foram isoladas de *Aspergillus terreus*. A citocalasina Z17 apresentou citotoxicidade moderada contra linhagens celulares humanas de carcinoma epidermóide com um valor de IC50 de 26,2 μM (Zhang et al., 2010). A outra substância inédita isolada de *A. felis* é um derivado da ardeemina, denominada 5-*N*-acetil-8-β-isopropil ardeemina (fig. 8G). Substâncias do grupo das ardeminas já foram isoladas de *A. terreus*, tais como, 5-*N*-acetil ardeemina (fig. 8H), 5-*N*-acetil 15b-β-hidroxi- ardeemina (fig. 8I), 5-*N*-acetil -15b-didehidro-ardeemina (fig. 8J) (Zhang et al., 2010).

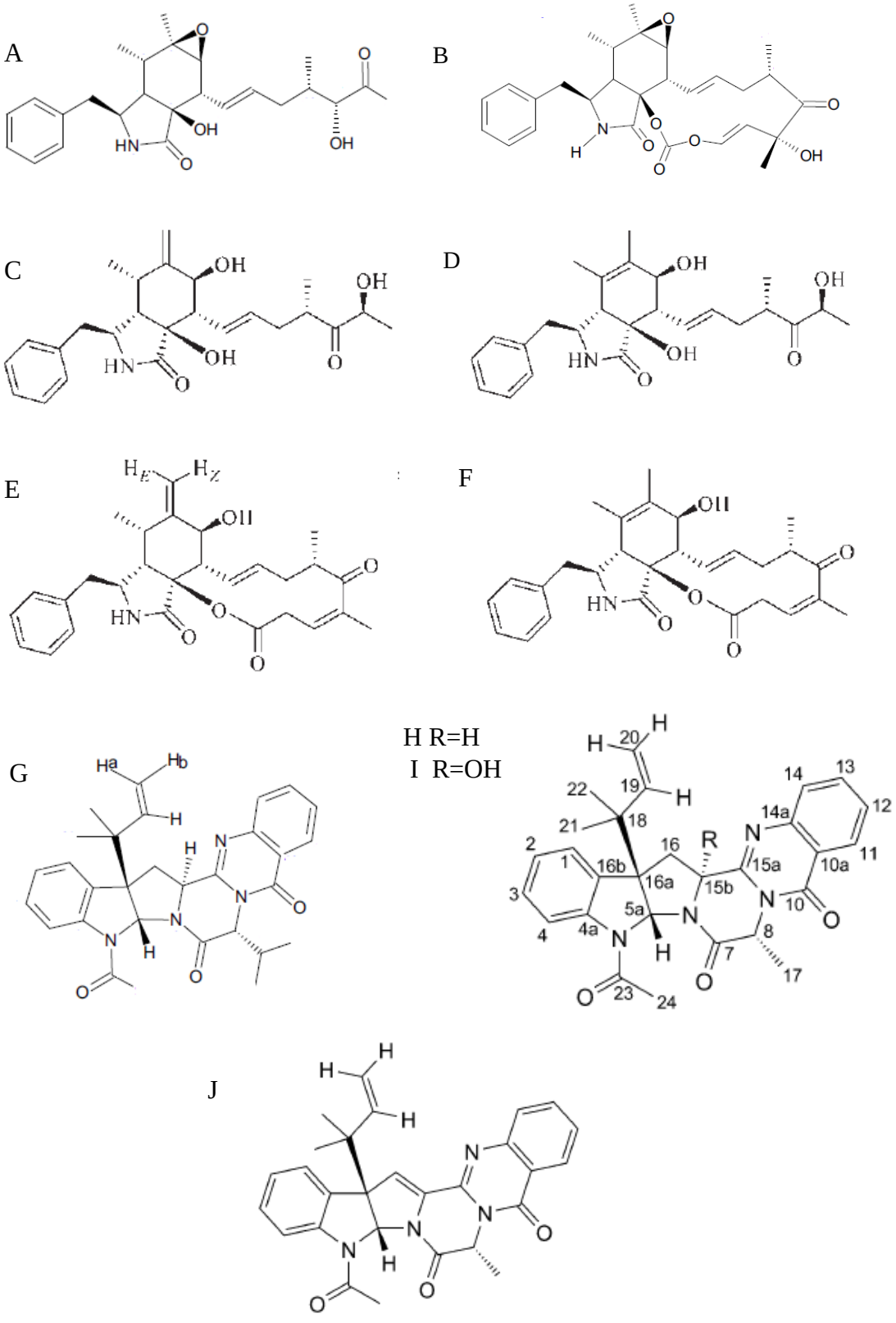

Figura 8: Estruturas da citolalasina Z $_{15E}$ (A), citocalasina E (B), citolalasina Z11 (C), Z13 (D), Z16 (E), Z17 (F), 5-*N*-acetil-8-*β*-isopropil ardeemina (G) 5-N-acetil ardeemina (H), 5-*N*-acetil 15b-*β*-hidroxi-ardeemina (I) e 5-*N*-acetil-15b-didehidro-ardeemina (J).

É importante ressaltar que o processo de desreplicação identificou previamente a presença de substâncias do grupo das citocalasinas, tais como, citocalasina E e roselicalasina, que foram, posteriormente, confirmadas pela elucidação das substâncias isoladas. Além disso, algumas substâncias contidas nas frações do extrato bruto não puderam ser identificadas mostrando que havia a possibilidade de se encontrar susbtâncias novas neste extato. Este fato realmente se confirmou visto que este extrato forneceu duas substâncias inéditas, citocalasina Z15E e 5-*N*-acetil-8-*β*-isopropil ardemina.

A atividade antifúngica contra o *P. brasiliensis* da substância inédita 5-*N*-acetil-8-*β*-isopropil ardemina (valor de CIM 62,5 μg/mL; 125,0 μM) foi melhor do que a apresentada pela outra substância inédita citocalasina Z15E (valor de CIM 250,0 μg/mL; 584,0 μM). Dentre as substâncias conhecidas, a citocalasina E apresentou o melhor valor de CIM (1,9 μg/mL; 3,60 μM). Este valor é mesmo apresentado pelo extrato bruto.

Neste trabalho, dentre as substâncias isoladas e já descritas pela literatura, a gancidina e a 2,4 -diidroxiacetofenona foram identificadas pela primeira vez no gênero *Aspergillus* e descritas como agentes antifúngicos. O alcalóide gancidina já foi identificado no fungo *Corollospora pulchella* CE demonstrou atividade antibacteriana (Furuya et al., 1985). A substância fenólica 2,4- diidroxiacetofenona, já foi isolada a partir de extratos de raízes da planta *Cynanchum wilfordii*, comumente usada na medicina Chinesa no tratamento de insônia, ansiedade, anemia e doenças geriátricas. Os autores sugerem que esta substância pode prevenir e tratar doenças de aterosclerose e inflamação vascular (Koo et al., 2015).

Pseurotina A já foi isolada de *Penicillium janczewskii* e apresentou atividade contra as bactérias *Erwinia carotovora* e *Pseudomonas syringae* com valores de IC50 220 e 112 μg/mL, respectivamente. Esse produto natural apresentou baixa citotoxicidade contra os fibroblastos de pulmão humano (Schmeda-Hirschmann et al., 2008). Além deste gênero, a pseurotina A e seus análogos (A1 e A2) foram isoladas do fungo *A. fumigatus* e apresentaram valores de IC50 67,0; 48,0 e 70,8 μM, respectivamente. Pseurotina A apresentou fraca citotoxidade contra células A549 e HL60, e A1 apresentou baixa citotoxicidade contra HL60 (Wang et al., 2011). No presente trabalho a pseurotina A1 apresentou CIM de 62,5 μg/mL (144,9 μM) contra o isolado Pb 18 de *P. brasiliensis.* Não foi encontrado nenhum registro desta substância como agente antifúngico.

O desenvolvimento de agentes antifúngicos é um desafio porque os fungos e o homem, por se tratarem de organismos eucariotos, compartilham alguns alvos enzimáticos relativos à toxicidade (Srinivasan et al., 2014). Apesar disso, a inibição de constituintes

que são exclusivos da membrana e parede celular fúngica constitui estratégia interessante para o desenvolvimento de novos antifúngicos com menos efeitos colaterais ao homem.

A investigação da ação da citocalasina E em células leveduriformes do isolado Pb 18 de *P. brasiliensis* apresentou efeitos na membrana celular e a parede celular principalmente. Além destes alvos, esta substância afetou a liberação dos brotos pelas células-mãe. Sabe-se que substâncias do grupo das citocalasinas inibem a polimerização da actina (Casella et al., 1981). Esta proteína globular forma microfilamentos que são fundamentais para a organização do citoesqueleto dos organismos eucariotos, ou seja, conferem forma as células. Além disso, está relacionada a funções de contração do citoplasma durante a divisão celular (Burke et al., 2014). Assim a inibição da polimerização da actina leva a não separação das células-filhas.

A ação da citocalasina E na membrana celular de *P. brasiliensis* PB 18 foi evidenciada pelos resultados obtidos no ensaio de perda de componentes, análises de microscopia óptica e de transmissão, além da atividade fungicida. Essa ação na membrana pode ter ocorrido pela produção de EROs e não por meio de interferência na síntese do ergosterol. A superprodução de EROs leva a degradação oxidativa dos lipídios resultando em dano celular (Ferreira et al., 2013).

Os danos provocados na parede celular foram visualizados por rachaduras na parede bem como pela diminuição brusca da camada externa mais eletrodensa desta estrutura. No primeiro caso, alterações na pressão interna da célula podem ter resultado em enfraquecimento, expansão e ruptura da parede (Santos et al., 2007). A presença de vacúlos grandes nessas células evidencia o aumento na pressão interna provavelmente causada por influxo de líquidos. A formação de vacúolos foi igualmente relatada por Santos et al 2007 em ensaios com *P. brasiliensis* Pb 01 na presença de oenoteína.

As alterações vistas na camada mais externa da parede, nitidamente fibrilar, na qual houve dano é composta principalmente pelo polissacarídeo α-1,3-glucano (Filho et al. 1987) a qual é coberta por camadas fibrilares de galactomanas (Puccia et al. 2011). Sugere-se que a ação nesta estrutura foi causada pela produção de EROs induzidos pela citocalasina E. EROs são capazes de degradar macromoléculas, incluindo proteínas, lípidos, ácido nucléico, bem como polissacarídeos e, presumivelmente, resultam na sua disfunção. Há relatos que o papel funcional de determinados polissacarídeos está ligado ao seu grau de polimerização, assim, a clivagem oxidativa dos polímeros resulta na geração de fragmentos menores que alteram a organização e a função da estrutura celular (Duan & Kasper, 2011). A ação da citocalasina E na parede celular de *P. brasiliensis* exclui os

outros componentes dessa estrutura, tais como, β-1,3-glucano e quitina, pelo resultado negativo no ensaio de marcação com o fluorocromo calcofluor-white das leveduras tratadas.

6- Conclusão

Este trabalho demonstrou que os fungos do Deserto do Atacama são fontes promissoras de extratos com propriedades antifúngicas contra o *P. brasiliensis.* O extrato de *A. felis* foi otimizado e demostrou nessa condição produzir substâncias antifúngicas e também substâncias inéditas. Dentre as substâncias isoladas, a citocalasina E apresentou melhor atividade antifúngica e nos tratamentos das leveduras induziu a produção de EROs que possivelmente atuaram na membrana e na parede celular causando danos morfológicos e possivlemente funcionais. Além disso, o tratamento com esta substância impediu que as células mães liberassem seus brotos.

7- Perspectivas

Estudar o mecanismo de ação da citocalasina E no tratamento de células leveduriformes de *P. brasiliensis.*

8- Referências bibliográficas

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9- Atividades desenvolvidas no período

9.1-Resumos publicados

Avaliação do efeito de compostos heterocíclicos tiazólios contra os fungos Cryptococcus gatti e Cryptococcus neoformans. Lima, C. M.; Sá, N. P.; **Mendes, G**.; Cruz, L.B.; Matos, T.T.S.; Oliveira, R.B.; Johann, S., no "II Simpósio de Microbiologia da UFMG – Microbiologia Translacional: Do ambiente natural às aplicações biotecnológicas", promovido pelo Programa de Pós-Graduação em Microbiologia do Departamento de Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. 2015.

Atividade Antifúngica de extratos de fungos da Antártica e do Atacama. **Mendes, G**.; Rosa, C. A.; Rosa, L. H.; Cota, B. B.; Johann, S. I Simpósio de Microbiologia: "A Microbiologia e a Sociedade" promovido pelo Programa de Pós-Graduação em Microbiologia do Departamento de Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. 2014.

9.2-Colaboração em artigos

Vivian N. Gonçalves, Camila R. Carvalho, Susana Johann, Graziele Mendes, Tânia M. A. Alves, Carlos L. Zani, Policarpo A. S. Junior, Silvane M. F. Murta, Alvaro J. Romanha, Charles L. Cantrell, Carlos A. Rosa, Luiz H. Rosa. *Antibacterial, antifungal and antiprotozoal activities of fungal communities present in different substrates from Antarctica*. Polar Biol (2014) 38:1143–1152. DOI 10.1007/s00300-015-1672-5.

9.3-Participação em eventos

IX Fórum de Microbiologia: Professor Romain Rolland Golgher, A Microbiologia e a Imunidade Inata.

9.4- Participação em bancas de Trabalhos de Conclusão de Curso

MENDES, G.. Participação em banca de Thays Perdigão Gonçalves. Avaliação da atividade antifúngica de compostos triazólicos. 2014. Trabalho de Conclusão de Curso (Graduação em Farmácia) - Universidade Federal de Minas Gerais.

MENDES, G.; Pôssa, A. P.. Participação em banca de Mariana Rezende de Sousa Lucas. Implantação da Micologia no Laboratório Clínico. 2013. Trabalho de Conclusão de Curso (Graduação em Farmácia) - Universidade Federal de Minas Gerais.

9.5- Co-orientação de Trabalho de Conclusão de Curso

Almeida, L. M.; Mendes, G.; Silva, S. L.. Avaliação do potencial antimicrobiano de fungos isolados da região amazônica. 2013 - Universidade Federal de São João Del-Rei.

Resende, E. F.; Mendes, G.; Silva, S. L.. Avaliação de atividade antimicrobiana do fungo *Aspergillus* sp. 2012. Trabalho de Conclusão de Curso (Graduação em Farmácia) - Universidade Federal de São João Del-Rei.