DÉRICA GONÇALVES TAVARES

ATIVIDADE ANTIOXIDANTE E ANTIBACTERIANA E COMPOSTOS FENÓLICOS TOTAIS DE EXTRATOS DE FUNGOS FILAMENTOSOS PRODUTORES DE PIGMENTOS

LAVRAS – MG

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Mestre.

Prof. Dra. Patrícia Gomes Cardoso
Orientadora

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Tavares, Dérica Gonçalves.
Atividade antioxidante e antibacteriana e compostos fenólicos totais de extratos de fungos filamentosos produtores de pigmento / Dérica Gonçalves Tavares. - 2017.
87 p. : il.

Orientador(a): Patrícia Gomes Cardoso.
Dissertação (mestrado acadêmico) - Universidade Federal de Lavras, 2017.
Bibliografia.

1. Fungos filamentosos. 2. Antibacteriano. 3. Antioxidante. I. Cardoso, Patrícia Gomes. . II. Título.

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ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY AND TOTAL PHENOLIC COMPOUNDS OF EXTRACTS FROM PIGMENT-PRODUCING FILAMENTOUS FUNGI

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Mestre.

APROVADA em 16 de março de 2017.

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LAVRAS-MG

2017

A Deus, que me dá força, que me guia e sem Ele nada seria possível.

À minha mãe, Hildenise Gonçalves Tavares, que sempre foi meu apoio e exemplo de mãe guerreira.

Ao meu pai, Daltro Dourado Tavares.

Às minha irmãs, Débora e Makellen, pelo carinho, apoio e amizade.

Dedico.

AGRADECIMENTOS

Agradeço a Deus, por sempre me dar forças, para superar todos os obstáculos, por sempre estar comigo, ajudando-me, guiando-me e dando-me coragem. Agradeço-Lhe por tudo.

À minha mãe Hildenise Gonçalves pelo exemplo de vida, dedicação, força e sacrifícios. Por ter me permitido chegar até aqui, pelas orações, por ser meu apoio e por tudo que fez e faz por mim e minhas irmãs.

Às minhas irmãs, Débora Gonçalves Tavares e Makellen Gonçalves Tavares, por todo o carinho, amizade, apoio e pela disponibilidade de sempre poderem me ajudar.

Ao meu pai Daltro Dourado Tavares por toda ajuda, principalmente, em momentos difíceis.

À minha tia Irineide por todas as orações, pelo carinho, por toda força que sempre me ofereceu. Ao meu tio Nóe e a meus primos Kátia, Elane, Maiara e Samuel, que são como irmãos, por todo apoio, conversas e amizade.

A toda minha família e amigos que sempre estão comigo me ajudando, apoiando e torcendo por mim.

À minha orientadora, Prof. Dra. Patrícia Gomes Cardoso, pelo apoio e orientação não apenas nas atividades acadêmicas, mas pela amizade e toda a disponibilidade em me ajudar, por todo carinho e confiança.

À Prof. Dra. Patrícia Nirlane da Costa Souza pelos ensinamentos, ajuda na condução de experimentos, pela amizade e pelos momentos de conversa.

Ao Prof. Dr. Whasley Ferreira Duarte por toda disponibilidade, orientação e ensinamentos.

À Prof. Dra. Roberta Hilsdorf Piccoli por disponibilizar o laboratório e os materiais e pela orientação na condução de parte dos experimentos.

Ao órgão de fomento, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos.

Ao Programa de Pós-Graduação em Microbiologia Agrícola da Universidade Federal de Lavras, e aos professores do programa pelos ensinamentos e por contribuírem para a minha formação profissional.

Aos funcionários do Setor de Microbiologia Agrícola do Departamento de Biologia pela prestação dos servisos, boa vontade e gentileza.

Aos amigos que fiz no Laboratório Biogen: Mônica Monteiro, Rafaela Merlo, Bárbara Godinho, Natálie Alves e Cibelly Ferreira pela ajuda, conversas, desabafos, todo apoio e valiosa amizade, Bárbara Viana, Peterson Nunes, Mauro Guilherme, Ítalo Férrer, Rafaela Corrêa pelas conversas e contribuições nas atividades.

Aos amigos do mestrado, em especial, Rafaela Merlo, Marília Santiago, Joice Raísa, Rafaela Andrade, Michelle Carlota e Natanne Santana pelas conversas, desabafos e amizade.

Muito Obrigada!

"Você nunca sabe que resultados virão da sua ação. Mas, se você não fizer nada, não existirão resultados" (Mahatma Gandhi).

RESUMO

Os objetivos deste trabalho foram avaliar a atividade dos extratos, em acetato de etila dos fungos filamentosos produtores de pigmento, na inibição de bactérias patogênicas, transmitidas por alimentos, determinando sua concentração mínima bactericida (CMB); avaliar a atividade antioxidante, utilizando os métodos de sequestro dos radicais livres DPPH e $ABTS^{\bullet+}$ e pelo sistema β-caroteno – ácido linoleico e determinar o conteúdo de compostos fenólicos totais pelo método do Folin-Ciocalteu. Doze isolados de fungos filamentosos, selecionados por sua produção de pigmentos, em meio de cultura sólido, foram identificados, utilizando as sequências dos genes espaçador transcrito interno (ITS) 5.8S do rDNA e subunidade maior da RNA polimerase II (RPB2). Foram identificadas as espécies *Aspergillus keveii* (CML 2968), *Aspergillus sydowii* (CML 2967), *Penicillium chermesinum* (CML 2966), *Epicoccum nigrum* (CML 2971, A2C32 e A2S61), *Periconia ignaria* A2C47) e *Lecanicillium aphanocladii* (CML 2970), e os gêneros *Arcopilus* sp. (A2C54) and *F. graminearum* lato sensu (CML 2969). Cinco isolados apresentaram atividade bactericida contra pelo menos uma cepa bacteriana patogênica. Os extratos de *A. sydowii* (CML 2967) e *A. keveii* (CML 2968) apresentaram CMB de 5 mg mL^{-1}, já o de *Fusarium* sp. (CML 2969) e as CMB variaram de 5 a 0,625 mg mL^{-1} e o de *P. ignaria* (A2C47) e as CMB variaram de 5 a 1.25 mg mL^{-1}. O extrato do fungo endofítico *Arcopilus* sp. (A2C54) exibiu a maior atividade bactericida, com CMB de 0,00244 e 0,0195 mg mL^{-1} para *Staphylococcus aureus* GL 8702 e GL 5674, respectivamente e 0,625 mg mL^{-1} para *Salmonella* Enteritidis. O extrato de *P. flavigenum* (CML 2965) exibiu a maior atividade antioxidante, em todos os métodos utilizados, com 98.22 % de inibição do radical DPPH e EC_{50} de 83.4 µg mL^{-1}; 29.50 % de inibição do radical $ABTS^{\bullet+}$ e EC_{50} de 862.2 µg mL^{-1}; e 72.23 % de proteção do β-caroteno. A concentração de compostos fenólicos totais foi de 201,43 mg de GAE/g de extrato. Com os resultados mostra-se o potencial desses fungos filamentosos como novas fontes de metabólitos antibacterianos e antioxidantes que podem, futuramente, ter aplicações biotecnológicas.

Palavras-chave: Metabólitos secundários. Potencial antioxidante. Antimicrobiano. Fungos endofíticos. Fungos de cavernas.

ABSTRACT

The objectives of this work were to evaluate the activity of the ethyl acetate extracts of pigment-producing filamentous fungi in the inhibition of pathogenic bacteria transmitted by food, determining their minimum bactericidal concentration (MBC). The evaluation of the antioxidant activity using the DPPH and the $ABTS^{\bullet+}$ free radical sequestration methods, the β-carotene-linoleic acid system and the content of total phenolic compounds by the Folin-Ciocalteu method were also carried out. Twelve isolates of filamentous fungi selected by their pigment production in solid culture medium were identified using the sequences of the internal transcribed spacer (ITS) 5.8S region of rDNA and RNA polymerase II second largest subunit (RPB2). The following species were identified: *Aspergillus keveii* (CML 2968), *Aspergillus sydowii* (CML 2967), *Penicillium chermesinum* (CML 2966), *Epicoccum nigrum* (CML 2971, A2C32 and A2S61), *Periconia ignaria* A2C47) and *Lecanicillium aphanocladii* (CML 2970). Furthermore, the genera *Arcopilus* sp. (A2C54) and *F. graminearum* sensu lato (CML 2969) were identified. Five isolates showed bactericidal activity against at least one pathogenic bacteria strain. The extracts of *A. sydowii* (CML 2967) and *A. keveii* (CML 2968) showed MBC of 5 mg mL^{-1}, while for *Fusarium* sp. (CML 2969) the MBC ranged from 5 to 0.625 mg mL^{-1} and for *P. ignaria* (A2C47) the MBC ranged from 5 to 1.25 mg mL^{-1}. The extract of the endophytic fungus *Arcopilus* sp. (A2C54) exhibited the highest bactericidal activity, with MBC 0.00244 and 0.0195 mg mL^{-1} against *Staphylococcus aureus* GL 8702 and GL 5674, respectively, and 0.625 mg mL^{-1} for *Salmonella* Enteritidis. The extract of *P. flavigenum* (CML 2965) showed the highest antioxidant activity in all the used methods with 98.22 % inhibition of the DPPH and EC_{50} of 83.4 μg mL^{-1}; 29.50 % inhibition of $ABTS^{\bullet+}$ and EC_{50} of 862.2 μg mL^{-1}; and 72.23% protection of β-carotene. The total concentration of phenolic compounds was 201.43 mg GAE g^{-1} extract. The results show the potential of these filamentous fungi as new sources of antibacterial and antioxidant metabolites that may have biotechnological applications in the future.

Keywords: Secondary metabolites. Antioxidant potential. Antimicrobial. Endophytic fungi. Cave fungi.

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PRIMEIRA PARTE

1 INTRODUÇÃO

Os fungos filamentosos são amplamente estudados pela sua capacidade de sintetizar uma infinidade de compostos que apresentam diversas aplicações biotecnológicas, como enzimas, antimicrobianos, antioxidantes e pigmentos naturais, além de serem utilizados na agricultura, para o controle de pragas e, na indústria alimentícia, para produção de queijos e outros alimentos.

Dentre os fungos mais estudados, destacam-se os endofíticos que são microorganismos que vivem no tecido de plantas sem causar dano aparente, podendo viver em simbiose com a planta hospedeira (BACON; WRITE, 2000). Muitos metabolitos bioativos já foram isolados e caracterizados, a partir de fungos endofíticos, apresentando atividades biológicas como antimicrobiana, antiparasitária, antioxidante, citotóxica, entre outras (ALY; DEBBAB; PROKSCH, 2011). Com isso, estudos demonstram que esses microorganismos são fontes prolíficas de novas substâncias com diversas atividades bioativas. Além dos fungos endofíticos, os fungos que crescem em cavernas são pouco estudados em relação ao seu potencial biotecnológico. As cavernas apresentam ambiente considerado de condições extremas, no qual se poderiam selecionar linhagens de microorganismos com características próprias e que podem produzir diversas substâncias com propriedades bioativas. Em alguns trabalhos ressalta-se que microorganismos de cavernas podem ter potencial para aplicações biotecnológicas como as actinobactérias que sintetizaram metabolitos com atividade antitumoral (NAKAEW; PATHOM-AREE; LUMYONG, 2009).

Tendo em vista a importância de metabolitos bioativos, a partir de microorganismos que podem ser aplicados, em benéfico da humanidade, destacam-se alguns fatos importantes como o desenvolvimento de resistência aos patógenos humanos, principalmente, às bactérias. Estima-se que cerca de 700 000 mortes ocorrem por ano, atribuídas à resistência a antimicrobianos, em todo o mundo, e a estimativa é que chegue a 10 milhões de mortes até 2050, principalmente, na Ásia e na África (HAMPTON, 2015). Esses dados demonstram a importância de pesquisas que visam a encontrar moléculas com atividade antimicrobiana e que possam, futuramente, serem utilizadas como novos fármacos. Outro campo importante de estudo é a demanda por compostos antioxidantes, principalmente, de origem natural, em razão da toxicidade que compostos sintéticos podem causar. Os antioxidantes inibem os radicais que são produzidos, com base em processos fisiológicos normais que acontecem, em todos os seres vivos e que, em excesso, podem se tornar tóxicos, pois reagem com moléculas

importantes como ácidos nucleicos, lipídeos, entre outras (CHANDRA; ARORA, 2014). Os danos oxidativos, causados pelos radicais livres, também, estão associados ao câncer, aterosclerose, imunossupressão, envelhecimento, inflamações, doença cardíaca isquêmica, diabetes, doenças neurodegenerativas como o Parkinson e Alzheimer (OLIVEIRA et al., 2008). Portanto torna-se cada vez mais necessária a busca por novos antimicrobianos e compostos antioxidantes, a partir de microorganismos, em especial, de fungos filamentosos, uma vez que seu potencial já é bem conhecido.

Dentre os metabólitos secundários sintetizados por fungos estão, também, os pigmentos que possibilitam ser alternativa, ao uso de corantes sintéticos e têm a possibilidade de agregar atividades biológicas com antimicrobiana e antioxidante. Nesse contexto, foi objetivo deste trabalho a prospecção de fungos endofíticos e de cavernas, produtores de pigmentos com potencial fonte de compostos antimicrobianos, antioxidantes e fenólicos.

2 REFERENCIAL TEÓRICO

2.1 Fungos filamentosos

Os fungos são encontrados, em diversos ambientes como a água, ar, solo, em animais e vegetais vivos, parasitando-os ou em simbiose, em material orgânico em decomposição e nos alimentos. São organismos eucarióticos leveduriformes ou pluricelulares, geralmente apresentando parede celular rígida, são heterotróficos e se alimentam por absorção, reproduzindo-se sexuada e assexuadamente (DEACON, 2006). Os fungos unicelulares são denominados leveduras e os pluricelulares são denominados filamentosos. Fungos filamentosos são compostos de hifas septadas ou cenocíticas que são responsáveis pela sua fixação ao substrato e à alimentação e se diferenciam para formar estruturas reprodutivas e de resistência (DEACON, 2006). Apresentam capacidade de degradar moléculas complexas tais como lignina, quitina, celulose, entre outros e podem crescer em condições de baixo pH, ambientes pobres de nutrientes e baixa atividade de água (MOLLEA; BOSCO; RUGGERI, 2005). Os fungos fazem parte de processos importantes como a reciclagem de nutriente. São utilizados, na indústria de alimentos e, na síntese de moléculas, como enzimas, antibióticos, ácidos orgânicos e outros metabólitos (DE OLIVEIRA; DE GRAAFF, 2011).

Na agricultura, alguns fungos filamentosos são utilizados, para o controle biológico de pragas e doenças, como alternativa para reduzir o uso de agrotóxico. O fungo *Metarhizium anisopliae* é um agente utilizado, no controle de pragas, que parasita mais de 300 espécies de insetos (SKALICKÝ et al., 2013). Os fungos filamentosos são bons produtores de enzimas com utilização em diversas áreas. Esses microorganismos são a principal fonte comercial de xilanases (POLIZELI et al., 2005), que são enzimas empregadas, para hidrólise de xilano, que é um tipo de hemicelulose presente na madeira e em resíduos agrícolas e agroindustriais. As xilanases obtidas de *Trichoderma* sp., *Humicola insolens* e *Aspergillus niger* são muito aproveitadas em processos industriais, para fabricação de pães, sucos e vinhos, como suplemento à alimentação animal, em produtos têxteis, para branqueamento da pasta de celulose, na produção de etanol e do adoçante xilitol (POLIZELI et al., 2005). Além disso, são aplicadas na produção de queijos e outros alimentos, além de serem fonte de metabólitos secundários e de alguns medicamentos como os antibióticos penicilina, produzido por *Penicillium notatum* e cefalosporina, a partir do fungo *Cephalosporium acremonium* (hoje renomeado *Acremonium chrysogenum*), redutores de colesterol mavastatina, produzida por *Penicillium citrinum* e lovastatina por *Aspergillus terreus* (MANZONI; ROLLINI, 2002) e os

imunossupressores ciclosporina produzida pelo fungo *Beauveria nivea* e rapamicina (CRAGG; NEWMAN, 2013).

Fungos filamentosos, também, estão sendo estudados como fonte de pigmentos naturais que abrange várias classes de moléculas como carotenoides, melaninas, flavinas, fenazinas, quinonas, monascinas e violaceína as quais apresentam potencial para serem utilizadas como corante natural de alimentos (DUFOSSÉ et al., 2014). A crescente busca por produtos de origem natural indica a importância dos pigmentso fúngicos que podem ser uma alternativa, para substituírem colorantes sintéticos, decorrentes de problemas de saúde que eles podem causar, como também por efeito do aumento de preço e complexidade do material, além da poluição ao meio ambiente (AKILANDESWARI; PRADEEP, 2016). Os colorantes naturais podem apresentar atividade antimicrobiana, menor probabilidade de serem alergênicos e são mais estáveis que os sintéticos (MEHRABIAN; MAJD; MAJD, 2000). Alguns pigmentos de fungos filamentosos já estão sendo comercializados como os pigmentos do fungo *Monascus*, Arpink red™ a partir de *Penicillium oxalicum*, riboflavina sintetizada por *Ashbya gossypii*, licopeno e β-caroteno do fungo *Blakeslea trispora* (DUFOSSÉ et al., 2014).

2.1.1 Fungos endofíticos

Uma das primeiras publicações sobre fungos endofíticos foi feita por Freeman em 1904, que relatou a presença desses microorganismos em sementes de *Lolium temulentum*. No entanto, esse grupo de micro-organismos não recebeu atenção até a demonstração da sua relevância ecológica e potencial para produção de metabólitos secundários com diversas atividades bioativas. No decorrer das últimas três décadas, fungos endofíticos vem atraindo a atenção de taxonomistas, biólogos, micologistas, ecologistas e químicos com o intuito de elucidar o potencial desses micro-organismos desde sua relação com a planta hospedeira até a produção de compostos bioativos e suas interações.

Endofíticos ou endófitos foi o termo descrito por De Bary em 1866, referindo-se a micro-organismos que vivem no interior de plantas diferindo-se dos epifíticos que vivem na superfície da mesma (CHAPLA; BIASETTO; ARAUJO, 2013). Muitas definições são encontradas na literatura e uma das mais utilizadas é a de Bacon e Write (2000) que define os endofíticos como micro-organismos que residem nos tecidos internos das plantas sem causar dano imediato ao seu hospedeiro (ALY; DEBBAB; PROKSCH, 2011). Entretanto, podem se tornar patogênicos no período de senescência da planta hospedeira ou devido a fatores desfavoráveis como desequilíbrio na troca de nutrientes e variações ambientais (KOGEL;

FRANKEN; HÜCKELHOVEN, 2006) podendo causar infecções externas. Geralmente, centenas de endofíticos podem ser isolados de uma mesma planta e acredita-se que cada planta é hospedeira de pelo menos uma ou várias espécies de endofíticos (STROBEL; DAISY, 2003). Algumas espécies de fungos endofíticos são encontrados colonizando varias espécies de plantas, entretanto, alguns endófitos são específicos ao hospedeiro, e a interação entre ambos podem variar entre hospedeiros e essas interações são pouco elucidadas (ALY; DEBBAB; PROKSCH, 2011; JALGAONWALA; MOHITE; MAHAJAN, 2011). São encontrados nos espaços intracelulares e intercelulares podendo colonizar diferentes tecidos da planta como, sementes, embrião, frutas, caule, raízes, folhas, tubérculos, botões florais, xilema e a casca. Muitos estudos mostraram que os fungos endofíticos são encontrados praticamente em todos os tipos de plantas tais como briófitas, pteridófitas, monocotiledônea e dicotiledônea (ZHANG; SONG; TAN, 2006).

A relação mutualística entre os fungos endofíticos contribui ou confere à planta hospedeira adaptação às condições adversas como estresses bióticos e abióticos. Dentre os fatores abióticos, podem atuar aumentando a tolerância da planta hospedeira a altas temperaturas e salinidades, e maior resistências a secas como também aumentando a tolerância ao estresse oxidativo pela produção de compostos antioxidantes (HERRERA-CARILLO et al., 2009). A adaptação a estresses bióticos muitas vezes está relacionada a produção de metabólitos bioativos com atividade antimicrobiana por parte dos fungos endofíticos. Podem atuar também no controle de microrganismos fitopatógenos, colonizando o mesmo local em que se encontram os patógenos nas plantas, competindo por espaço e nutrientes, além de produzirem substâncias com ação antibiótica (JALGAONWALA; MOHITE; MAHAJAN, 2011). Em contrapartida, a planta fornece ao endofítico estrutura espacial, proteção contra dessecação, nutrientes, compostos essenciais para o seu crescimento, como na indução do seu estado teleomorfo e sua transmissão vertical, promovemdo a disseminação desse para a próxima geração da planta (SAIKKONEN et al., 1998). Alguns desses fungos responsáveis por produzir enzimas que atuam no crescimento e regulação de metabólitos secundários envolvidos na síntese de antibióticos. Além disso, a interação com o endofítico pode induzir genes da planta responsáveis pela resistência e tolerância a fatores adversos (BAILEY et al., 2006). Portanto, a associação entre planta e endofítico mostra que esses organismos coevoluiram juntos apresentando uma relação de benefício mútuo para ambas as partes.

Segundo Ownley et al. (2010) os micro-organismos endofíticos são uma fonte potencial de produtos naturais com mais de 20000 substâncias descritas onde 51% apresentam

novas estruturas químicas e 80% são substâncias com atividade biológica. Entre 2008 e 2009 foram descobertos mais de 100 novos produtos naturais mostrando a importância dos fungos endofíticos para bioprospeção nos últimos anos (ALY; DEBBAB; PROKSCH, 2011). Dentre os metabolitos secundários bioativos sintetizados pelos fungos endofíticos foram encontradas diversas classes químicas como alcaloides, terpenoides, esteroides, quinonas, isocumarinas, ácidos fenólicos, fenilpropanoides, lignanas, e outros (ZHANG; SONG; TAN, 2006). Compostos produzidos podem apresentar atividades biológicas como antimicrobiana, antiparasitária, neuroprotetiva, antioxidante, antidiabética, imunossupressoras, anticolinesterásica, antineoplástica e citotóxica (ALY; DEBBAB; PROKSCH, 2011; JALGAONWALA; MOHITE; MAHAJAN, 2011; ZHANG; SONG; TAN, 2006).

2.1.2 Fungos de cavernas

Os microorganismos são capazes de habitar diversos locais da biosfera, incluindo as cavernas, nas quais é encontrada uma variedade de microorganismos, mesmo apresentando um ecossistema pobre em nutrientes, pela ausência de luz que impede a produção primária por meio da fotossíntese e apresentando parâmetros constantes, ao longo do ano, como temperaturas amenas e alta umidade (BINDSCHEDLER et al., 2010; JURADO et al., 2009). Os microorganismos podem estar associados a diversos processos de formação e composição do ecossistema cavernícola, principalmente a espeleogênese. Podem atuar, por meio de fungos patogênicos, no controle de determinadas populações, como base da cadeia alimentar e assumindo papéis importantes em processos biogeoquímicos como a ciclagem de nutrientes (BARTON, 2006).

Em pesquisas realizadas percebe-se a importância do estudo de fungos no interior de cavernas como fonte de alimentos para invertebrados, além do isolamento de microorganismos raros como o fungo *Pidoplitchkoviella terricola* e descoberta de novas espécies como o fungo *Chrysosporium speluncarum* (NOVÁKOVÁ, 2009). Além disso, estudo, para identificar fungos patogênicos presentes no interior de cavernas como fonte de infecção e alergia em humanos, foi realizado e encontradas espécies de *Fusarium*, *Mucor circinelloides* e esporos de *Aspergillus* e *Penicillium* (DOCAMPO et al., 2011). Nas cavernas, são encontrados muitos fungos entomopatogênicos que parasitam artrópodes que acabam por disseminar os esporos fúngicos, no interior de cavernas, o que ajuda a compreender um pouco sobre a colonização de fungos nesses locais (JURADO et al., 2009).

Nakaew et al. (2009) isolaram actinobactérias de amostras de solo, presentes em algumas cavernas localizadas no Norte da Tailândia, as quais apresentaram compostos

bioativos com atividade anticâncer. O estudo de microorganismos de ambientes extremos, como são consideradas as cavernas (BINDSCHEDLER et al., 2010), é de grande importância, em virtude da resistência que eles podem apresentar, e podem ser fonte de moléculas bioativas novas. O conhecimento sobre a biodiversidade e o estudo da microbiota cavernícola, como a identificação e caracterização dos microorganismos, podem levar à identificação de novas espécies e estudos aprofundados podem identificar linhagens de interesse biotecnológico.

2.2 Identificação de fungos a partir de técnicas moleculares

Desde 1990, o sequenciamento de DNA vem sendo uma grande ferramenta para determinar os microorganismos, no estudo das relações entre espécies, ao eliminar problemas relacionados ao cultivo de microorganismos, em diferentes meios, culturas que não esporulam e dão mais segurança à identificação das espécies. A identificação molecular utiliza pequenos fragmentos de DNA padronizado e permite a comparação das sequências obtidas a sequências depositadas, em bancos de dados certificados, além de identificar qualquer organismo (VISAGIE et al., 2014).

Atualmente, o espaçador transcrito interno (ITS - Internal Transcribed Spacer) do DNA ribossômico é aceito como o "código de barras" de microorganismos, sendo o mais utilizado para a identificação de fungos (SCHOCH et al., 2012). Embora a região ITS seja transcrita, ela não codifica nenhuma função no organismo e acumula mutações neutras, sendo utilizada para diferenciar organismos estritamente relacionados. Contudo, para alguns gêneros de ascomicetos, o ITS não é suficientemente variável para distinguir todas as espécies estritamente relacionadas (SCHOCH et al., 2012; SEIFERT et al., 2007), como espécies dos gêneros *Penicillium*, *Aspergillus* e *Fusarium*. Com isso, um marcador secundário se faz necessário, para identificação de fungos ao nível de espécie e o qual deve ser fácil para amplificar e distinguir as espécies estritamente relacionadas (VISAGIE et al., 2014). A fim de identificar espécies de fungos, são utilizados, além do ITS, a segunda maior subunidade da RNA polimerase (RPB2), gene β-tubulina, sequências do fator de elongação parcial 1-α ou alguns outros genes codificadores de proteínas (SEIFERT et al., 2007). Para as espécies do gênero *Penicillium* e *Aspergillus*, por exemplo, os genes secundários mais indicados, na identificação, são β-tubulina, calmodulina e RPB2, os quais apresentam poder discriminatório, visto que o gene RPB2 tem vantagem de não conter íntrons no amplicon, proporcionando alinhamento robusto e fácil, quando usado filogeneticamente (VISAGIE et al., 2014).

Identificações corretas são necessárias, pois possibilitam informações valiosas sobre o microorganismo, como papéis ecológicos, características fisiológicas e bioquímicas, riscos e/ou benefícios à população (SEIFERT et al., 2007). Em casos de isolamento de novas espécies, permitem diferenciar as espécies relacionadas e podem assegurar a identificação da nova estirpe, além de contribuir para uma maior credibilidade do trabalho realizado.

2.3 Atividade antibacteriana

Algumas bactérias fazem parte da microbiota natural dos animais, cobrindo o trato intestinal, revestindo a pele e mucosas em que são benéficas ao seu hospedeiro, protegendo-o contra patógenos e doenças (SILVA, 2014). Entretanto outras bactérias são maléficas, causando doenças que podem levá-los à morte.

Nos últimos anos, tem aumentado os casos de doenças veiculadas por alimentos. Muitas bactérias são responsáveis pela contaminação de alimentos estocados e industrializados, principalmente, quando manipulados incorretamente, levando a problemas sérios de toxinfecções alimentares, como as bactérias Gram-positivas *Staphylococcus aureus*, *Listeria monocytogenes* e Gram-negativas *Salmonella* Cholerasius e patótipos *Escherichia coli* (OLIVEIRA, 2015).

A utilização indiscriminada de antibióticos tem levado à resistência dos microorganismos, principalmente de bactérias, que apresentam tempo de geração curto adaptando-se rapidamente ao ambiente. O aparecimento de linhagens bacterianas multirresistentes a drogas tem aumentado recentemente e, dentre essas bactérias, citam-se *E. coli*, *S. aureus*, *Salmonella*, *Shigella*, *Enterococcus* sp. e *Pseudomonas aeruginosa* (SILVA, 2014).

Atividade antibiótica tem sido encontrada, a partir do extrato de culturas de fungos filamentosos. O extrato bruto do fungo *Acremonium* sp. isolado de sedimentos marinho e testado contra bactérias patogênicas apresentou alta atividade antibacteriana contra *Bacillus subtilis*, *Pseudomonas putida*, *Vibrio cholarae* e *Micrococcus* sp. (SAMUEL et al., 2011). Os extratos de alguns fungos endofíticos isolados da planta medicinal *Nerium oleander* L. apresentaram atividade antibacteriana maior que o extrato da planta hospedeira, em que o fungo leveduriforme *Torula* sp. demonstrou boa atividade contra *L. monocytogenes* e *S. aureus* (HUANG et al., 2007). O extrato do fungo filamentoso *Guignardia* sp. proporcionou atividade contra *E. coli* e *S. aureus* (RODRIGUES et al., 2000). Também os extratos de *Penicillium citrinum* e *Aspergillus ochraceus* inibiram 80 e 100% do crescimento de microorganismos patogênicos, principalmente *P. aeruginosa* (MACHALSKIS et al., 2007).

Os fungos *Lewia infectoria* e *Chaetomium globosum*, isolados como endofíticos, apresentaram atividade inibitória contra *S. aureus*. Foram identificadas as substâncias pirrocidine C e cochiodinol a partir do extrato com atividade antibiótica contra essa bactéria (CASELLA et al., 2013). O composto phomopsichalasin obtido, a partir do fungo endofítico *Phomopsis* sp., exibiu atividade antibacteriana contra *B. subtillis*, *Salmonella enterica* sorotipo Gallinarum e *S. aureus* (HORN et al., 1995). As dicetopiperazinas sintetizadas por alguns fungos dificulta o inibidor-1 do ativador do plasminogênio, apresentando atividade antitumoral, antiviral, antibacteriana, antifúngica, agente antidiabético e ação sobre os neurotransmissores GABA (4-aminobutirato) e 5-HT (5-hidoxitriptamina) (MARTINS; CARVALHO, 2007)

Dentre os metabólitos secundários de microorganismos isolados, no Brasil, 85% são de origem fúngica e entre os locais de isolamento de microorganismos que mais apresentaram substâncias do metabolismo secundário, 66% são de endofíticos (IÓCA et al., 2014). Com isso, observa-se o potencial dos fungos filamentos, na síntese de compostos, que apresentam diversas atividades biológicas, entre elas a antibacteriana, visto sua importância decorrente de fatores como resistência a antibióticos e ao aumento na utilização de compostos naturais.

2.3.1 *Escherichia coli*

A bactéria *Escherichia coli* pertence à família *Enterobacteriaceae*, Gram-negativa, anaeróbia facultativa, não apresenta esporos e fermenta lactose com produção de gás (SILVA; JUNQUEIRA; SILVEIRA, 2007). É habitante natural do trato gastrointestinal de humanos e animais e a maioria das cepas não são patogênicas, no entanto alguns sorotipos podem causar doenças graves (FRATAMICO; GEHRING, 2014), provocar infecções gastrointestinais, como diarreia, colite hemorrágica e síndrome urêmica hemolítica e infecções extraintestinais, como infecção do trato urinário e meningite neonatal (YANG; WANG, 2014). As cepas de *E. coli* que causam infecções entéricas são classificadas em grupos, incluindo *E. coli* enteropatogênica (EPEC), *E. coli* enterotoxigênica (ETEC), *E. coli* enteroinvasiva (EIEC), *E. coli* enteroagregativa (EAEC), *E. coli* aderente difusivamente (DAEC) e *E. coli* produtora de toxina Shiga (FRATAMICO; GEHRING, 2014). As infecções intestinais causadas por *E. coli* estão ligadas ao consumo de alimentos e à água contaminados (YANG; WANG, 2014).

Dentre os grupos virulentos de *E. coli*, a ETEC é responsavel, principalmente, por causar diarreia em humanos, geralmente resultante da ingestão de alimentos e água contaminados (DUBREUIL, 2014). Na ETEC, os genes de virulência estão envolvidos na adesão ao tecido e células do intestino delgado e produção de moléculas tóxicas que afetarão a

homeostase intestinal (DUBREUIL, 2014). *E. coli* enteropatogênica (EPEC) foi o primeiro patovar da espécie associado à diarreia de verão em lactentes. A EPEC causa, principalmente diarreia aguda, em crianças pequenas, e os principais sintomas são diarreia aquosa profusa, vômitos e febre (BRÜSSOW, 2014). EPEC tem sido isolada de frutos do mar, na Korea, vegetais no México, frutas e laticíneos, incluindo leite pasteurizado, no Brasil (BRÜSSOW, 2014). Essas bactérias, além de causar toxinfecção, diminuem vida útil dos alimentos, causando perdas econômicas (MOREIRA et al., 2005).

2.3.2 *Staphylococcus aureus*

Staphylococcus aureus é uma espécie que pertence à família *Staphylococcaceae* (LUDWIG; SCHLEIFER; WHITMAN, 2009) com forma de cocos Gram-positivos, arranjados em cachos irregulares, que lembram cachos de uva, podendo apresentar ou não cápsulas e são anaeróbios facultativos. São microorganismos mesófilos com temperatura de crescimento de 7 a 47 °C. O gênero *Staphylococcus* é composto por 47 espécies contendo algumas patogênicas (LAMERS et al., 2012). Estão presentes na pele, fossas nasais, virilha e axilas de pessoas saudáveis, mas podem causar desde infecções simples, como espinhas e furúnculos, a graves como pneumonia, meningite, entre outras (SILVA, 2014). São bactérias conhecidas por desenvolver resistência a vários antibióticos.

Algumas cepas de *S. aureus* produzem enterotoxinas termoestáveis, que resistem ao tratamento térmico e podem causar vômito e diarreia (BROOKS; BUTEL; MORSE, 2000). Por estarem presentes na pele, são facilmente passadas para os alimentos, durante seu processo de manipulação (MURRAY et al., 2004). *Staphylococcus aureus*, também, está associado a quadros contagiosos de mastite bovina, sendo um dos principais agentes etiológicos dessa doença (FONTANA et al., 2010). Isolados de *S. aureus*, a partir de casos de mastite bovina, apresentam resistência à ampla variedade de antimicrobianos como as penicilinas e cefalosporinas, por produzirem enzimas que inativam esses antibióticos (PERINI, 2013). A mastite leva ao impacto negativo, na produção leiteira; no Brasil, as perdas variam de 12 a 15% por causa da grande prevalência dessa doença nos rebanhos (SILVA et al., 2010).

2.3.3 *Salmonella* Typhimurium e *Salmonella* Enteritidis

Segundo a Organização Mundial de Saúde, a *Salmonella* é o principal agente causador de doenças alimentares, em muitas partes do mundo (WORLD HEALTH ORGANIZATION

– WHO, 2005), inclusive, no Brasil. *Salmonella* é uma bactéria pertencente à família *Enterobacteriaceae*, Gram-negativa, anaeróbia facultativa, com forma de bastonete curto, não produz esporos, a maioria é móvel e com flagelos peritríquos. Apresenta crescimento na faixa de pH de 4,5 a 9,5 e temperatura ótima de cerca de 38°C e mínima de 5°C, com termossensibilidade relativa sendo destruída a 60°C por 15 a 20 minutos (FORSYTHE, 2002).

No gênero *Salmonella*, são consideradas duas espécies: *Salmonella enterica* e *Salmonella bongori* (GUIBOURDENCHE et al., 2010). *Salmonella enterica* é dividida em seis subespécies denominadas *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* e *indica* (BAUMLER, 1997). Usualmente, para designação dos sorotipos, é escrito o gênero *Salmonella* em itálico e, subsequente, o nome do sorotipo, em letra romana, iniciando com letra maiúscula (*S. enterica* subsp. *enterica* sorotipo Enteritidis ou *S.* Enteritidis).

Alguns subespécies são mais encontradas, envolvidas em doenças humanas como a *S. enterica* subsp. *Entérica*, correspondendo a 99% das salmoneloses em humanos, em que os principais sorotipos envolvidos na transmissão de salmoneloses, em animais, inclusive, no homem, são *S.* Enteritidis e *S.* Typhimurium (SILVA et al., 2007), que causam infecções gastrointestinais de severidade variável no homem e outros animais, sendo, também, transmitidas por alimentos. Alimentos de origem animal crus e mal cozidos, principalmente carne de frango e ovos, são usualmente fontes de contaminação (HUMPHREY, 2004). As enterocolites (salmoneloses) provocam febre, cólicas abdominais, dor de cabeça, diarreia, náusea e pode ocorrer vômito; os sintomas aparecem entre 6 e 48 horas (SILVA et al., 2007).

2.3.4 *Listeria monocytogenes*

O gênero *Listeria* pertence à família *Listeriaceae* que compreende bacilos delgados e curtos, Gram-positivos, anaeróbias facultativas, não esporulam, são móveis com flagelos peritríquios, halotolerantes e desprovida de cápsula. Esses microorganismos podem ser encontrados no solo, na água, na ração animal, nos alimentos, na microbiota natural e fezes de animais e humanos saudáveis (SILVA, 2014). Dentro do gênero *Listeria*, duas espécies são patogênicas ao homem e outros mamíferos: *Listeria monocytogenes* e *Listeria ivanovii* (GASANOV; HUGHES; HANSBRO, 2005); *L. monocytogenes* é um dos principais patógenos de humanos transmitido por alimentos.

Listeria monocytogenes causa a listeriose, doença invasiva que acomete, principalmente os grupos de riscos, como imunodeprimidos, gestantes e idosos, podendo causar meningite, septicemia, aborto e morte, em 20 a 30% dos casos, sendo considerada um

problema de saúde pública (PAPARELLA et al., 2008). Bactérias do gênero de *Listeria* podem ser encontradas, em alimentos como o leite cru ou pasteurizado, queijos, sorvetes, vegetais crus, peixes, aves, frutos do mar, carne e seus derivados (HARVEY; CHAMPE; FISHER, 2008). Em razão da resistência a condições, como elevadas concentrações de sais e baixas temperaturas, a gravidade da doença e a transmissão da listeriose por alimentos, os órgãos de saúde pública como também industrias de alimentos têm aumentado o interesse sobre esses microorganismos.

2.3.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa é bactéria Gram-negativas, aeróbia, não fermentativa, que se difunde no solo, água e ambientes úmidos, sendo a espécie frequentemente associada a doenças em seres humanos (LOVEDAY et al., 2014). Pode causar muitas infecções, atuando como patógeno oportunista, podendo causar infecções, em quase todos os órgãos e tecidos, principalmente, em pacientes comprometidos por doença, idade avançada ou deficiência imunológica (LOVEDAY et al., 2014). É capaz de crescer, em uma gama de temperaturas, explorar ambientes relativamente pobres em nutrientes, formar biofilmes e apresenta resistência inata e adquirida a muitos antimicrobianos e desinfetantes (LOVEDAY et al., 2014). Em microbiologia alimentar, é um dos principais deteriorantes, sendo um marcador de higiene, qualidade de alimentos e água para consumo humano (NEVES et al., 2014). Atualmente, o surgimento de *P. Aeruginosa*, produzindo metalo-beta-lactamases, tem sido um dos principais problemas de saúde pública, em todo o mundo, sendo isolada de pacientes, em vários países e, também, em águas residuais, água da torneira e rios urbanos, denotando o potencial para ser transmitida por meio de alimentos e água (NEVES et al., 2014).

2.3.6 *Aeromonas hydrophila*

O gênero *Aeromonas* pertence à família *Aeromonadaceae*, são Gram-negativos, anaeróbios facultativos, maioria móveis com flagelo polar, fermentam D-glicose, catalase e oxidase positivos e podem crescer em ambientes com 3% de NaCl. São muito encontradas, em ambientes aquáticos, como patogênicos de peixes (KIRKAN et al., 2003), e muitas infecções estão sendo associadas a esse gênero, ocasionando preocupações para a saúde pública. Esses microorganismos sintetizam exoenzimas, como DNases, RNases, proteases, lipases, gelatinases, amilases e elastases e algumas dessas enzimas estão relacionadas aos fatores de virulência (POPOFF, 1984).

A. hydrohila é considerada a espécie mais patogênica dentro desse gênero, causando septicemias primárias e secundárias, em imunocomprometidos, feridas graves em indivíduos saudáveis e, principalmente gastroenterites, que geram diarreia secretória, disentérica, crônica, semelhante à colérica e a dos viajantes (ABBOTT et al., 2003), afetando, principalmente crianças, idosos e imunocomprometido. A água é fonte potencial de *Aeromonas*, o qual em contato com a pele lesionada leva à formação de feridas, e as doenças entéricas ocorrem pela ingestão de água não tratada corretamente e alimentos contaminados como vegetais crus e carne crua ou mal cozida (BAUAB et al., 2003).

2.3.7 *Cronobacter sakazakii*

Bactérias do gênero *Cronobacter* pertencem à família *Enterobacteriaceae*, são Gram-negativas, anaeróbias facultativas, móveis e não formadoras de esporos (YAN; GURTLER, 2014). Espécies de *Cronobacter* podem provocar doenças como infecções nosocomiais, incluindo pneumonia, septicemia, infecções de feridas e osteomielite (HEALY et al., 2010) e doença invasiva em lactentes e neonatos. Dentre as espécies de *Cronobacter* de maior importância, encontra-se *C. sakazakii* por causar infecções fatais em neonatos. A enterocolite necrosante neonatal, por exemplo, causa pneumatoses intestinais, necroses, vômitos, distensão abdominal, hematoquezia, podendo passar para peritonite, pneumoperitonite e choque (VIEIRA; LOPES, 2003). Esses microorganismos podem ser transmitidos por alimentos, sendo isolados, também, do ambiente, clínicas e, principalmente de fórmulas em pó, utilizadas para alimentação infantil (HIMELRIGHT et al., 2002). Mais de 120 casos de doença relacionada com *C. sakazakii* foram relatados e a maioria dos casos são infecções, potencialmente fatais, muitos destes surtos têm sido associados ao consumo de fórmula infantil em pó contaminada (YAN; GURTLER, 2014).

2.4 Atividade antioxidante

Os radicais livres são átomos, moléculas ou íons que apresentam elétrons de valência desemparelhados o que os torna altamente reativos, podendo associar-se a átomos isolados, como o hidrogênio ou íons metálicos e a macromoléculas como carboidratos, lipídeos, proteínas e ácidos nucleicos. Em decorrência dessa característica, são denominados agentes oxidantes. Os radicais livres são produzidos, naturalmente, durante os processos bioquímicos celulares que envolvem reações de oxidação-redução (SHAMI; MOREIRA, 2004). Essas substâncias apresentam função importante na defesa do organismo como na fagocitose, em

que são produzidos para matar agentes invasores como fungos e bactérias. Entretanto, quando há acentuada produção de radicais livres, ocorre o estresse oxidativo pelo desequilíbrio entre os níveis de antioxidantes e oxidantes.

O oxigênio tem atividade fundamental no metabolismo de aeróbicos. Entretanto forma compostos reativos, à medida que ganha ou perde elétrons, durante os processos metabólicos celulares, sendo chamados espécies reativas do oxigênio (ERO), que são ou geram radicais livres. Pela configuração eletrônica do oxigênio, que tende a receber um elétron por vez, forma, assim, compostos intermediários altamente reativos, como o ânion radical superóxido ($O_2^{\cdot -}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($OH^{\cdot}$). Os radicais livres são resultantes de processos biológicos que envolvem várias organelas como mitocôndrias, retículo endoplasmático, peroxissomos, lisossomos, membranas e núcleo, como também, a partir de fontes externas como radiações, poluição do ar, tabagismo, anestésicos, solventes orgânicos e pesticidas (SOARES, 2002). Os danos causados pelos radicais livres nas células podem ser prevenidos ou reduzidos, por meio da atividade de antioxidantes que podem agir de forma direta na neutralização da ação dos radicais livres ou indireta por sistemas enzimáticos (SHAMI; MOREIRA, 2004). Os antioxidantes são substâncias que, em concentrações baixas com relação ao substrato, retardam ou inibem a oxidação do substrato (DUARTE-ALMEIDA et al., 2006). Os antioxidantes podem ser classificados como enzimáticos sendo compostos por várias enzimas como a superóxido-dismutase (SOD), a catalase (CAT) e a glutationa peróxidase (GPx) e não enzimáticos como os compostos fenólicos, vitaminas (C, A e E), minerais, pigmentos naturais, carotenoides entre outros compostos (MESSIAS, 2009).

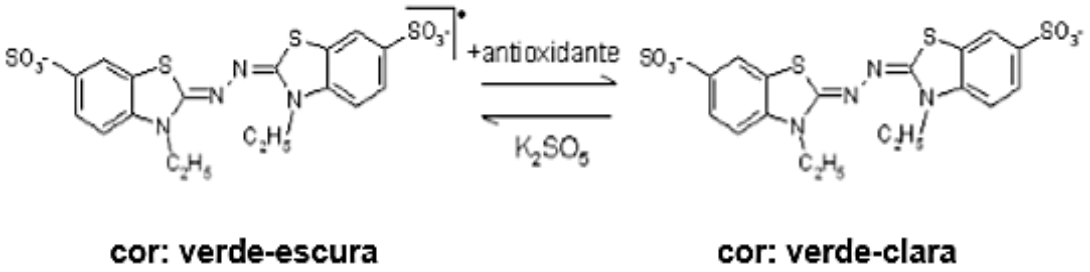
Os compostos antioxidantes podem ser de origem natural, como o ácido ascórbico, α-tocoferol e compostos fenólicos, como também de origem sintética. Antioxidantes sintéticos são muito utilizados, a fim de impedir a oxidação lipídica de produtos alimentares como o 6-hidroxi-2,5,7,8-tetrametilchroman-2-ácido carboxílico (Trolox), butil-hidroxi-tolueno (BHT) e butil-hidroxi-anisol (BHA), sendo o Trolox mais utilizado por ser análogo à vitamina E e de natureza hidrossolúvel (RUFINO, 2006). Contudo Chalamaiah et al. (2012) relatam os efeitos tóxicos que os sintéticos podem causar à saúde dando como alternativa os antioxidantes naturais. Alguns compostos antioxidantes já foram isolados de fungos endofíticos tais como ácidos fenólicos e seus derivados (HUANG et al., 2007), isobenzofuranonas e isobenzofuran (HARPER et al., 2003), obtidos do fungo *Pestalotiopsis microspora*. Chandra e Arora (2014), por meio de análises cromatográficas, encontraram vários compostos com atividade antioxidantes, a partir de extratos do fungo *Aspergillus terreus*.

Muitos métodos são utilizados, para avaliar a capacidade antioxidante, dentre eles estão:

2.4.1 Atividade sequestrante do radical ABTS$^{\bullet+}$

A atividade antioxidante pode ser verificada por métodos de descoloração que podem ser aplicados a antioxidantes lipofílicos e hidrofílicos (RE et al., 1999). Um método muito utilizado, para medir a atividade antioxidante, é pela captura do radical 2,2-azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), a fim de se obter o radical por reação química eletroquímica ou enzimática (RUFINO et al, 2007), em que o radical livre pode ser formado pela oxidação do ABTS com persulfato de potássio (FIGURA 1).

Figura 1 – Estabilização do radical ABTS$^{\bullet+}$ e sua formação pelo persulfato de potássio.



Fonte: Rufino et al. (2007).

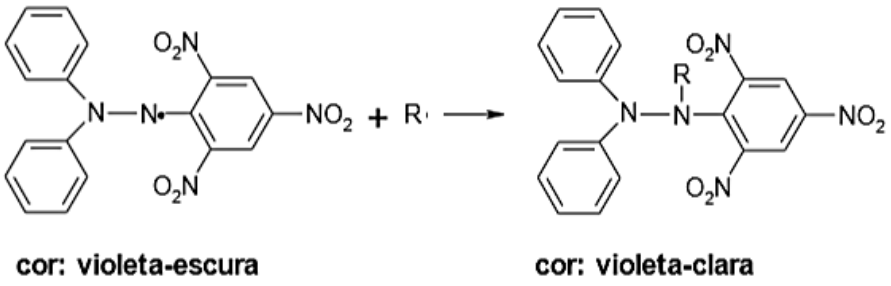
O radical ABTS$^{\bullet+}$ é solúvel, em água e solventes orgânicos, dessa forma, pode-se avaliar a atividade antioxidante de compostos hidrofílicos e lipofílicos (KUSKOSKI, 2005), observando o decréscimo da absorbância e consequente mudança de cor, da solução de verde-escura para uma cor mais clara, de acordo a capacidade antioxidante do composto teste.

2.4.2 Atividade sequestrante do radical DPPH

É um teste muito utilizado por ser fácil e preciso, na avaliação de compostos de origem natural com atividade antioxidante, sendo um ensaio muito aplicado, pois o DPPH possui alta estabilidade e a coloração violeta que é perdida no processo de redução (HRISTEA et al., 2006). O método DPPH, inicialmente descrito por Brand-William et al., (1995), baseia-se, no sequestro do radical DPPH (1,1-difenil- 2-picrilidrazil), por compostos antioxidantes. Isso ocorre, quando o DPPH recebe um elétron ou um radical hidrogênio de um composto antioxidante ou de um radical livre, levando a um decréscimo na absorbância, em

que a coloração violeta-escura torna-se mais clara, de acordo com a capacidade antioxidante do composto testado. O radical livre de DPPH (FIGURA 2) pode ser obtido pela dissolução do reagente em meio orgânico (SANTOS et al, 2011).

Figura 2 – Estabilização do radical livre DPPH.



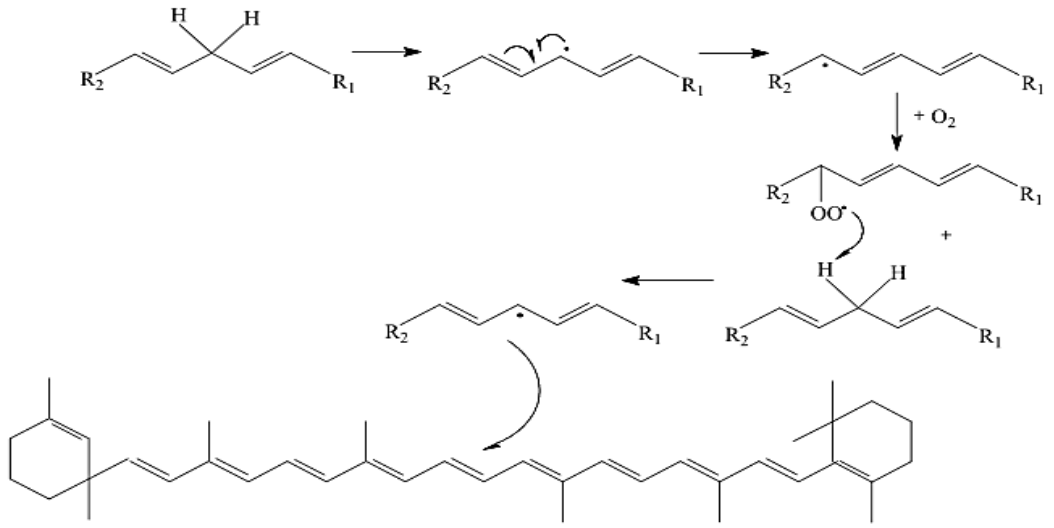
Fonte: Santos et al. (2011).

A redução do DPPH pode ocorrer por duas vias: por transferência de um átomo de hidrogênio ou por transferência de elétrons por compostos antioxidantes (EBRAHIMABADI et al., 2010).

2.4.3 Sistema β-caroteno/ácido linoleico

Os carotenoides são compostos naturais que apresentam propriedades lipofílicas e, em sua maioria, apresentam um extenso sistema de duplas ligações conjugadas que é responsável pela atividade antioxidante como o β-caroteno. Substâncias como o β-caroteno inibem o oxigênio singleto e interagem sinergicamente com a vitamina E, inibindo, assim, a peroxidação lipídica (RUFINO, 2006). Esse método avalia a capacidade de um composto em proteger um substrato lipídico da oxidação (DUARTE-ALMEIDA et al., 2006). No sistema β-caroteno/ácido linoleico, desenvolvido por Marco (1968), modificado por Miller (1971), utilizam-se o ácido linoleico, monopalmitato de polioxietileno sorbitan (Tween 40) e o β-caroteno, em que os radicais livres, oriundos da oxidação do ácido linoleico, agem sobre as moléculas insaturadas do β-caroteno que perde suas características cromóficas (FIGURA 3), com isso, ocorre o decréscimo da absorbância a 470 nm.

Figura 3 – Reação de oxidação do sistema β-caroteno/ácido linoleico.



Fonte: Oliveira (2015).

Compostos com atividade antioxidante neutralizam a ação dos radicais livres, formados como o linoleato formado no sistema, inibindo a extensão de descoloramento do β-caroteno. Assim, na presença de antioxidantes, a absorbância e, consequentemente, a coloração amarelo-alaranjada permanecem por mais tempo (OLIVEIRA et al., 2008).

2.5 Quantificação de compostos fenólicos totais

Os compostos fenólicos são substâncias, que contêm um anel aromático com um ou mais componentes hidrofílicos e seus grupos funcionais, apresentam grande variedade de compostos, compreendendo diversas categorias, como fenóis simples, ácidos fenólicos, flavonoides, cumarinas, entre outros (NACZK, 2004). Substâncias fenólicas são uma das classes de compostos antioxidantes naturais que auxiliam ou retardam a velocidade da oxidação pela inibição de radicais livres ou complexação de metais (DUARTE-ALMEIDA et al., 2006). Muitos benefícios são atribuídos aos compostos fenólicos, além da atividade antioxidante, como anti-inflamatória, antimicrobiana e anticarcinogênica (DE BEER, 2003).

A quantificação de compostos fenólicos pode ser realizada por várias técnicas e uma das mais utilizadas é a que utiliza o reagente Folin-Ciocalteu. O reagente Folin-Ciocalteu é uma mistura do ácido fosfomolibídico e ácido fosfotunguístico, em que esses ácidos são reduzidos por agentes redutores como as substâncias fenólicas, e a redução dos compostos mostra uma coloração azulada em uma faixa de absorvância de luz de até 765 nm (CARVALHO, 2010). O teste que utiliza o Folin-Ciocalteu é um método colorimétrico, em

que a coloração obtida pela redução dos ácidos fosfomolibídico e fosfotunguístico aos óxidos de tungstênio e molibdênio, possibilita a determinação das substâncias redutoras, em que a concentração dos fenóis é proporcional à intensidade da absorção da luz a um comprimento de onda (WATERHOUSE, 2002).

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 – MOLECULAR IDENTIFICATION AND ANTIBACTERIAL ACTIVITY OF EXTRACT FROM PIGMENT-PRODUCING FILAMENTOUS FUNGI *

ARTIGO FORMATADO DE ACORDO COM A NORMA NBR 6022 (ABNT 2003)

Dérica Gonçalves Tavares **

Prof. Dra. Patrícia Gomes Cardoso (Orientador) ***

ABSTRACT

In view of the increasing in bacterial resistance to many different drugs, the search for new antimicrobial molecules has been growing recently. The filamentous fungi are invaluable sources of bioactive matabolites and their antimicrobial properties may be associated with molecules that color the organic fungal extract. Therefore, the objective of this work was to use molecular techniques to identify fungal isolates and to evaluate the activity of EtOAc extract against pathogenic bacteria, determining their minimum bactericidal concentration (MBC). Twelve filamentous fungi were selected based on their production of pigments in solid culture media. These fungi were identified using the sequences of the ITS 5.8S genes of rDNA, Larger Subunit of RNA Polymerase (RPB2), Calmodulin and β-tubulin. Five fungi extracts presented bactericidal activity against at least one pathogenic bacterial strain.. The extracts of *Aspergillus sydowii* (CML 2967) and *A. keveii* (CML 2968) showed MBC of 5 mg mL^{-1}, while *Fusarium* sp. (CML 2969) the MBC ranged from 5 to 0.625 mg mL^{-1} and *Periconia ignaria* (A2C47) the MBC ranged from 5 to 1.25 mg mL^{-1}. The extract of the endophytic fungus *Arcopilus* sp. (A2C54) exhibited the highest bactericidal activity, with MBC 0.00244 and 0.0195 mg mL^{-1} for *Staphylococcus aureus* GL 8702 and GL 5674, respectively, and 0.625 mg mL^{-1} for *Salmonella* Enteritidis. The extract of *Arcopilus* sp. (A2C54) presents a strong pigmentation and high antibacterial activity and these results show the potential of this filamentous fungus as new sources of antimicrobial metabolites.

Keywords: Endophytic fungus. Cave fungi. Antimicrobial. Foodborne pathogenic bacteria.

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* Artigo Apresentado para a conclusão do curso de pós-graduação em Microbiologia Agrícola da Universidade Federal de Lavras.

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1 INTODUCTION

The large development of antibiotic resistance of pathogenic microorganisms, especially bacteria, has increased the demand for new classes of antimicrobials. An estimated of 700,000 deaths per year have been attributed to antimicrobial resistance worldwide and about 10 million deaths are predicted by 2050, especially in Asia and Africa (HAMPTON, 2015). Among the pathogens that most urgently need new drugs are: *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumamii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Eterobacter* species, for both hospital and community infections (HAMAD, 2010).

Fungal metabolites are exploited in different contexts, from agriculture to medicine. Studies with filamentous fungi and their antimicrobial potential were boosted from the accidental discovery of penicillin by Fleming in 1928 (TULP; BOHLIN, 2004). Since then, these microorganisms represent sources of new molecules that are used for the production of medicines, presenting great importance for humanity. β-lactam antibiotics originating from filamentous fungi are the most used by the population, such as penicillin, accounting for 19 % and cephalosporins with 28 % of the world antibiotic market, and moving $ 7.9 billion and $ 11.9 billion in 2009, respectively (HAMAD, 2010).

In addition to the isolated filamentous fungi of different environments such as *Penicillium* and *Aspergillus* genus, which synthesize many different bioactive compounds, interest in the isolation of plants with endophytic fungi has increased. Studies demonstrate the potential of these endophytes and many antimicrobial molecules have been isolated and characterized. Endophytic fungi of the genus *Chaetomium,* for example, synthesize many classes of substances that exhibit several active properties such as antitumor, antimalaria, antioxidants, antibiotics and with more than 200 types and structures of metabolites identified (ZHANG et al., 2012). The natural pigments of *Chaetomium* species that is identified as azaphilones are poliketide derivatives that also have many active antimicrobial properties (WANI; SOUMYA; SHARMILA, 2016). Some authors found antimicrobial and antioxidant activity of natural pigments of fungal origin (DUFOSSÉ et al., 2014; GEWEELY, 2011; MAPARI; MEYER; THRANE, 2008; VENDRUSCOLO et al., 2013)

The presence of medicinal properties in molecules of natural origin, which confer color, are of great interest for medicines production and also in foods and cosmetics, since the association of these benefits in natural compounds can substitute synthetic compounds. Therefore, the objective of this work was to use molecular techniques for the identification of

pigment-producing filamentous fungi and to evaluate the antibacterial activity of EtOAc extract from these fungi, determining their minimum inhibitory concentration on pathogenic bacteria.

2 METHODS

2.1 IDENTIFICATION OF THE FILAMENTOUS FUNGI

The filamentous fungi used for this work were selected by the production of pigments in culture media. The fungi used are part of the collection of the Laboratório de Bioprospecção e Genética de Fungos Filamentosos (BIOGEN) from the Universidade Federal de Lavras, Brazil. The fungi *Aspergillus keveii* (CML 2968), *Aspergillus sydowii* (CML 2967), *Penicillium chermesinum* (CML 2966), *Penicillium flavigenum* (CML 2965), *Lecanicillium aphanocladii* (CML 2970) and *Epicoccum nigrum* (CML 2971) were isolated from Brazilian caves. *Fusarium* sp. (CML 2969) was isolated as a laboratory contaminant. These fungi were previously identified (SOUZA et al., 2016) using the ITS (Internal Transcribed Spacer) and deposited in the Coleção Micológica de Lavras (CML) do Departamento de Fitopatologia da Universidade Federal de Lavras, Brazil.

The fungi A2C32, A2C47, A2C54 and A2S61 were isolated as endophytes from the *Eremanthus* sp. plant popularly known as Candeia (GODINHO, 2016). The total DNA was extracted from the mycelium grown in PD broth (200 g L^{-1} potato; 20 g L^{-1} dextrose) using the Wizard Genomic DNA Purification Kit®. The internal transcribed spacer (ITS)-5.8S region of rDNA was amplified: ITS 4 (5'-TCCTCCGCTTTATTGATATGC-3') was the forward primer and ITS 5 (5'- GGAAGTAAAAGTCGTAACAAGG-3') was the reverse primer (WHITE et al., 1990). The polymerase chain reaction (PCR) conditions for the ITS region were: initial denaturation at 95 °C for 2 min, 35 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and final extension at 72 ºC for 7 min. The RNA polymerase II second largest subunit (RPB2) region was amplified: 5F (5'-GAYGAYMGWGATCAYTTYGG-3') was the forward primer and 7CR (5'-CCCATRGCTTGYTTRCCCAT-3') was the reverse primer (LIU; WHELEN; HALL, 1999). The PCR conditions for the RPB2 region were: initial denaturation at 95 °C for 2 min, 35 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and final extension at 72 ºC for 7 min. The PCR was prepared with a final volume of 30 µl containing 15 µL of the TopTaq Master Mix Kit©, 12 µL of sterile ultrapure water, 1 µl of each primer (10 pmol) and 1 µl of DNA. Samples were sequenced at Macrogen in South

Korea. Consensus sequences were assembled from forward and reverse sequences using SeqAssem ver. 07/2008 (SequentiX - Digital DNA Processing, Klein Raden, Germany). Additional sequences were obtained from Genbank (TABLE 4, APPENDIX A). Phylogenetic analyses were performed using MEGA 5 (Tamura et al. 2011). Sequences were aligned using CLUSTAL W as implemented in MEGA 5. Maximum Likelihood phylogenetic trees were constructed for each gene and for the combined dataset using the Kimura 2-parameter model with gamma-distributed rate heterogeneity for nucleotide substitution. Clade support was inferred from 1000 bootstrap replications. The sequences generated in this study will be deposited in Genbank (TABLE 4, APPENDIX A).

To complement the molecular identification, it was observed the characteristic structures such as conidiophore, fruiting body, spores of the fungi that showed identity less than 98% with the reference isolates from the sequences of the RPB2, calmodulin and β-tubulin genes. For this, the fungi were grown in five different culture media and incubation temperatures (TABLE 1) for 15 days, in order to cause sporulation of the fungi.

Table 1 – Culture media used for sporulation of endophytic fungi.

Medium* Incubation at 15, 20, 25, 30 e 35 ºC	Composition	Reference
Malt Extract Agar (MEA)	20 g L^{-1} malt extract; 20 g L^{-1} glucose; 1 g L^{-1} peptone; 20 g L^{-1} agar.	Markovskaja and Kacergius (2014)
Oatmeal Agar (OA)	30 g L^{-1} oatmeal; 15 g L^{-1} agar	Rodríguez; Stchigel; Guarro (2002)
Corn Meal Agar (CMA)	60 g L^{-1} corn meal;15 g L^{-1} agar	Thompson; Aveling; Prieto (2013)
Sabouraud Agar (SDA)	40 g L^{-1} glucose; 10 g L^{-1} peptone; 15 g L^{-1} agar	Rajendran et al. (1990)
Potato Dextrose Agar (PDA)	200 g L^{-1} potato; 20 g L^{-1} glucose; 15 g L^{-1} agar	Wang et al. (2014)

Source: Tavares (2017).

2.2 EXTRACTION OF FUNGAL CULTURES

The methodology used was according to Souza et al. (2016). Disks of approximately 9 mm in diameter from the fungal colonies were transferred to 1 L of PD broth, which was

incubated at 30 ºC in the dark on shaker at 150 rpm for 7 days. After that, the cultures were filtered and extracted twice by liquid-liquid partition with volume 0.5 ethyl acetate (EtOAc). The extracts were concentrated in a rotary evaporator (RV10 digital; IKA) at 80 ºC and 150 rpm and the negative control was obtained by extraction from the PD broth without the inoculum.

2.3 ANTIBACTERIAL ACTIVITY

2.3.1 Screening by disk diffusion method

The pathogenic bacterial species used in this study were enterotoxigenic *Escherichia coli* 055, enteropathogenic *E. coli* ATCC 35401, *Salmonella* Enteritidis S64, *S.* Typhimurium S190, *Listeria monocytogenes* ATCC 19117, *Staphylococcus aureus* GL 8702, *S. aureus* GL 5674, *Cronobacter sakazakii* ATCC 29004, *Pseudomonas aeruginosa* ATCC 25853 and *Aeromanas hydrophila* ATCC 7966. Stock cultures were stored in freezing medium (150 mL L^{-1} glycerol; 5 g L^{-1} bacterial peptone; 3 g L^{-1} yeast extract; 5 g L^{-1} NaCl; pH 7.0). Cultures were thawed at room temperature and reactivated by inoculating 200 µL in 10 mL Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 h. After reactivation, 200 µL of the inoculum were transferred to 10 ml Tryptone Soy Broth (TSB) and incubated at 37 °C for 24 h. After that period, 1 mL of the inoculum was centrifuged at 5000 rpm for 5 min to separate the bacterial cells from the TSB medium, which were resuspended in saline medium (8.5 g L^{-1} NaCl). The inoculum was standardized by McFarland scale at 0.5 ($1.5x10^8$ CFU mL^{-1}). The screening was done by the disk diffusion technique (NCCLS, 2000). Soybean Tryptone Agar (TSA) medium was used in 120 mm diameter Petri dishes. Approximately $1.5x10^8$ UFC mL^{-1} of each bacterial strain was inoculated and 6 mm diameter sterile filter paper disks were added. The filter paper disks were filled with 5 µL of each 5 mg mL^{-1} fungal extract resuspended in DMSO (dimethylsulfoxide) and the plates incubated at 37 °C for 24 h. After this period, the formation of the inhibition halo was observed and its diameter was measured. The experiment was performed in triplicate with a control consisting of Chloramphenicol at the concentration of 5 mg mL^{-1}.

2.3.2 Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration was determined using the microdilution technique (M7-A6) (NCCLS, 2003) with adaptations. In this method, a total of 150 µL of the TSB plus 0.5% Tween 80 and the fungal extracts at concentrations: 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 e 0.039 mg mL^{-1}, were added to the wells of 96-well polystyrene plates. For the *S. aureus* strains the isolate A2C54 fungus extract was further diluted to the concentrations 0.0195, 0.00976, 0.00488, 0.00244 mg mL^{-1}. Ten microliters were inoculated of the standardized bacterial cultures. Plates were incubated at 37 °C for 24 h. After this period, 5 µL of the fungal extratct were plated in TSA and incubated at 37 °C for 24 h. The experiment was performed in triplicate with a negative control, containing TSB plus 0.5% Tween 80 and a positive control, containing TSB plus 0.5% Tween 80 and inoculum.

2.4 STATISTICAL ANALYSIS

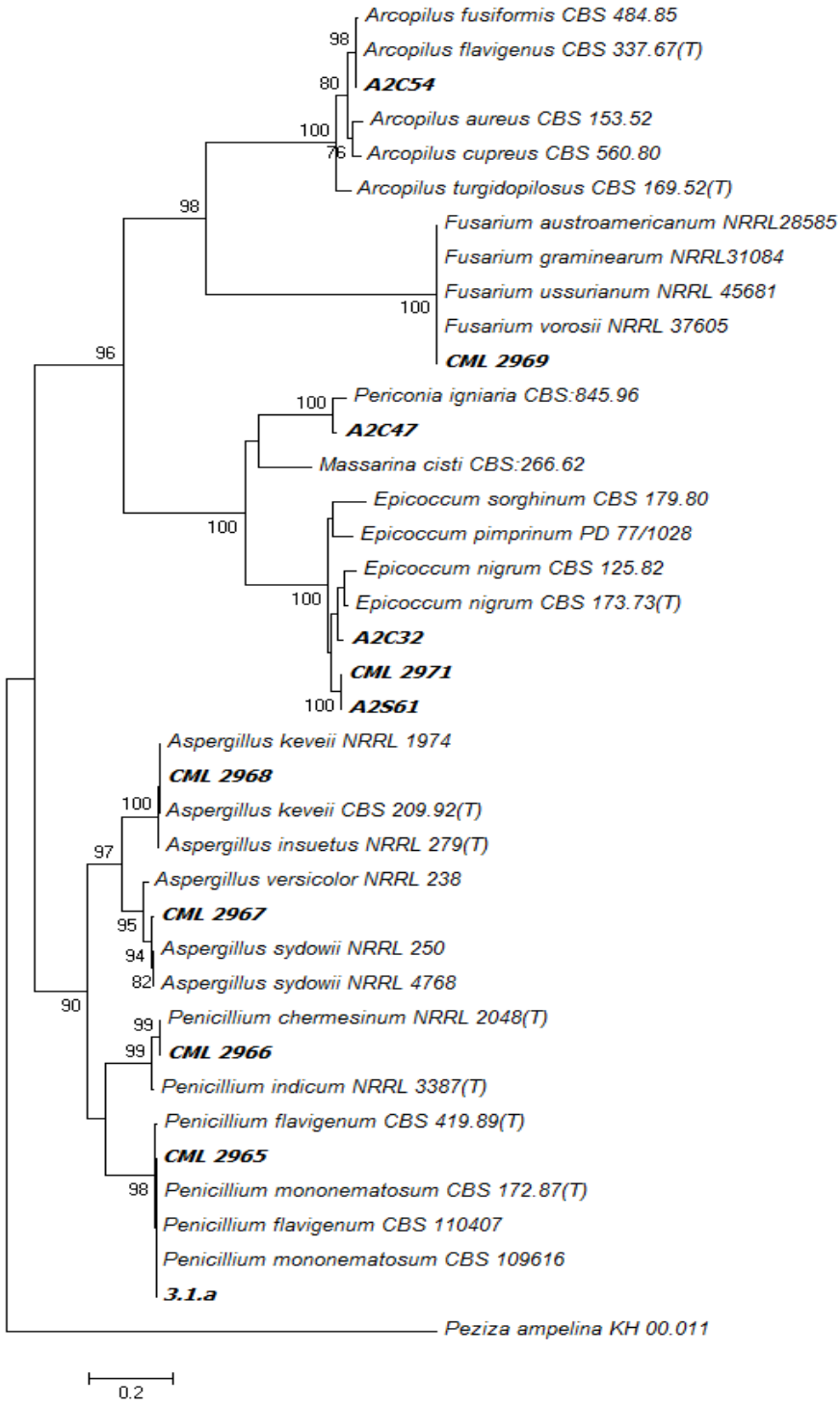
The assay was composed of twelve treatments with three replicates and the results of the test by the disc diffusion method were submitted to analysis of variance by the SISVAR (FERREIRA, 2000) program and the means were compared by the Scott and Knott test at 5 % significance level.

3 RESULTS AND DISCUSSION

3.1 IDENTIFICATION OF THE FILAMENTOUS FUNGI

Individual phylogenetic trees were constructed for each gene region (FIGURE 4, APPENDIX B). The combined phylogenetic tree used the ITS and RPB2 regions of all genus, with the exception of *Lecanicillium*, is shown in Figure 1.

Figure 1 – Maximum Likelihood analysis with the evolutionary model K2+G, tree formed from ITS and RPB2 genes was constructed from the sequences of fungal isolates and reference.



Numbers above branches are bootstrap values obtained from 1000 replications; only branches supported by more than ≥ 70 % were shown. *Peziza ampelina* (KH 00.011) was used with outgroup. T: ex-type isolated.
Source: Tavares (2017).

The isolates CML 2966, CML 2968 and CML 2967 were identified as *Penicillium chermesinum* (99% bootstrap), *Aspergillus keveii* (70 % bootstrap) and *Aspergillus sydowii* (75 % bootstrap), respectively. For these isolates the use of the RPB2 region confirm the identification found by Souza et al. (2016), which used only the ITS, confirming the found species. The isolates CML 2965 and 3.1.a are grouped with the *P. flavigenum* and *P. mononematosum* species. In this work, the ITS and RPB2 regions did not present sufficient polymorphism to separate this species. However, Souza et al. (2016), using the ITS region, identified these isolates as *P. flavigenum*. Therefore, studies using other gene regions for the confirmation of this species should be performed. The RPB2 region used in this work is commonly used for taxonomy of *Penicillium* and *Aspergillus* and proved to be a good marker for species identification of these genera (PETERSON, 2008; BARRETO et al., 2011). The combination of ITS, RPB2, CaM and BenA are useful for characterization of *Penicillium* species (VISAGIE, 2014). To identify new *Aspergillus* species it is recommended to use the ITS, RPB2, β-tubulin, calmodulin and actin genes that have a good database (SAMSON et al., 2007). Aguileta at al. (2008), using a bioinformatics approach, observed that the RPB1, RPB2, TEF1α, β-tubulin and γ-actin genes do not show the best performance in fungal systematics.

The isolate CML 2969 was grouped with strain of species of the complex *F. graminearum*. However, the ITS and RPB2 regions were not sufficiently variable to separate the species within the complex. The *F. graminearum* species complex comprises at least 16 phylogenetically distinct species (HAO et al., 2016). The genus *Fusarium* is one of the largest genus of fungi filaments and the characterization and phylogeny can be complex due to existing differences that are morphologically imperceptible (O'DONNELL et al., 2004). In order, to separate species from this complex, other gene regions may be used, such as 3-O-acetyltransferase (*Tri101*), partial translation elongation factor (*EF-1α*) and ammonia ligase (*URA*) (O'DONNELL et al., 2008; HAO et al., 2016).

The isolates CML 2971, A2C32 and A2S61 were grouped with the ex-type species of *Epicoccum nigrum* (CBS 173.73) and *E. nigrum* (CBS 125.82). The species of *E. nigrum* are highly variable and may present different morphological and physiological types (ARENAL et al., 2002; FÁVARO et al., 2011). Fávaro et al. (2011) proposed the reclassification of *E. nigrum* based on a polyphase analysis, as well as the reclassification of many sequences deposited in GenBank and collection of cultures. The isolates of *E. nigrum* (CBS 125.82 and CBS 173.73) used in the phylogenetic tree differ by 20 bp in RPB2, requiring further studies to confirm if they are of the same species (CHEN et al., 2015). The A2C32, A2S61 and CML

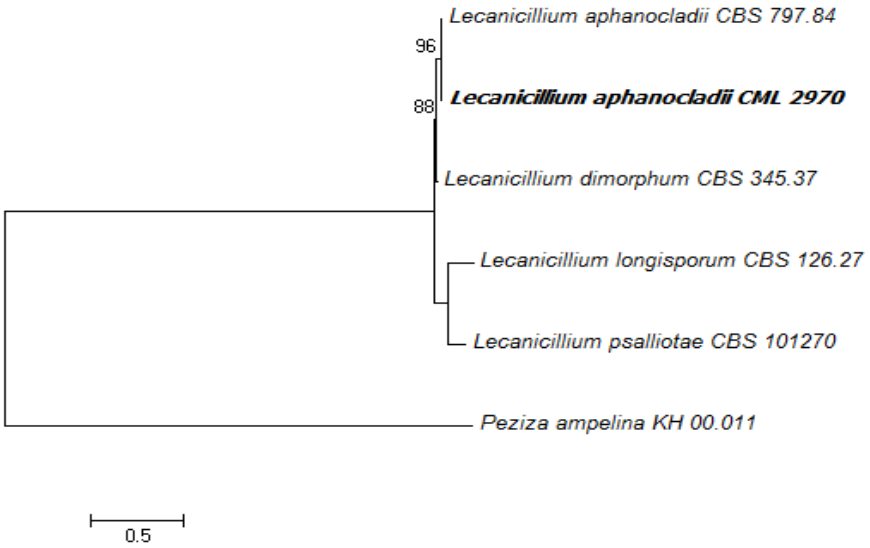
2971 isolates differ by more than 20 bp for this same gene region, so further molecular and morphological studies are needed to confirm the found species.

Isolate A2C47 was identified as *Periconia ignaria* (*Massarina ignaria*), presenting a well-supported clade with 100% bootstrap. The genus *Periconia* is a polyphyletic that present a complicated taxonomy and little clarified phylogeny (SEIFERT; GAMS, 2011). Some authors treat the genus *Periconia* as members of *Massarinaceae*, based on the topology of the genealogical trees (ZANG et al., 2012; HYDE et al., 2013). However, *Periconia* was again segregated for *Periconiaceae*, because they form a clade strongly supported by the SSU and LSU of nrDNA gene regions and tef1 (TANAKA et al., 2015), bringing back this family (NANNIZZI, 1934). More than 185 species are described as *Periconia*, with only reports of two species that present sexual phase (TANAKA et al., 2016).

The isolate A2C54 was grouped within the *Arcopillus* genus, being close to the strain of *A. fusiformis* (CBS 484.85) and the ex-type isolate of *A. flavigenum* (CBS 337.67), family *Chaetomiaceae*. Wang et al. (2016) proposed the genus *Arcopilus* due to the clade *A. aureus* (= *Chaetomium aureum*) to be phylogenetically distant from the *Chaetomium globosum* species complex, possibly representing a new genus. The gene regions that strongly support this clade are: RPB2, Tub2, ITS and LSU. The five species previously referred to as *Chaetomium* were renamed as *A. aureus*, *A. cupreus*, *A. fusiformes*, *A. flavigenum* and *A. turgidopilosus*. Phylogenetics analysis of six loci was found in the 28S large subunit (LSU) and 5.8S ITS gene regions were not reliable to reach the species, but β-tubulin (Tub2) and RPB2 were shown to be promising to differentiate species within *Chaetomiaceae* (WANG et al., 2016).

Phylogeny based on the RPB2 gene confirmed the identification of the CML 2970 isolate as *L. aphanocladii* (FIGURE 2), presenting a well-supported clade with 96 % bootstrap. The sequence of ITS and mitochondrial gene were used to classify species of *Lecanicillium*, and the ITS region was able to discriminate most of the isolates; the mitochondrial gene contributed to solve some ambiguities and relationships of the proposed new species of *Lecanicillium* (KOUVELIS; SIALAKOUMA; TYPAS, 2008).

Figure 2 – Maximum Likelihood analysis with the evolutionary model K2+G, tree formed from RPB2 gene was constructed from the sequences of *L. aphanocladii* CML 2970 and reference isolates.

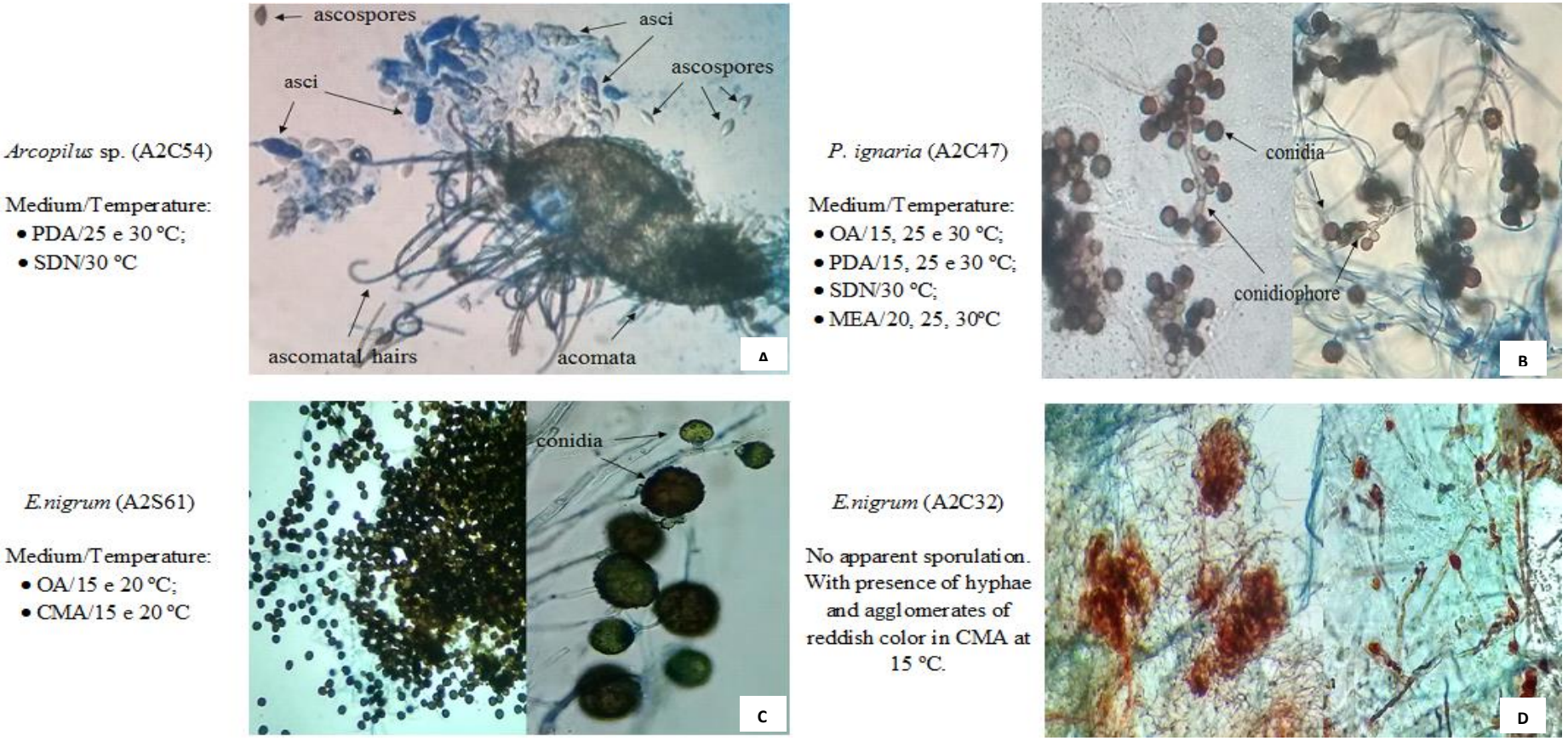


Numbers above branches are bootstrap values obtained from 1000 replications; only branches supported by more than ≥ 70 % are shown. *Peziza ampelina* KH 00.011 was used with outgroup.
Source: Tavares (2017).

In order to complement the molecular identification, structures of the genus of endophytic fungi were observed after 15 days of incubation, as shown in Figure 3. The structures of *Arcopilus* sp. (A2C54) present ascomata with brown wall, ascomata hairs with arched apex, and ascos with 8 ascospores that turn brown when mature and present colonies with red to rust exudates. Ascomata on the sufarce was present only in PDA medium at temperatures of 20 and 30 °C (FIGURE 3. A). Due to the lack of information, many species were defined by morphology based on ascomat hairs and also by the morphology of the ascos, ascospores and structural surface of the ascomas wall (WANG et al., 2016).

The microscopy of *Periconia ignaria* (A2C47) shows the presence of conidiophores with dark brown globular verrucose conidia (FIGURE 3. B). Abundant sporulation occurred mainly in OA medium at 15, 25 and 30 °C. The genus *Periconia* produces macronematous conidiophores with apical conidial stipe and head, but sometimes apices are sterile and conidiogenic cells with conidia appear in the lower and basal part of the stipe, being known as micronematous conidiophores (MARKOVSKAJA; KACERGIUS, 2014), as observed in Figure 3.B.

Figure 3 -Structures of endophytic fungi. A) Ascomata of *Arcopilus* sp. (A2C54) of the perithecium type with asci and ascospores. B) Conidiophore and conidia of *P. ignaria* sp. (A2C47). C) Conidia of *E. nigrum* (A2S61). D) Hyphae and agglomerates with reddish color of *E. nigrum* (A2C32). Sporulation medium: PDA (Potato Dextrose Agar), SDN (Sabouraud Agar), OA (Oatmeal Agar), MEA (Malt Extract Agar) and CMA (Corn Meal Agar).



Source: Tavares (2017).

The fungus *E. nigrum* (A2S61) sporulated only in OA and CMA media at temperatures of 15 and 20 ºC, presenting globular spores of dark brown coloration (FIGURE 3. C). Sporulation also occurs in PDA medium when the incubation period is increased. *E. nigrum* (A2C32) isolate did not sporulate in the tested media during the 15 days of incubation, only occurring in the CMA medium the presence of hyphae and reddish agglomerates at 15 ºC (FIGURE 3. D). Schol-schwarz (1959) characterized 70 isolates of *Epicoccum* and found that a total of 47 fungi sporulated. This author observed abundant mycelial development with different colors in the isolates; from pink, red, purple, and yellow, with few olivaceous and brown olives. Pigments diffusing through agar could present different colorations, and sporulation for some isolates was delayed. Arenal et al. (2002) comparing different genotypic and phenotypic techniques founsnd great variability among the 36 *E. nigrum* isolates studied.

3.2 ANTIBACTERIAL ACTIVITY

3.2.1 Screening by disk diffusion method

From the 12 fungal extracts, at the concentration of 5 mg mL-1, five presented antibiotic activity against at least one pathogen bacterial strain (TABLE 2). The PD broth was used as negative control, and all bacterial strains tested were grown. The antibiotic Chloramphenicol was used as positive control. The extract of the endophytic fungus *Arcopilus* sp. (A2C54) showed significant antibiotic activity against strains of *S. aureus* ATCC 8702 and *S. aureus* ATCC 5674 showing inhibition halos of 23 and 23.3 mm and against strains of Gram-negative S. Typhimurium S190 and S. Enteritidis S64 with halos of 8 and 7 mm, respectively. The EtOAc extract of *A. cupreus* (*Chaetomium cupreum*) at the concentration of 2 mg mL^{-1} showed a zone of inhibition of 18 and 17 mm, against *S. aureus* and *S.* Typhi, respectively (WANI, SOUMYA; SHARMILA, 2016). Sadrati et al. (2013) found antibiotic activity of the EtOAc of the endophyte *Chaetomium* sp. against *S. aureus* and *S.* Typhi of 9.7 and 11.7 mm respectively, but had no activity against *P. aeruginosa*, which is a result similar to the present study.

Table 2 – Antibacterial activities of extracts from filamentous fungi (5 mg mL^{-1}) by disc diffusion method on different bacteria causing alimentary toxinfections.

Pathogenic organisms	Inhibition zone (mm)				
	Arcopilus sp. (A2C54)	*P. ignaria* (A2C47)	*Fusarium* sp. (CML 2969)	*A. keveii* (CML 2968)	*A. sydowii* (CML 2967)
S. aureus GL 8702	23 ± 0^{Aa}	17.7 ± 0.58^{Ab}	7 ± 0^{Ac}	0	0
S. aureus GL 5674	23.3 ± 0.58^{Aa}	17.3 ± 0.58^{Ab}	7 ± 0^{Ac}	0	0
S. Typhimurium S190	8 ± 0^{Ba}	7 ± 0^{Ba}	0	7 ± 0^{Aa}	7 ± 0^{Aa}
S. Enteritidis S64	7 ± 0^{Ca}	0	0	7 ± 0^{Aa}	7 ± 0^{Aa}
E. coli EPEC 055	0	0	0	7 ± 0^{Aa}	8 ± 1.73^{Aa}
E. coli ETEC ATCC 35401	0	0	0	7 ± 0^{Aa}	7.3 ± 0.58^{Aa}
L. monocytogenes ATCC 19117	0	7 ± 0^{Ba}	7 ± 0^{Aa}	7 ± 0^{Aa}	0
P. aeruginosa ATCC 25853	0	7 ± 0^{Ba}	0	7 ± 0^{Aa}	7 ± 0^{Aa}
A. hydrophila ATCC 7966	0	7 ± 0^{Ba}	0	7 ± 0^{Aa}	0
C. sakazakii ATCC 29004	0	7 ± 0^{Ba}	7 ± 0^{Aa}	7 ± 0^{Aa}	7 ± 0^{Aa}

Data expressed as means of triplicates ± standard deviation. Means with diferent letters are significantly diferent at $P<0.005$.
Capital letters represent the treatments in the columns.
Lowercase letters represent the treatments on the lines.
Source: Tavares (2017).

The extract of the endophytic *P. ignaria* (A2C47) was also significant active against *S. aureus* strains with halos of 17.7 and 17.3 mm and also against *S.* Typhimurium S190, *L. monocytogenes* ATCC 19117, *P. aerugimosa* ATCC 25853, *A. hydrophila* ATCC 7966 and *C. sakazakii* ATCC 29004 with inhibition halos of 7 mm. Bhilabutra et al. (2007) evaluated EtOAc extracts of *Periconia siamensis* obtained from different culture media, finding zones of inhibition between 14.3 and 6.8 mm against *S. aureus, L. monocytogenes* and *P. aeruginosa*. In comparison with the present study, the extract of *P. ignaria* (A2C47) presented higher antibacterial activity with halo of 17.7 mm against *S. aureus*.

The extract of the fungus *A. keveii* (CML 2968) showed activity against 8 of the 10 tested bacterial strains, showing the greater spectrum of action among evaluated fungal extracts with inhibition halos of 8 and 7 mm in diameter. However, this extract did not present activity against the strains of *S. aureus*. The extract of *A. sydowii* (CML 2967) exhibited antibiotic action only against Gram-negative bacteria tested, except for *A. hydrophila*. The extract of *A. fumigatus* presented antibacterial activity with zone of inhibition of 13.33 mm against *S. aureus* (FURTADO et al., 2002). The extract of *Aspergillus* sp. showed inhibition halos of 13.3, 21.7 and 11.3 mm against Gram-negative *S.* Typhi, *E. coli* and *Pseudomonas* sp., respectively, and 11.7 mm against Gram-positive *S. aureus* (SADRATI et al., 2013). *A. flavus* was isolated as a marine derived fungus, and its extract showed antibiotic activity against *E. coli* and *P. putida* with zone of inhibition of 5 and 10 mm, respectively (SAMUEL; PRINCE; PRABAKARAN, 2011).

Fusarium sp. (CML 2969) had antibiotic action only against the tested Gram-positive strains of *S. aureus* and *L. monocytogenes* and for Gram-negative *C. sakazakii*. Deoxynivalenone synthesized by *F. graminearum* and exhibits antibiotic activity against *E. coli* and *S. aureus* with 61 and 46 mm inhibition, respectively, as well as the extract of this fungus that presented halos of 52 and 27 mm against the same bacteria, respectively (PRAVEENA; PADMINI, 2011). The five fungal extracts that showed antibiotic activity in the disk diffusion test were determined to their minimum bactericidal concentration (MBC).

3.2.2 Determination of Minimum Bactericidal Concentration (MBC)

The MBCs presented by extract from *Arcopilus* sp. (A2C54) were 0.00244 and 0.0195 mg mL^{-1} for *S. aureus* strains (GL 8702 and GL 5674), respectively (TABLE 3). For this same extract with respect to *S.* Typhimurium S190 and *S.* Enteritidis S64, the MBCs were 0.625 and 2.5 mg mL^{-1}, respectively. The antibiotic activity observed in this work was higher

than the one found in EtOAc extract for *A. cupreus* (*C. cupreum*), which presented MBC of 1 and 0.062 mg mL^{-1} against *S. aureus* and *S.* Typhi, respectively (WANI, SOUMYA; SHARMILA, 2016). The compound cochliodinol, which is synthesized by several species of *Chaetomium*, belonging to the class of quinones, has shown inhibition in the growth and metabolism of many bacterial genera (BREWER et al., 1984). *Chaetomium* species synthesize pigments that are secondary metabolites that have biological activities. Azaphilones have been identified as colored metabolites that are derived from polyketides and exhibit various biological properties, such as antimicrobial, antiviral, antioxidant, cytotoxic, and anti-inflammatory properties (WANI; SOUMYA; SHARMILA, 2016).

Table 3 – Minimum bactericidal concentration (MBC) of different extracts of filamentous fungi on bacteria causing alimentary toxinfections.

Pathogenic organisms	MBC (mg mL^{-1})				
	Arcopilus sp. (A2C54)	*P. ignaria* (A2C47)	*Fusarium* sp. (CML 2969)	*A. keveii* (CML 2968)	*A. sydowii* (CML 2967)
S. aureus GL 8702	0.00244	1.25	0.625	*	*
S. aureus GL 5674	0.01953	2.5	5	*	*
S. Typhimurium S190	0.625	5	*	5	5
S. Enteritidis S64	2.5	*	*	5	5
E. coli EPEC 055	*	*	*	5	5
E. coli ETEC ATCC 35401	*	*	*	5	5
L. monocytogenes ATCC 19117	*	2.5	2.5	5	*
P. aeruginosa ATCC 25853	*	5	*	5	5
A. hydrophila ATCC 7966	*	5	*	5	*
C. sakazakii ATCC 29004	*	5	5	5	5

* Did not present antibacterial activity in 5 mg mL^{-1}.
Source: Tavares (2017).

The extract from *P. ignaria* (A2C47) showed MBC values of 1.25 and 2.5 mg mL^{-1} against *S. aureus* strains (GL 8702 and GL 5674), respectively, and 2.5 mg mL^{-1} for *L. monocytogenes*. *Periconia* spp. is known to produce chlorine-containing compounds that may have antibiotic activity (SELIM et al., 2012). From the endophytic fungus *Periconia* sp. periconicins were isolated, which is an antibacterial compound that present activity against *S. aureus* e *S.* Typhimurium (KIM et al., 2004). The compounds Modiolide A and 4-Cromanone, 6-hydroxy-2-methyl- (5Cl) of *P. siamensis* were isolated from grasses and identified (BHILABUTRA et al., 2007). These compounds showed activity against pathogenic bacteria transmitted by food as *E. coli*, *L. monocytogenes*, *S. aureus* (resistant to methicillin) and *P. aeroginosa*, the highest activity was found in Modiolide A compound with MIC of 50, 6.25, 25 and 12.50 µg mL^{-1}, respectively.

The endophytic fungi evaluated in this study were isolated from the *Eremanthus* spp., popularly known as Candeia. From the stem of Candeia, an essential oil rich in α-bisabolol is extracted, presenting anti-inflammatory, antimicrobial properties and the ability to improve the absorption of trans-epidermal drugs (LIMA et al., 2013; KADIR; BARRY, 1991). From the leaves is extracted an essential oil rich in β-caryophyllene, β-pinene, β-mircene and germacrene, and oil with high concentrations of β-caryophyllene that inhibits the growth of bacteria and certain fungi (LIMA et al., 2013).

For the extract from *Fusarium* sp. (CML 2969), the MBC was 0.625 and 2.5 mg mL^{-1} against *S. aureus* ATCC 8702 and *L. monocytogenes*, respectively, and 5 mg mL^{-1} against *S. aureus* ATCC 5674 and *C. sakazakii*. *Fusarium* genus fungi are also known to produce mycotoxins. Members of this genus may present more than 30 secondary metabolites in clusters of genes (SONDERGAARD et al., 2016). Deshmukh; Mathew; Purohit (2014) identified the Bikaverin compound from the EtOAc extract of the fungus *Fusarium* sp., which presented antibacterial action against *P. aeruginosa* and *S. aureus* and other multiresistant bacteria, and is already known for its antifungal and antitumor activities.

The antibiotic activity of extracts from the endophytic fungi studied and *Fusarium* sp. (CML 2969) showed lower MBC against Gram-positive bacterial strains than Gram-negative strains. Gram-positive bacteria are generally more susceptible to drugs. The absence of an outer membrane to the Gram-positive cell wall facilitates the entry of antibiotic by the cell wall and also due to compounds targeting the peptidoglycan which is present in greater amount in Gram-positive (KOHANSKI; DWYER; COLLINS, 2010). On the other hand, Gram-negative bacteria present membrane efflux systems as in *E. coli* and *P. aeruginosa* that act by exporting antibiotic compounds as well as large protein toxins that are inside the cell,

decreasing their concentration intracellular (ESWARAN et al., 2004; KORONAKIS; ESWARAN; HUQHES, 2004).

Caves are poorly exploited ecosystems that can provide microorganisms with biotechnological applications (BARTON, 2006). The MBC of the EtOAc extracts of the fungi isolated from caves *A. keveii* (CML 2968) and *A. sydowii* (CML 2967) were 5 mg mL^{-1} for all bacteria except strains of *S. aureus* for both extratcs and *L. monocytogenes* and *A. hydrophila* for the extract of *A. sydowii* (CML 2967). Fungi of the genus *Aspergillus* present one of the major contributions as source of antimicrobial compounds (BARAKAT; GOHAR, 2012). Three new alkaloids from the EtOAc extract with significant antimicrobial activity against *E. coli*, *B. subtilis* and *Micrococcus lysoleikticus* (ZHANG et al., 2008) were isolated from the marine-derived *A. sydowii* fungus (same species in this work). Barakat and Gohar (2012) found a potent marine isolate of *A. terreus* var. *africanus* that presents compounds with activity against the bacterial pathogens *A. hydrophila*, *Vibrio ordalli* and *V. angularium*. *A. fumigatus* isolated from soils produces antimicrobial compounds identified as 3,4-dimethoxyphenol and 1,3,5-trimethoxybenzene that inhibited the growth of *S. aureus* (FURTADO et al., 2002). Other bioactive substances have also been identified from *Aspergillus* species. Ophiobolin A isolates of fungi of the genus *Aspergillus* among them *A. kevei* (same species in this work) exhibits inhibitory activity against various types of cancer cells (CHEN et al., 2012). These results are interesting because of the high demand for drugs because of the rapid development of antibiotic resistance by Gram-negative bacteria. Since the extracts of the fungi, mainly of *A. keveii* (CML 2968), showed activity against all Gram-negative bacteria tested.

4 CONCLUSION

Phylogenetics analysis of ITS and RPB2 allowed the identification of the following species: *A. keveii* (CML 2968), *A. sydowii* (CML 2967), *P. chermesinum* (CML 2966), *E. nigrum* (CML 2971, A2C32 and A2S61), *P. ignaria* A2C47) and *L. aphanocladii* (CML 2970). The genus *Arcopilus* sp. (A2C54) and *F. graminearum* sensu lato (CML 2969) were also found. Among the fungal isolates, the EtOAc extract from *Arcopilus* sp. (A2C54) showed a high antibacterial potential against pathogenic strains of *S. aureus* and *Salmonella*, exhibiting the lowest CMB of 0.00244 mg mL^{-1}.

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APPENDIX A

Table 4 – Sequences of the fungal isolates that were used to construct the phylogenetic trees and their respective access numbers in GenBank.

Isolated	Collection nº.	GenBank accession nº.		Reference
		ITS	RPB2	
Arcopilus aureus	CBS 153.52	KX976582.1	KX976806.1	WANG et al., 2016
Arcopilus aureus	CBS 538.73	KX976583.1	KX976806.1	WANG et al., 2016
Arcopilus cupreus	CBS 560.80	KX976584.1	KX976806.1	WANG et al., 2016
Arcopilus fusiformis	CBS 484.85	KX976585.1	KX976806.1	WANG et al., 2016
Arcopilus flavigenus	CBS 337.67	KX976587.1	KX976806.1	WANG et al., 2016
Arcopilus turgidopilosus	CBS 169.52	KX976588.1	KX976806.1	WANG et al., 2016
Epicoccum nigrum	CBS 125.82	FJ426995.1	KT389631.1	CHEN et al., 2015
Epicoccum nigrum	CBS 173.73 (T)	FJ426996.1	KT389632.1	CHEN et al., 2015
Epicoccum sorghinum	CBS 179.80	FJ427067.1	KT389635.1	CHEN et al., 2015
Epicoccum pimprinum	PD 77/1028	FJ427050.1	KT389633.1	CHEN et al., 2015
Epicoccum nigrum	CML 2971	KR261452.1	*	SOUZA et al., 2016
Massarina cisti	CBS:266.62	LC014568.1	FJ795464.1	TANAKA et al., 2015
Periconia igniaria	CBS:845.96	LC014586.1	GU371793.1	SCHOCH et al., 2009; TANAKA et al., 2015
Aspergillus keveii	CBS 209.92	NR 137492.1	KU866938.1	CHEN et al., 2016
Aspergillus keveii	NRRL 1974	EF652432.1	EF652168.1	PETERSON, 2008
Aspergillus keveii	CML 2968	KR261449.1	*	SOUZA et al., 2016
Aspergillus insuetus	NRRL 279	NR 131292.1	EF652193.1	CHEN et al., 2016
Aspergillus versicolor	NRRL 238	EF652442.1	EF652178.1	JURJEVIC; PETERSON; HORN, 2012
Aspergillus sydowii	NRRL 250	EF652450.1	EF652186.1	JURJEVIC; PETERSON; HORN, 2012
Aspergillus sydowii	NRRL 4768	EF652473.1	EF652209.1	JURJEVIC; PETERSON; HORN, 2012
Aspergillus sydowii	CML 2967	KR261448.1	*	SOUZA et al., 2016

Table 4 – (Continued).

Isolated	Collection nº.	GenBank accession nº.		Reference
		ITS	RPB2	
Penicillium chermesinum	NRRL 2048(T) = CBS 231.81	NR 121310.1	JN406600.1	HOUBRAKEN; SAMSON, 2011
Penicillium chermesinum	CML 2966	KR261447.1	*	SOUZA et al., 2016
Penicillium flavigenum	CBS 419.89(T)	NR 103695.1	JN406551.1	HOUBRAKEN et al., 2012
Penicillium flavigenum	CBS 110407	JX997064.1	JX996692.1	HOUBRAKEN et al., 2012
Penicillium flavigenum	3.1.a	KR261455.1	*	SOUZA et al., 2016
Penicillium flavigenum	CML 2965	KR261446.1	*	SOUZA et al., 2016
Penicillium indicum	NRRL 3387(T)	NR 121311.1	EU427256.1	PETERSON; HORN, 2009
Penicillium mononematosum	CBS 172.87(T)	NR 111817.1	JX996709.1	HOUBRAKEN et al., 2012
Penicillium mononematosum	CBS 109616	JX997062.1	JX996690.1	HOUBRAKEN et al., 2012
Fusarium graminearum	NRRL31084	DQ459823.1	JX171644.1	O'DONNELL et al., 2008; AOKI et al., 2015;
Fusarium austroamericanum	NRRL28585	DQ459839.1	KM361661.1	O'DONNELL et al., 2008; AOKI et al., 2015;
Fusarium ussurianum	NRRL 45681	FJ240312.1	KM361666.1	O'DONNELL et al., 2008; AOKI et al., 2015;
Fusarium vorosii	NRRL 37605	DQ459865.1	KM361665.1	O'DONNELL et al., 2008; AOKI et al., 2015;
Fusarium sp.	CML 2969	KR261450.1	*	SOUZA et al., 2016
Lecanicillium aphanocladii	CBS 797.84	**	KM283853.1	Unpublished
Lecanicillium longisporum	CBS 126.27	**	KM283862.1	Unpublished
Lecanicillium psalliotae	CBS 101270	**	EF469113.1	Unpublished
Lecanicillium dimorphum	CBS 345.37	**	KM283854.1	Unpublished
Peziza ampelina	KH 00.011	AF491629.1	AY500492.1	SOUZA et al., 2016

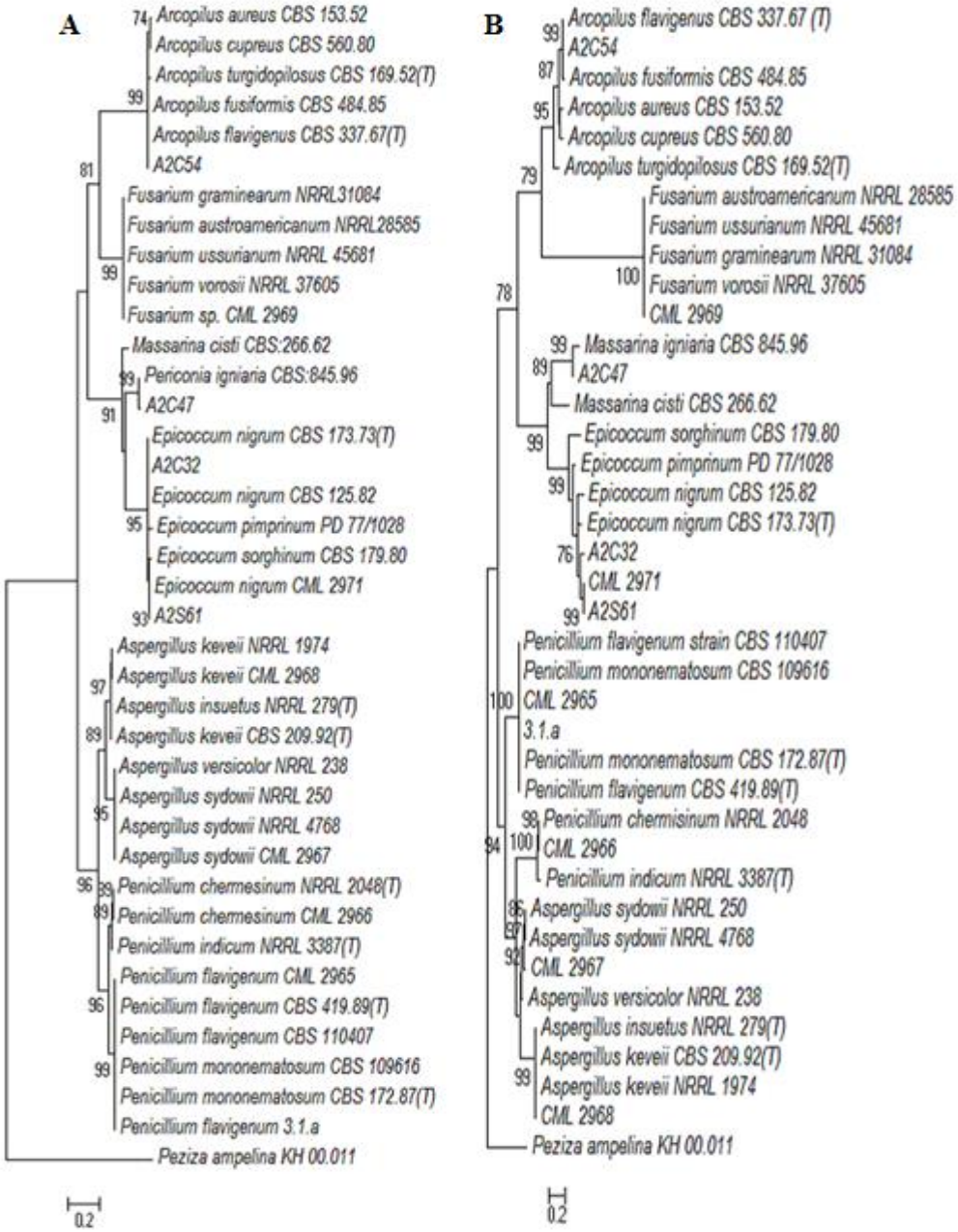
* Sequences that have not yet been deposited in GenBank.

** Access sequences that were not used for phylogenetic tree construction.

Souce: Tavares (2017)

APPENDIX B

Figure 4 – Maximum Likelihood analysis with the evolutionary model K2+G+I. Phylogenetic trees constructed from the sequences of fungal and reference isolated. A. Tree formed from the 5.8S region of rDNA (ITS). B. Tree formed from the RPB2 gene.



Numbers above branches are bootstrap values obtained from 1000 replications; only branches supported by more than ≥ 70 % are shown. *Peziza ampelina* KH 00.011 was used with outgroup.
Source: Tavares (2017).

ARTIGO 2 ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC COMPOUNDS OF EXTRACT FROM PIGMENT-PRODUCING FILAMENTOUS FUNGI *

ARTIGO FORMATADO DE ACORDO COM A NORMA NBR 6022 (ABNT 2003)

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ABSTRACT

Among the secondary metabolites of filamentous fungi, pigments-producing ones may present bioactive properties as antioxidant activity, and may be used in the food, cosmetic and pharmaceutical industries. In this context, the objective of this work was to evaluate the antioxidant activity using the free radical scavenging methods DPPH and $ABTS^{\bullet+}$ and the β-carotene-linoleic acid system. Moreover, this work aimed at determining the content of total phenolic compounds by the Folin-Ciocalteu method of extract of pigment-producing filamentous fungi. The pigmented extract of the fungus *Penicillium flavigenum* (CML 2965) exhibited the highest antioxidant activity in all used methods and the results were compared with the activity of the commercial antioxidant Trolox. In the DPPH assay *P. flavigenum* (CML 2965) showed 98.22 % inhibition of this radical, and this activity was greater than Trolox activity of 95.72 %, corresponding to EC_{50} of 83.4 μg mL^{-1}. The percent inhibition of the $ABTS^{\bullet+}$ radical by Trolox was 47.09% and the extract of *P. flavigenum* (CML 2965) was 29.50% with EC_{50} of 862.2 μg mL^{-1}. Moreover, the percentage of protection against bleaching β- carotene was 72.23% and Trolox 82.62%. The content of total phenolic compounds of EtOAc extract of *P.flavigenum* (CML 2965) was 201.43 mg of GAE/g of extract. The results indicate that the antioxidant substance of the pigment extract from *P. flavigenum* (CML 2965) is more hydrophilic in nature due to high activity in the DPPH assay and the high phenolic content may also have contributed to the antioxidant capacity of the extract. Therefore, this fungus shows biotechnological potential since the crude extract has high antioxidant activity and total phenolic compounds.

Keywords: Fungal secondary metabolites. Antioxidant potential. Phenolic compounds. Dye.

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* Artigo Apresentado para a conclusão do curso de pós-graduação em Microbiologia Agrícola da Universidade Federal de Lavras.

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1 INTODUCTION

Currently, there is an increasing use and demand for natural products from natural dources due to the adverse effects that many synthetic compounds can cause to human health and the environment. In many food products, the antioxidant compounds that are used can inhibit, control or delay their oxidation and deterioration, preserving their quality and increasing the shelf life. In addition, antioxidants neutralize free radicals resulting from normal biological activities to living organisms, which in excess can cause serious damage such as cancer, atherosclerosis, immunosuppression, aging, inflammation, ischemic heart disease, diabetes, neurodegenerative diseases such as Parkinson's and Alzheimer's (OLIVEIRA et al., 2008)

Synthetic antioxidants are widely used to prevent lipid oxidation of food and pharmaceutical products such as butyl-hydroxy-toluene (BHT), butyl-hydroxy-anisole (BHA), and propyl (GP) (KIM; WIJESEKARA, 2010). Studies on the toxicology of synthetic antioxidants show that they may exhibit carcinogenic effects, and some countries even restrict their use (WHANG, 2013). Many natural antioxidants used in foods are plant-derived from the classes of phenolic and polyphenolic compounds, carotenoids and antioxidant vitamins (SHAHIDE, 2015). Factors such as raw material availability and growing time, which in turn depend on climatic conditions and seasonality, may limit the use of these metabolites.

On the other hand, microbial metabolites do not depend on climatic and seasonal factors, and can be produced faster since their growth is relatively faster. Other advantages are the genetic manipulation and the production of compounds by fermentative processes that are faster. Fungi produce many extracellular enzymes and secondary metabolites such as organic acids, pigments and other food additives (AKILANDESWARI; PRADEEP, 2016). Furthermore, they may present antioxidant, antimicrobial, immunosuppressive, and other properties (TAKAHASHI; CARVALHO, 2010). Among the antioxidant compounds isolated from filamentous fungi are carotenoids and flavonoids (AKILANDESWARI; PRADEEP, 2016), phenolic acids and their derivatives (HUANG et al., 2007a), isobenzofuranones (STROBEL et al., 2002), isobenzofuran (HARPER et al., 2003). Fungal pigments can be an alternative to the use of synthetic dyes in the food, cosmetics and pharmaceutical industry, and may even add biological properties, such as antioxidant, that contribute to human health. In this context, the objective of this work was to evaluate the antioxidant activity by free radical capture methods and protection against bleaching of β-carotene, and total phenolic compounds of extract of filamentous fungi producing pigment.

2 METHODS

2.1 FUNGAL ISOLATES AND INOCULUM PREPARATION

The filamentous fungi used for this work were selected by the production of pigments in solid culture media. The fungi used are part of the collection of the Laboratório de Bioprospecção e Genética de Fungos Filamentosos (Biogen) from the Universidade Federal de Lavras, Brazil. The fungi *Aspergillus keveii* (CML 2968), *Aspergillus sydowii* (CML 2967), *Penicillium chermesinum* (CML 2966), *Penicillium flavigenum* (CML 2965), *Fusarium* sp. (CML 2969), *Lecanicillium aphanocladii* (CML 2970) and *Epicoccum nigrum* (CML 2971) were isolated from caves and deposited in the Coleção Micológica de Lavras (CML) from the Phytopathology department from the Universidade Federal de Lavras, Brazil. *Periconia ignaria* (A2C47), *Arcopilus* sp. (A2C54) (formerly called *Chaetomium* sp.), *E. nigrum* (A2C32) and *E. nigrum* (A2S61) were isolated as endophytes from the *Eremanthus* sp. Plant, popularly known as Candeia (GODINHO, 2016). The fungi were grown in PDA culture medium (200 g L^{-1} potato, 20 g L^{-1} dextrose and 15 g L^{-1} agar) and were incubated at 25 ºC for 7 days prior to use.

2.2 EXTRACTION OF FUNGAL CULTURES

The methodology used was according to Souza et al. (2016). Disks of approximately 9 mm in diameter from the fungal colonies were transferred to 1 L of PD broth, which was incubated at 30 ºC in the dark on shaker at 150 rpm for 7 days. The cultures were filtered and extracted twice by liquid-liquid partition with 0.5 ethyl acetate (EtOAc). The extracts were concentrated in a rotary evaporator (RV10 digital; IKA) at 80 ºC and 150 rpm and stored in a freezer at -20 ºC until use.

2.3 DETERMINATION OF ANTIOXIDANT ACTIVITY

2.3.1 DPPH and ABTS assays

The method of scavenging the DPPH radical was performed according to Milardović et al. (2006), with modifications (SANTOS et al., 2011). The extracts in the concentrations of

500, 250 and 125 μg mL^{-1} were diluted with ethyl alcohol. In a dark environment, 100 μL of each dilution of the extracts were transferred to the test tube and 900 μL of the DPPH solution was added at a concentration of 0.004 %. The negative control used was ethyl alcohol while the antioxidant Trolox 0.05 % was used as positive control. The samples were incubated for 30 min in total absence of light. The readings were performed in a spectrophotometer at 517 nm. The experiment was carried out in triplicate.

The scavenging method of the $ABTS^{\bullet+}$ radical was performed according to Rufino et al. (2007). The $ABTS^{\bullet+}$ radical was prepared from the reaction of 5 mL of the 7 mM ABTS reagent solution with 88 μL of the 140 mM potassium persulfate solution, which was kept in the dark for 16 h. Then, 1 mL of this reaction was diluted in ethyl alcohol until it reached an absorbance of 0.7 nm ± 0.05 nm at 734 nm. The extracts in the concentrations of 500, 250 and 125 μg mL^{-1} were diluted with ethyl alcohol. In a dark environment, 30 μL of each dilution of the extract were transferred to test tubes with 3 mL of the $ABTS^{\bullet+}$ radical and homogenized in a tube shaker. For the negative control ethyl alcohol was used, and for the positive control the antioxidant Trolox 2 mM. After 6 min in the dark, the reading was carried out at 734 nm. The experiment was carried out in triplicate.

The percentage of inhibition (I%) of the DPPH and $ABTS^{\bullet+}$ free radicals were calculated using the expression below (Equation 1)**.**

$$I\ \% = [(A_{blank} - A_{sample})/A_{blank}] \times 100 \quad (\textbf{Eq. 1})$$

where A_{blank} is the absorbance of the negative control and A_{sample} is the absorbance of the tested extracts. The results were also presented in EC_{50} (50 % effective concentration), which expresses the minimum antioxidant concentration in reducing 50 % of the initial concentration of the DPPH and $ABTS^{\bullet+}$ radicals. For that, a curve with the values of I % plotted on the y-axis and the concentration of the extracts (mg mL^{-1}) on the x-axis was obtained, and the equation of the line (Equation 2) was determined.

$$y = ax + b \quad (\textbf{Eq. 2})$$

where, "y" is the percentage inhibition of the DPPH and $ABTS^{\bullet+}$ radicals and "x" is the EC_{50} (mg mL^{-1}).

2.3.2 β-Carotene-linoleic acid assay

The methodology used in this essay was proposed by Miller (1971), with some modifications (RUFINO et al, 2006). The β-carotene solution was prepared in a 2 mL light-shielded tube, adding 20 mg of β-carotene and 1 mL of chloroform. For the β-carotene-linoleic acid solution, 40 µL of linoleic acid, 530 µL of Tween 40, 50 µL of β-carotene solution and 1 mL of chloroform was added in a light-erlenmeled flask. The system solution was homogenized and the chloroform was evaporated. Then, treated water was added with oxygen until an absorbance was obtained between 0.6 nm and 0.7 nm at 470 nm. The extracts in the concentrations of 500, 250 and 125 µg mL^{-1} were diluted with ethyl alcohol. In a dark environment, 0.4 mL of each dilution of the extract was mixed with 5 mL of the system solution in test tubes, homogenized on a shaker and after 2 min run at zero time at 470 nm. The tubes were kept in a water bath at 40 °C for 2 h after the final reading at 470 nm. The spectrophotometer was calibrated with water. The blank was read as the β-carotene / linoleic acid system without extracts and as a positive control Trolox (200 mg mL^{-1}). The experiment was carried out in triplicate.

The antioxidant activity in β-carotene bleaching model in percentage (A %) was calculated according to the following equation (Equation 5):

$$A\ \% = [1 - (A_S - A_B)/(A_{S'} - A_{B'})] \times 100 \quad \textbf{(Eq. 5)}$$

where, A_S and A_B are the absorbances of the sample and the blank at time zero, and $A_{S'}$ and $A_{B'}$ are the absorbances of the sample and the blank of the final reading, measured after 2 h.

2.4 DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

Total phenolic compounds were determined by the Folin-Ciocalteu's method, according to Waterhouse (2002). A total of 0.5 mL of the fungal extract (5 mg mL^{-1}) diluted in ethyl alcohol was mixed with 2.5 mL of Folin-Ciocalteu 10% (v / v) solution and 2 mL of sodium carbonate 4% (w / v) solution. The mixture was then homogenized in a tube shaker and allowed to stand for 2 h, protected from light. The spectrophotometer was set to a wavelength of 750 nm, which corresponds to the absorption peak of the molybdenum and tungsten oxides. To calibrate the spectrophotometer, the solution of the blank composed of the reagents and 0.5 mL of ethyl alcohol was used. The total phenolic content was calculated from the straight line equation (Equation 6) of the standard curve of gallic acid (5-40 µg mL^{-1})

and the results were expressed as mg of gallic acid equivalent (GAE) per g of extract. The experiment was carried out in triplicate.

$$y = 0.0142x - 0.0158 \text{ (\textbf{Eq. 6})}$$

$$R^2 = 0.9971$$

2.5 STATISTICAL ANALYSIS

The antioxidantes activities were composed of twelve treatments with three replicates. The results of inhibition of free radical in percentage (I%), EC_{50} and percentage of protection β-caroteno by the antioxidant compound (A%) were submitted to analysis of variance by the SISVAR (FERREIRA, 2000) program and the means were compared by the Scott and Knott test at **($p < 0.05$)** level of significance. The quantitative variables of the antioxidant activity were submitted to regression analysis.

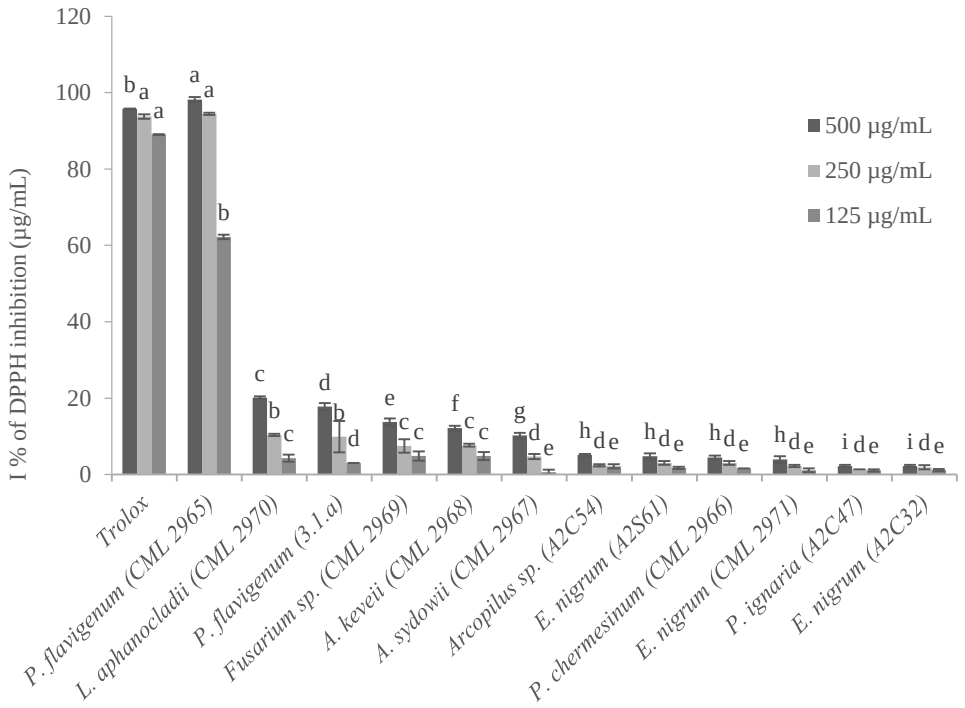
2 RESULTS AND DISCUSSION

3.1 DETERMINATION OF ANTIOXIDANT ACTIVITY

3.1.1 DPPH assay

The antioxidant activity of fungal extracts at concentrations of 500, 250 and 125 μg mL^{-1} was expressed as inhibition of DPPH free radical in percentage (I%) and is shown in Graphic 1. The data show that different concentrations result in different percentages of inhibition with decreased inhibition of the DPPH radical directly related to the decrease in concentration, as expected. At the concentration of 500 μg mL^{-1}, the extract of *P. flavigenum* (CML 2965) exhibited 98.22% inhibition of the DPPH radical, which was significantly higher than the Trolox antioxidant with 95.72% and being statistically equal in the concentration of 250 μg mL^{-1} with 94.47% and Trolox with 93.74%. The fungus *P. flavigenum* (CML 2965) showed significantly higher percent inhibition in the three concentrations while the other extracts fungi. *L. aphanocladii* (CML 2970) exhibited the second best percentage at the concentrations of 500 and 250 μg mL^{-1}, but at 125 μg mL^{-1} presented the fourth best inhibition.

Graphic 1 – Free radical scavenging capacity DPPH of extract from filamentous fungi with different concentrations.



Data expressed as means of triplicates ± standard deviation. Means with different letters are significantly different at P<0.005.
Source: Tavares (2017).

Moreover, the extracts at lower concentrations did not follow the same pattern for the other fungal extracts. The isolate *P. flavigenum* (3.1.a) presented 17.18, 9.90 and 3.03% inhibition of the DPPH radical, differing significantly from *P. flavigenum* (CML 2965) extract, which are of the same species. The extracts from *Fusarium* sp. (CML 2969), *A. sydowii* (CML 2967) and *A. keveii* (CML 2968) presented 13.74, 10.23 and 12.12% at the concentration of 500 µg mL^{-1}, respectively. The highest endophytic fungi activity was found in the extract of *Arcopilus* sp. (A2C54), with 5.08, 2.41, 2.14%, respectively. The fungi isolated from caves showed a greater capacity to inhibit the DPPH radical, with the exception of *P. chermesinum* (CML 2966) and *E. nigrum* (CML 2971), in relation to the endophytic fungi tested.

The data obtained from different concentrations of extracts were also expressed in $EC_{50,}$ which refers to the concentration in µg mL^{-1} capable of inhibiting 50% of the DPPH

radical (TABLE 1). Among the fungal extracts studied, *P. flavigenum* (CML 2965) presented EC_{50} of 83.4 μg mL^{-1}, which was significantly lower than the other extracts, exhibiting strong sequestering capacity of the DPPH radical. However, *P. flavigenum* (3.1.a) despite being of the same species obtained, EC_{50} was 1,333.7 μg mL^{-1}.

Table 1 – EC_{50} values of extract from filamentous fungi, which inhibit 50 % of free radicals DPPH and $ABTS^{\cdot+}$.

Fungi extracts (500 μg mL^{-1})	EC_{50} (μg mL^{-1})	
	DPPH	$ABTS^{\cdot+}$
P. flavigenum (CML 2965)	83.4 ± 2.35[a]	862.2 ± 84.2[a]
L. aphanocladii (CML 2970)	1,211.5 ± 53.7[b]	6,075.9 ± 242.9[b]
Arcopilus sp. (A2C54)	5,967.1 ± 343[c]	15,031.2 ± 7270.5[c]
P. chermesinum (CML 2966)	6,740 ± 1013[c]	20,565 ± 2095.2[c]
E. nigrum (A2C32)	21,755.6 ± 10208[d]	20,641.7 ± 5261.9[c]
Fusarium sp. (CML 2969)	2,050.1± 267.7[b]	8,164 ± 2487.1[b]
A. keveii (CML 2968)	2,552.5 ± 531.1[b]	6,297 ± 1598.6[b]
E. nigrum (A2S61)	6,651.7 ± 2364[c]	27,867.2 ± 15854[c]
A. sydowii (CML 2967)	2,090.95 ± 208.7[b]	1,249.51 ± 75[a]
E. nigrum (CML 2971)	7,280.2 ± 2520[c]	17,753 ± 6624.5[c]
P. ignaria (A2C47)	16,030.1 ± 2496[d]	9,830.9 ± 11.5[b]
P. flavigenum (3.1.a)	1,333.7 ± 58.8[b]	7,514.7 ± 11.5[b]

Data expressed as means of triplicates ± standard deviation. Means with different letters are significantly different at $P<0.005$.
Source: Tavares (2017).

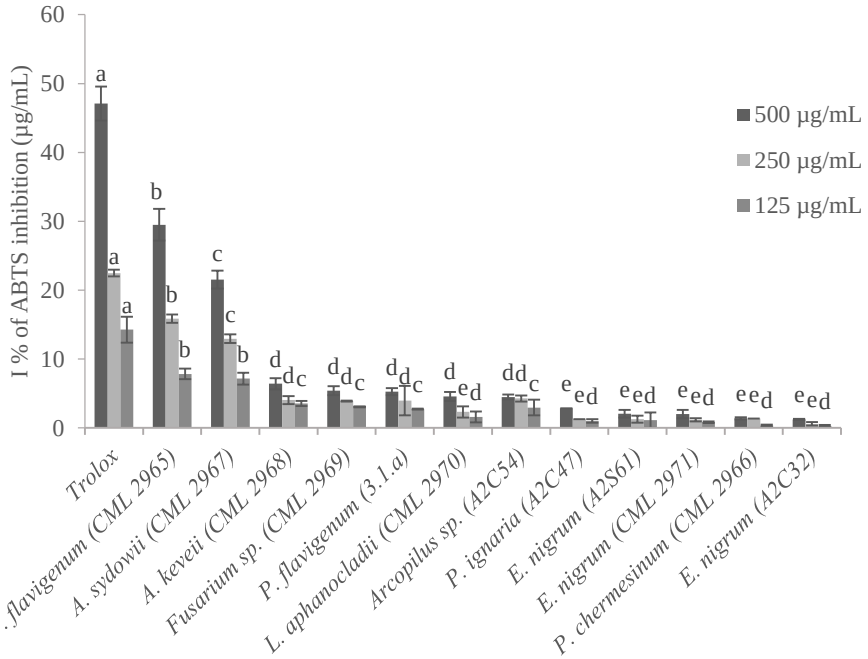
Three polysaccharides isolated from the marine fungus *Penicillium* sp. showed a capacity of inhibition of the DPPH radical in the concentration of 6.4 mg mL^{-1}, presenting a scavenging effect of 44.2 to 79.9% and EC_{50} of 2.53 to 6.81 mg mL^{-1} (SUN et al., 2009). The farnesylhydroquinone compound was isolated from the mycelium of marine fungi of the genus *Penicillium* and showed high scavenging activity of the DPPH radical (SALEEM et al., 2007). Nine new compounds derived from gentisyl alcohol isolated from *P. terrestre* showed moderate antioxidant activity using the DPPH method, with EC_{50} in the range of 2.6 to 8.5 μM (CHEN et al., 2008). The EtOAc extract from *P. citrinum* isolated two new compounds: pennicitrinone C and penicitrinol B, the antioxidant capacity in EC_{50} was 0.8 to 59 μM, using the DPPH method (LU et al., 2008).

The second best value of EC_{50} was exhibited by *L. aphanocladii* (CML 2970) with 1,211.5 μg mL^{-1}, but differs significantly from the antioxidant potential of *P. flavigenum* (CML 2965) for this method. The extracts from *Fusarium* sp. (CML 2969), *A. sydowii* (CML 2967) and *A. keveii* (CML 2968) presented 2,050.1, 2,090.95 and 2,552.5 μg mL^{-1}, respectively. The exopolysaccharide synthesized by the endophytic fungus *Aspergillus* sp. showed a strong antioxidant capacity by the DPPH method with EC_{50} of 1.45 mg mL^{-1} (CHEN et al., 2011). Alkaloid diketopiperazine compound was isolated from *Aspergillus* species, which has the antioxidant ability to sequester the DPPH radical with IC_{50} of 20 μM (SALEEM et al., 2007). Furthermore, the extracellular polysaccharide of *A. versicolor* presented capacity of inhibition of the radical DPPH with EC_{50} of 2.05 mg mL^{-1} (CHEN et al., 2012). The highest antioxidant activity among endophytic fungi was found in *Arcopilus* sp. (A2C54) with EC_{50} of 5,967.1 μg mL^{-1}. Among the three fungi of the genus *Epicoccum* the extract of *E. nigrum* (A2S61) exhibited a higher EC_{50} of 6,651.7 μg mL^{-1}.

3.1.2 ABTS assay

The antioxidant activity of EtOAc extracts in the concentrations of 500, 250 and 125 μg mL^{-1} was expressed by inhibition of the $ABTS^{\bullet+}$ free radical in percentage (I%) and is presented in Graphic 2. The antioxidant Trolox had a higher percentage of inhibition with 47.09, 22.48 and 14.27%. *P. flavigenum* (CML 2965), exhibiting the second highest percent inhibition at all concentrations, with 29.50, 15.84, 7.82% than the other EtOAc extracts. The fungus *A. sydowi* (CML 2967) showed a percentage of sequestering the free radical $ABTS^{\bullet+}$ greater than the radical DPPH, being the second best percentage with 21.54, 15.84 and 7.82%, respectively, suggesting that its antioxidant activity is related to a compound of a more lipophilic nature since the ABTS method also responds to lipophilic compounds. In contrast to the results observed with *L. aphanocladii* (CML 2970), which showed a lower percentage in the ABTS test, with 4.58, 2.31 and 1.59, respectively, the DPPH assay suggests that the antioxidant compound is more hydrophilic in nature.

Graphic 2 – Free radical scavenging capacity ABTS$^{\bullet+}$ of extract from filamentous fungi with different concentrations.



Data expressed as means of triplicates ± standard deviation. Means with different letters are significantly different at P<0.005.
Source: Tavares (2017).

Arcopilus sp. (A2C54) presented the best antioxidant activity among endophytic fungi, with 4.48, 4.26 and 2.94% ABTS$^{\bullet+}$ radical sequestration and inhibition values close to those of the DPPH assay. The isolated fungi of the cave also showed a greater capacity of sequestration of the ABTS$^{\bullet+}$ radical, except for *E. nigrum* (CML 2971) and *P. chermesinum* (CML 2966), when compared with the endophytes and with the DPPH method. These results show that because the ABTS and DPPH methods have the same principle of free radical sequestration, the found values of antioxidant activity may be equivalent, although differences, probably due to the nature of the compounds, were observed.

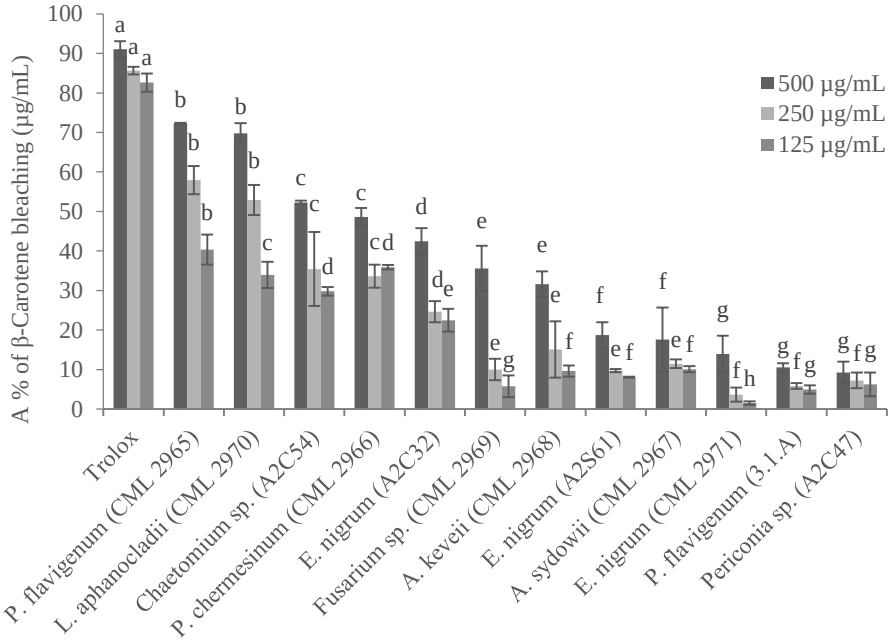
The data obtained from different concentrations of the extracts were also expressed in EC_{50}, which refers to concentration in µg mL^{-1} capable of inhibiting 50% of the ABTS$^{\bullet+}$ free radical (TABLE 1). The EC_{50} values of extracts from the studied fungi showed a variation from 862.2 to 27,867.2 µg mL^{-1}. The extract of the fungus *P. flavigenum* (CML 2965) showed a lower EC_{50} among the extracts studied, with 862.2 µg mL^{-1}, showing a great antioxidant capacity, even showing a higher EC_{50} value in relation to the DPPH method. The second best activity was exhibited by the *A. sydowii* (CML 2967) fungus with EC_{50} of 15031.2 µg mL^{-1} followed by *A. keveii* (CML 2968), *Fusarium* sp. (CML 2969), *P. flavigenum* (3.1.A) and *L.*

aphanocladii (CML 2970). The extract of the fungus *Arcopilus* sp. (A2C54) presented higher antioxidant power among the four endophytes studied, with EC_{50} of 15031.2 μg mL^{-1} followed by *P. ignaria* sp. (A2C47). The methanolic extract of the fungus *Chaetomium* sp. showed high antioxidant activity by the ABTS method (HUANG et al., 2007b), which was higher than that found in the present study using the ethyl acetate extract. This difference can be related to the extraction of different compounds by the solvents used. The three fungi of the genus *Epicoccum* showed the lowest antioxidant activities for this method with EC_{50} from 17753 to 27867.2 μg mL^{-1}, being the largest of the extract of *E. nigrum* sp. (A2S61). *P. chermesinum* (CML 2966) showed the lowest antioxidant activity among the fungi of the genus *Penicillium* studied in this work, in both DPPH and ABTS methods.

3.1.3 β-Carotene-linoleic acid assay

In the β-carotene-linoleic acid system, the free radical linoleic acid attacks the unsaturations of β-carotene. Antioxidant substances can neutralize the linoleate radical formed in the system, leading to a decrease in bleaching extent of β-carotene (YANISHLIEVA-MASLAROVA, 2001), which is expressed as a percentage of protection by the antioxidant compound (A%). The percentage of protection of the β-carotene-linoleic acid system by the extracts at the concentrations of 500, 250 and 125 μg mL^{-1} are presented in Graphic 3. As expected, there is a directly proportional variation to the extracts concentration in the protection percentage of the system. The antioxydant Trolox presented a higher percentage of β-carotene protection in the three concentrations with 91.05, 85.64 and 82.62%, respectively. *P. flavigenum* (CML 2965) and *L. aphanocladii* (CML 2970) exhibited the highest antioxidant activities among the extracts and did not differ statistically between them. *P. flavigenum* (CML 2965) obtained 72.23, 57.93 and 40.36% and *L. aphanocladii* (CML 2970) 69.77, 52.89 and 33.94% protection of β-carotene for the three concentrations respectively, differing statistically only in the concentration of 125 μg mL^{-1}. *Fusarium* sp. (CML 2969), *A. keveii* (CML 2968), *E. nigrum* sp. (A2S61), *A. sydowii* (CML 2967) and *E. nigrum* (CML 2971) exhibited 35.57, 31.61, 18.50, 17.56 and 13.91% β-carotene protection, respectively at the concentration of 500 μg mL^{-1}. Nevertheless, *P. flavigenum* (3.1.a) had one of the lowest protection percentages of 10.57% followed by the endophytic *P. ignaria* (A2C32) with 9.25% at the concentration of 500 μg mL^{-1}.

Graphic 3 – Antioxidant activity (A %) of extract from filamentous fungi with different concentrations measured in β-carotene – linoleic acid assay.



Data expressed as means of triplicates ± standard deviation. Means with different letters are significantly different at P<0.005.
Source: Tavares (2017).

The endophytic *Arcopilus* sp. (A2C54) with 52.30, 35.45 and 29.79%, *P. chermesinum* (CML 2966) with 48.61, 33.62 and 30.16% and *E. nigrum* (A2C32) with 42.44, 24.62 and 22.48% presented β-carotene protection at the three concentrations, respectively. The extracts of these three fungi showed low sequestering ability of the DPPH and $ABTS^{\bullet+}$ radicals, but showed better antioxidant activity by the β-carotene-linoleic acid method. This strongly suggests that these extracts present antioxidant compounds of a lipophilic nature that are better detected by the β-carotene-linoleic acid method and also shows the importance of using different methods to evaluate the antioxidant activity of extracts.

Srinivasan et al. (2010) used both DPPH and ABTS methods to evaluate the antioxidant activity of the ethanolic extract of endophyte *Phyllostic* sp. and found EC_{50} of 2030.25 and 580.02 µg mL^{-1}, respectively, showing different percent inhibition values, as occurred in this study. There is no correlation between the activity of inhibition of DPPH and $ABTS^{\bullet+}$ with the oxidant activity performed by the β-carotene-linoleic acid method (LIU et al., 2007). However, it may be attributed to the β-carotene-linoleic acid system being more specific to lipophilic compounds (LIU et al., 2007). Sadrati et al. (2013) found antioxidant activity of EtOAc from *Penicillium* sp. and *Aspergillus* sp. of 78.96 and 73.98% protection of

β-carotene, respectively and *Chaetomium* sp. with 50% system protection. In this study, *P. flavigenum* (CML 2965) and *Chaetomium* sp. exhibited 72.23 and 52.30% β-carotene protection, showing values close to those found by Sadrati et al. (2013). Liu et al. (2007) found in the methanolic extract of the endophytic *Xylaria* sp. 72.9% protection against β-carotene bracketing and 82.42% inhibition of the DPPH radical and also concluded that this difference may be due to the specificity of the β-carotene-linoleic acid assay for lipophilic compounds.

3.2 DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

Phenolic compounds play an important role in the stabilization of lipid oxidation and are associated with antioxidant activity (YANISHLIEVA-MASLAROVA, 2001). The content of phenolic compounds of extracts at the concentration of 500 μg mL^{-1} is shown in Table 2.

Table 2 – Total phenolic content in extract from flilamentous fungi expressed as gallic acid equivalents (mg of GAE/g of extract).

Fungi extracts (500 μg mL^{-1})	Total phenolic (mg of GAE/g of extract)
P. flavigenum (CML 2965)	201.43 ± 5^{a}
A. sydowii (CML 2967)	52.74 ± 1.33^{b}
Arcopilus sp. (A2C54)	23.73 ± 0.43^{c}
P. ignaria (A2C47)	21.99 ± 0.46^{c}
P. flavigenum (3.1.A)	18.84 ± 0.21^{d}
A. keveii (CML 2968)	18.56 ± 0.25^{d}
Fusarium sp. (CML 2969)	18.23 ± 0.21^{d}
L. aphanocladii (CML 2970)	15.75 ± 0.49^{d}
P. chermesinum (CML 2966)	7.30 ± 0.14^{e}
E. nigrum (A2S61)	6.36 ± 0.18^{e}
E. nigrum (CML 2971)	6.36 ± 0.12^{e}
E. nigrum sp. (A2C32)	5.37 ± 0.16^{e}

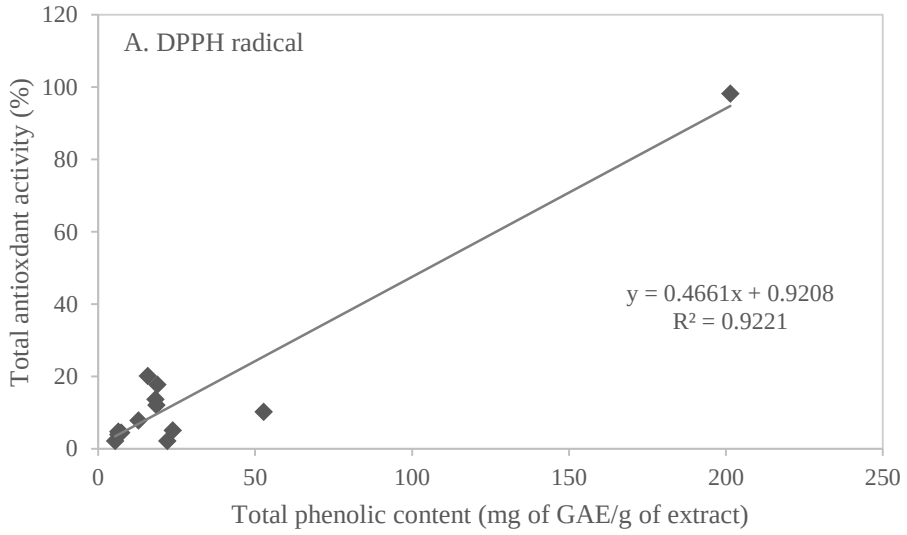
Data expressed as means of triplicates ± standard deviation. Means with different letters are significantly different at $P<0.005$.
Source: Tavares (2017).

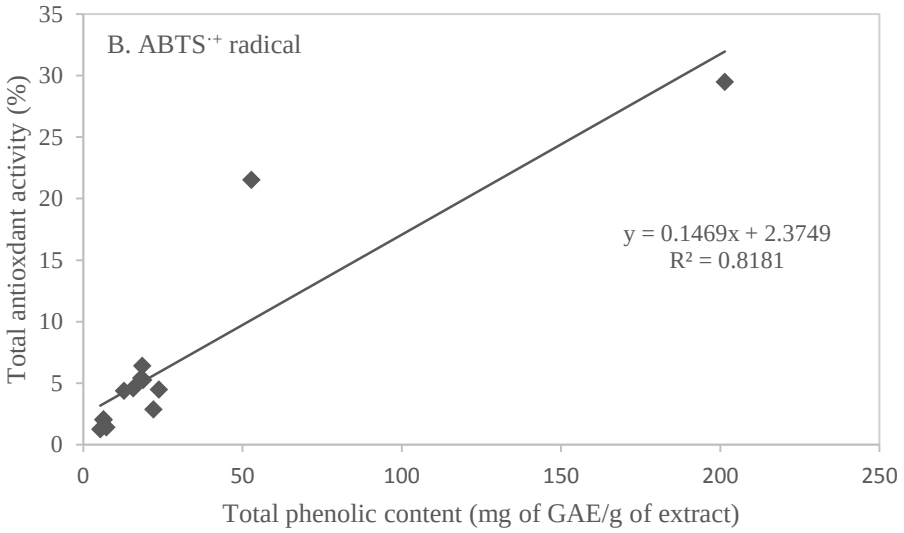
As shown in previous essay, the extract from *P. flavigenum* (CML 2965) shows strong antioxidant activity, which can also be demonstrated by the high concentration of phenolic compounds in the extract with 201.43 mg of GAE/g of extract, differing statistically from other extracts. *P. flavigenum* (3.1.a), although of the same species, showed only 18.84 mg of GAE/g of extract. The second largest result was the extract of *A. sydowii* (CML 2967) with

52.74 mg of GAE/g of extract, followed by the endophytic fungi *Arcopilus* sp. (A2C54) and *P. ignaria* (A2C47) with 23.73 and 21.99 mg of GAE/g of extract, respectively. For the fungus *P. ignaria* (A2C47), although it did not show high antioxidant capacity, it exhibited considerable phenolic content without statistically differing of the fungus *Arcopilus* sp. (A2C54). This shows that the antioxidant activity also depends on other factors such as phenolic profile type, extract concentration, and nature of the antioxidant. In addition, it is suggested that endophytic fungi can synthesize the host plant compounds, and plants are known to produce many phenolic compounds. The extracts of *A. keveii* (CML 2968), *Fusarium* sp. (CML 2969) and *L aphanocladii* (CML 2970) presented 18.56, 18.23 and 15.75 mg of GAE/g of extract, respectively. The lowest phenolic contents were found in *P. chermesinum* (CML 2966) with 7.30, followed by *E. nigrum* (CML 2971 and A2S61) with 6.36 and *E. nigrum* (A2C32) with 5.37 mg of GAE/g of extract. It is observed that the content of phenolic compounds in the extracts of the isolates of the genus *Epicoccum* exhibited amounts that did not differ statistically between them.

Graphic 4 shows that extracts from filamentous fungi that showed low antioxidant activity, showed a lower phenolic content while those that obtained higher antioxidant capacity also exhibited a higher phenolic content.

Graphic 4 – Relationships between total antioxidant capacities and phenolic contents of A) DPPH radical and of B) $ABTS^{\cdot+}$.





Source: Tavares (2017).

The high positive linear correlation between the total antioxidant activity (y) and the total phenolic content (x) was found by both the DPPH radical capture method (y = 0.4661x + 0.9208; R2 = 0.9221) in Graphic 4.A and by the $ABTS^{\bullet+}$ radical capture method (y = 0.1469x + 2.3749; R2 = 0.8181) in Graphic 4.B. This positive relation shows that phenolic compounds act on the antioxidant activity of the EtOAc extracts. However, it is not a general rule. Observing Graphic 8 A and B, the antioxidant potential of the *P. flavigenum* fungus (CML 2965) is clear, both in the antioxidant capacity and in the production of phenolic compounds.

Phenolic compounds are products of secondary metabolism, and have metal chelating ability and capture of free radicals. Different phenolic compounds may have a difference in their effectiveness with antioxidant agent. This relationship is still unclear, but the antioxidant activity of the extract depends on its concentration and on the structure and interaction between the phenolic compounds present (LIU et al., 2007). Extracts that exhibit high antioxidant activity by the DPPH radical capture method present compounds that donate hydrogen leading to the neutralization of this free radical (SHON; KIM; SUNG, 2003). Liu et al. (2007) found a positive correlation between the phenolic content and the scavenging activity of the DPPH radical, similar to the results found in this work. Other authors also concluded that phenolic compounds contribute to the antioxidant activity (KANDASAMY, KANDASAMY, 2014; LIU et al., 2007; CAKIR et al., 2003; PROESTOS et al., 2006). Yadav et al. (2014) investigated 21 EtOAc extracts from endophytic fungal and found a content of phenolic compounds between 4.20 and 60.13 mg of GAE/g of extract, with the

highest values for the fungi *Chaetomium* sp. and 4 isolates of *Aspergillus* that also exhibited antioxidant activity with values of 80% inhibition of DPPH.

In the present work, *P. flavigenum* (CML 2965) extract showed high antioxidant activity in all the tested methods and a high content of phenolic compounds and could become a potential source of natural antioxidants. This isolate produces yellow-colored pigments, and the yellow-colored molecule found in the extract of *P. flavigenum* (CML 2965) – dihydrotrichodimerol - was identified by Souza et al. (2016). This compound is a bisorbicillinoid polyketide that has a rare structure in nature (SOUZA et al., 2016) and has biological activities as antioxidant and antitumor capacity (LEE et al., 2005; LIU et al., 2005).

Huang et al. (2007a) using the ABTS method observed that the extract of endophytic fungi showed high total antioxidant activity also presented high total phenolic content and extracts that showed low antioxidant activity also presented low total phenolic content. The methanolic extract of the endophytic fungus *Chaetomium* sp. showed high antioxidant capacity by the ABTS method and also high content of phenolic compounds (HUANG et al., 2007b). The positive correlation found between the total antioxidant activity and the phenolic content suggest that the phenolic compounds present in the extracts contribute to its antioxidant activity. The results of antioxidant activity and total phenolic compounds show that the filamentous fungi, especially *P. flavigenum* (CML 2965) isolated from caves and the endophytic *Arcopilus* sp. (A2C54) have great potential for the production of natural compounds.

4 CONCLUSION

Among the endophytic fungi, the *Arcopilus* sp. (A2C54) showed the highest antioxidant activity and significant phenolic content. The extract from fungus *P. flavigenum* (CML 2965) isolated from caves showed strong antioxidant potential in the three methodologies used and high total phenolic content can be used in the future with natural antioxidant and phenolic compound producer. Further work is needed to better elucidate the composition of the extract and to identify the molecules with antioxidant activity.

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