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EXTRAÇÃO COM FLUIDO SUPERCRÍTICO E EXTRAÇÃO ASSISTIDA POR ULTRASSOM NA OBTENÇÃO DE COMPOSTOS ANTIOXIDANTES PRODUZIDOS POR *Diaporthe schini*

Santa Maria, RS
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RESUMO

EXTRAÇÃO COM FLUIDO SUPERCRÍTICO E EXTRAÇÃO ASSISTIDA POR ULTRASSOM NA OBTENÇÃO DE COMPOSTOS ANTIOXIDANTES PRODUZIDOS POR *Diaporthe schini*

AUTORA: Barbara Vargas da Rosa
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O bioma Pampa que abrange o sul do Brasil e parte do Uruguai e Argentina possuiu uma das biodiversidades mais ricas do mundo, a qual ainda não foi completamente descrita pela ciência. Os fungos endofíticos fazem parte desta biodiversidade, sendo ultimamente explorados para a produção de metabólitos com diferentes propriedades bioativas. Uma destas propriedades é a capacidade de uma molécula de proteger contra o ataque de radicais livres, sendo interessante para diversos seguimentos como as indústrias de alimentos e farmacêuticas. Portanto, o objetivo principal deste trabalho foi extrair compostos antioxidantes a partir da biomassa obtida da fermentação do fungo endofítico *Diaporthe schini* utilizando extração com fluído supercrítico (EFS) e extração assistida por ultrassom (EAU). A extração utilizando dióxido de carbono (CO_2) em estado supercrítico foi realizada utilizando CO_2 puro e CO_2 com etanol como co-solvente. Para a extração supercrítica foram avaliadas as condições de pressão (150-250 bar), temperatura (40-60 ºC) e quantidade de co-solvente (razão biomassa: co-solvente, 1:0, 1:0,75, e 1:1,5 (m/v)). De acordo com os dados experimentais, as curvas cinéticas e as taxas de transferência de massa, a melhor condição foi obtida utilizando 250 bar, 40 ºC e razão biomassa: co-solvente de 1:1,5 (m/v), sendo o rendimento de extração e a atividade antioxidante frente ao radical 2,2-difenil-1-picrilhidrazilo (DPPH) de 3,24 % e 96,62 %, respectivamente. A extração assistida por ultrassom foi realizada utilizando etanol como solvente e as condições avaliadas foram a intensidade de ultrassom (17-85 $W.cm^{-2}$) e o ciclo de pulso (0,50-1,00) as quais foram analisadas de acordo com um Delineamento Composto Central Rotacional (DCCR) para a obtenção de uma maior quantidade de extrato. A condição de validação foi realizada utilizando intensidade de ultrassom de 85 $W.cm^{-2}$ e ciclo de pulso de 0,93, resultando em 22,30 ± 0,47 % de rendimento de extrato e 91,35 ± 0,27 % de atividade antioxidante. Já, a técnica de extração assistida pelo calor foi realizada para comparação, apresentou um rendimento de extrato de 8,34 ± 0,38 % e atividade antioxidante de 91,32 ± 0,89 %. As análises dos extratos por cromatografia gasosa/ espectroscopia de massa (CG/MS) indicaram a presença dos compostos 1,4-diaza-2,5-dioxo-3-isobutil biciclo [4.3.0] nonano, benzeno etanol, 9,12 ácido octadecadienoico (Z, Z), éster metílico, ácido hexadecanóico, e octadec-9-enoato de etila os quais podem ser atribuídas a atividade antioxidante encontrada. Os resultados demonstraram que a extração utilizando fluido supercrítico apresentou-se mais seletiva, devido a maior atividade antioxidante em relação aos demais métodos de extração avaliados. Na extração assistida por ultrassom o rendimento foi superior e o tempo de extração foi reduzido mantendo alta a atividade antioxidante, sendo assim o método mais interessante.

Palavras-chave: *Diaporthe schini*. Fermentação submersa. Atividade antioxidante. Extração supercrítica. Extração assistida por ultrassom.

ABSTRACT

SUPERCRITICAL FLUID EXTRACTION AND ULTRASOUND-ASSISTED EXTRACTION OF ANTIOXIDANT COMPOUNDS FROM *Diaporthe schini*

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The Pampa biome that covers southern Brazil and part of Uruguay and Argentina has one of the richest biodiversity in the world, which has not yet fully described by science. Part of this biodiversity are the endophytic fungus, being exploited for the production of metabolites with different bioactive properties. One of these properties is the ability of a molecule to protect against attack by free radicals, providing interesting several segments such as the food, and pharmaceutical industries. Therefore, the main objective of this work was to extract antioxidant compounds from the biomass obtained from the fermentation of endophytic fungus *Diaporthe schini* using supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE). The extraction using carbon dioxide (CO_2) in the supercritical state was performed using pure CO_2 and CO_2 plus ethanol as cosolvent. For the supercritical extractions pressure (150-250 bar), temperature (40-60 ºC) and cosolvent [biomass: cosolvent ratio, 1:0, 1:0.75 and 1:1.5 (w/v)] were evaluated. According to experimental data, kinetic curves and mass transfer rates, the best condition was obtained using 250 bar, 40 ºC and biomass: cosolvent ratio of 1:1.5 (w/v), with extraction yield and the antioxidant activity against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical of 3.24 % e 96.62 %, respectively. The ultrasound-assisted extraction was performed using ethanol as solvent and the parameters ultrasound intensity (17-85 $W.cm^{-2}$) and pulse cycle (0.50-1.00) were investigated according to a Central Composite Rotatable Design (CCRD), to obtain a highest extract yield. The validation condition was performed using an ultrasound intensity of 85 $W.cm^{-2}$ and a pulse cycle of 0.93, resulting in an extraction yield of 22.30 ± 0.47 % and an antioxidant activity of 91.35 ± 0.27 %. The heat-assisted extraction which was performed for comparison, presented an extract yield of 8.34 ± 0.38 % and an antioxidant activity of 91.32 ± 0.89 %. Analysis of the extracts by gas chromatography/ mass spectroscopy (GC/MS) indicated the presence of the compounds 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane, benzeneethanol, 9,12 octadecadienoic acid (Z, Z), methyl ester, hexadecanoic acid, and ethyl octadec-9-enoate which could be attributed to the antioxidant activity found in this study. The results demonstrated that supercritical fluid extraction was more selective, due to the greater antioxidant activity in relation to the other extraction methods evaluated. However, in the ultrasound-assisted extraction, the yield was higher, the extraction time was reduced, keeping the antioxidant activity high, thus being the most interesting method.

Keywords: *Diaporthe schini*. Submerged fermentation. Antioxidant activity. Supercritical extraction. Ultrasound-assisted extraction.

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1 INTRODUÇÃO

O bioma brasileiro Pampa por conta de sua localização geográfica apresenta características distintas de vegetação, solo e clima, tornando-se um ecossistema único no planeta. Entretanto, sua biodiversidade é a menos explorada dos biomas brasileiros, especialmente em relação à biodiversidade microbiana (LUPATINI et al., 2013). A bioprospecção de metabólitos fúngicos pelo isolamento de microrganismos do bioma Pampa pode ser realizada para a exploração de novas fontes de compostos bioativos, como a obtenção de compostos antioxidantes a partir da fermentação do fungo endofítico *Botriosphaeria dothidea* (DRUZIAN et al., 2019) e a produção de metabólitos com atividade bioherbicida a partir do fungo *Diaporthe* sp. (SOUZA et al. 2017).

A obtenção de moléculas que desempenham funções biológicas a partir de fungos é uma vantagem em relação às demais fontes, por conta do fato de que microrganismos podem ser cultivados em larga escala em fermentadores, não havendo prejuízo ao ecossistema, como pode ocorrer com a retirada de plantas e algas de áreas naturais. Além disso, evita problemas éticos como os que podem advir da prospecção de metabólitos bioativos a partir de insetos, anfíbios e outras espécies animais (TAKAHASHI e LUCAS, 2008). Embora pouca estudada, a biodiversidade microbiana do bioma Pampa pode ser um modelo eficaz para a produção de compostos bioativos de alto valor, da mesma maneira que ajuda na conservação dessas espécies (SOUZA et al., 2015).

Estes microrganismos, muitas vezes, produzem compostos com atividade antioxidante, os quais servem como proteção contra radicais livres, que são os principais elementos que levam a uma variedade de efeitos patológicos, como por exemplo, danos ao DNA, doenças como câncer, Alzheimer e degeneração celular (ZHANG et al., 2018). Na literatura diversos trabalhos realizam a bioprospecção destas moléculas (MOU et al., 2013; ZHAO et al., 2012; ZHAO et al., 2014).

Um dos desafios para a extração destes compostos é encontrar métodos alternativos que aumentem o rendimento e diminuam o tempo de extração (LEITE et al., 2019). Ultimamente, dois métodos têm se destacado para a extração de biocompostos, a extração com fluido supercrítico (EFS) e a extração assistida por ultrassom (EAU). As duas técnicas apresentam vantagens quanto à seletividade, redução no consumo de solvente e tempo de extração. Sua principal diferença é que na EFS é gerado um extrato sem a necessidade de etapas para a remoção do solvente, enquanto que a EAU possui alto rendimento de extração por conta de mecanismos de bolhas de cavitação (CHEMAT et al., 2017; ŞAHIN et al., 2011).

Os fungos do gênero *Diaporthe,* encontrados no bioma Pampa, produzem metabólitos secundários com ampla aplicação industrial, agrícola e farmacêutica (TANAPICHATSAKUL et al., 2018). Alguns trabalhos da literatura identificaram a presença de compostos com propriedades antitumoral (ASGHAR et al., 2017; SHARMA et al., 2018), antifúngica (EL-GERDY et al., 2018; TANNEY et al., 2016), antibacteriana (LI et al., 2015; SEBASTIANES et al., 2012; TANAPICHATSAKUL et al., 2018), bioherbicida (SOUZA et al., 2015) e antioxidante (TANAPICHATSAKUL et al., 2018). Entretanto, nenhum trabalho na literatura, até o momento, explora a otimização da extração através de métodos não convencionais, como a extração assistida por ultrassom e a extração com fluido supercrítico para estes fungos. Sendo assim, o objetivo deste estudo foi avaliar os parâmetros de extração, atividade antioxidante e a caracterização química dos extratos obtidos a partir da extração com CO_2 supercrítico com e sem co-solvente e a extração assistida por ultrassom da biomassa produzida pela fermentação submersa do fungo *Diaporthe schini*.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Este trabalho teve como objetivo extrair compostos antioxidantes a partir da biomassa obtida da fermentação do fungo endofítico *Diaporthe schini* utilizando extração com fluído supercrítico e extração assistida por ultrassom.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar as melhores condições de extração utilizando gás carbônico supercrítico como solvente e etanol como co-solvente;

- Avaliar as melhores condições de extração utilizando ondas ultrassônicas em diferentes intensidades e pulsos utilizando etanol como solvente;

- Avaliar a extração assistida pelo calor em diferentes temperaturas;

- Identificar os extratos através de cromatografia gasosa (CG/MS).

3 REVISÃO DA LITERATURA

Com uma área de aproximadamente 750 mil km^2, o bioma dos Pampas está localizado na região sul da América do Sul, distribuído entre Brasil, Uruguai e Argentina (BRASIL, 2019). No Brasil ocupa uma área aproximada de 2,07% do território nacional que abrange a metade meridional do estado do Rio Grande do Sul (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2019), como pode ser visto na Figura 1.

Figura 1 – Biomas brasileiros

Fonte: (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2019)

O bioma Pampa, um conjunto de vida vegetal e animal caracterizado pelo tipo de vegetação dominante é classificado como Estepe no sistema fitogeográfico internacional (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2012) e formado por quatro conjuntos principais de vegetação de campos, compostas por ervas e arbustos, situadas nas áreas geográficas conhecidas como Planalto da Campanha, Depressão Central, Planalto Sul-Rio-Grandense e Planície Costeira. É caracterizado por clima chuvoso, sem período seco, mas com

temperaturas negativas no inverno, que influenciam a vegetação (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2019).

Por ser um conjunto de ecossistemas muito antigos, o Pampa apresenta flora e fauna próprias (BRASIL, 2019) e é considerado como um dos campos mais férteis e com uma das biodiversidades mais ricas do mundo (PILLAR et al., 2009). Contudo, este bioma ainda não é completamente descrito pela ciência (BRASIL, 2019).

3.1 Fungos endofíticos

Os microrganismos são uma importante parte de um ecossistema e contribuem significativamente para sua biodiversidade. Englobam uma enorme variedade de organismos, incluindo bactérias, fungos, vírus, algas e protozoários. A grande maioria destes microrganismos não representa uma ameaça real para seres humanos, plantas ou animais. Fungos endofíticos são um exemplo, vivem sistematicamente no interior das plantas, sem causar aparentemente dano e beneficiando seus hospedeiros enquanto produzem metabólitos secundários biologicamente ativos (BILAL et al., 2018; EMBRAPA, 2019; SUDHA et al., 2016).

Fungos endofíticos são relativamente pouco estudados, contudo são potenciais fontes de novos produtos naturais para a exploração na medicina, agricultura e indústria (STROBEL e DAISY, 2003). A Penicilina é um composto com ação antibacteriana produzida pelo fungo *Penicillium chrysogenun* (TORTORA et al., 2012) e é um dos primeiros exemplos que se tem, que mostram como este tipo de fonte pode contribuir para o desenvolvimento da ciência.

Metabólitos secundários são compostos frequentemente associados com a cessação do crescimento, e são definidos como produtos naturais que não possuem função no crescimento celular, e são sintetizados por células que pararam de se dividir (BENNETT e BENTLEY, 1989). Geralmente possuem baixo peso molecular, são bioativos e sua produção frequentemente esta correlacionada com um estágio específico de diferenciação morfológica (KELLER et al., 2005).

3.1.1 Gênero *Diaporthe*

Espécies de *Diaporthe* ocorrem na natureza como endófitos ou sapróbios, mas também como patógenos humanos e outros mamíferos, contudo algumas espécies de *Diaporthe* podem ser endófitos patogênicos ou inofensivos dependendo do hospedeiro e sua saúde (GOMES et

al., 2013). Espécies dentro deste gênero representam endófitos multi hospedeiros e/ou fungos associados a plantas que podem ser observados em diferentes localizações geográficas (PIRTTILÄ e FRANK, 2011). A produção de metabólitos secundários biologicamente ativos por este gênero pode ser atribuída a um fator proeminente de interação planta-endófito (EL-GENDY et al., 2018).

O gênero *Diaporthe* compreende um grupo de fungos estudados extensivamente por sua capacidade de produzir metabólitos para diversas aplicações (FLORES et al., 2013) alguns exemplos destes estudos podem ser observados na Tabela 1.

Tabela 1 – Compostos com ação biológica a partir de diferentes espécies de *Diaporthe*

Microrganismo	**Atividade biológica**	**Referência**
Diaporthe sp.	Bioherbicida	DE SOUZA et al., 2015
Diaporthe sp.	Antibacteriana	LI et al., 2015
Diaporthe spp.	Antibacteriana e Antioxidante	TANAPICHATSAKUL et al., 2018
Diaporthe schini	Antifúngica e antibacteriana	DOS REIS et al., 2019
Diaporthe phaseolorum	Anticâncer	CARVALHO et al., 2012
Diaporthe maritima	Antifúngica	EL-GENDY et al., 2018
Diaporthe phaseolorum	Antibacteriana	SEBASTIANES et al., 2012
Diaporthe sp.	Anticâncer (antitumor), antioxidante e citotóxico	SHARMA et al., 2018

3.2 Compostos antioxidantes

O oxigênio é um componente essencial para os organismos vivos (AHMAD, 2012), no entanto, espécies reativas de oxigênio quando sobrecarregam o sistema de defesa antioxidante celular podem causar estresse oxidativo resultando em danos nas células (RAY et al., 2012). Espécie reativa de oxigênio é o termo utilizado para dar nome as moléculas e espécies derivadas do oxigênio molecular, porém não são radicais livres. Essas espécies são popularmente conhecidas pelo termo oxidante (THOMAS, 1998).

Radicais livres são considerados qualquer espécie capaz de existir de forma independente que contenha um ou mais elétrons desemparelhados o que pode lhes conferir uma alta reatividade, apesar de existir uma grande variedade na reatividade química desses radicais (HALLIWELL e GUTTERIDGE, 2015). Essas moléculas são instáveis e são formadas constantemente como subproduto do metabolismo (BAJEROVÁ et al., 2014).

Ao longo dos anos, pesquisas relacionadas com estresse oxidativo têm crescido constantemente, aumentando assim o conhecimento da importância do status redox celular e auxiliando no reconhecimento do estresse oxidativo como um processo com implicações para um grande número de estados fisiopatológicos (ÖZBEN, 2013).

Compostos antioxidantes são substâncias naturais ou sintéticas que protegem biomoléculas do ataque de radicais livres (ALVEZ et al., 2013; BAJEROVÁ et al., 2014; NAM e KIM, 2013;), retardando a velocidade de oxidação através de um ou mais mecanismos (PIETTA, 2000). De uma forma geral os compostos antioxidantes podem ser classificados como modo de ação primários e secundários. Nos antioxidantes de ação primária a atividade antioxidante engloba uma transição redox envolvendo a doação de um único elétron (ou átomo H, equivalente à doação de um elétron e um H +) para uma espécie de radical livre (ÖZBEN, 2013), como o mecanismo do ácido ascórbico que pode ser observado pelas Equações 1-4.

$$AH^- + \cdot OH \rightarrow H_2O + A^{\cdot -} \quad (1)$$

$$AH^- + O_2^{\cdot -} + H^+ \rightarrow H_2O_2 + A^{\cdot -} \quad (2)$$

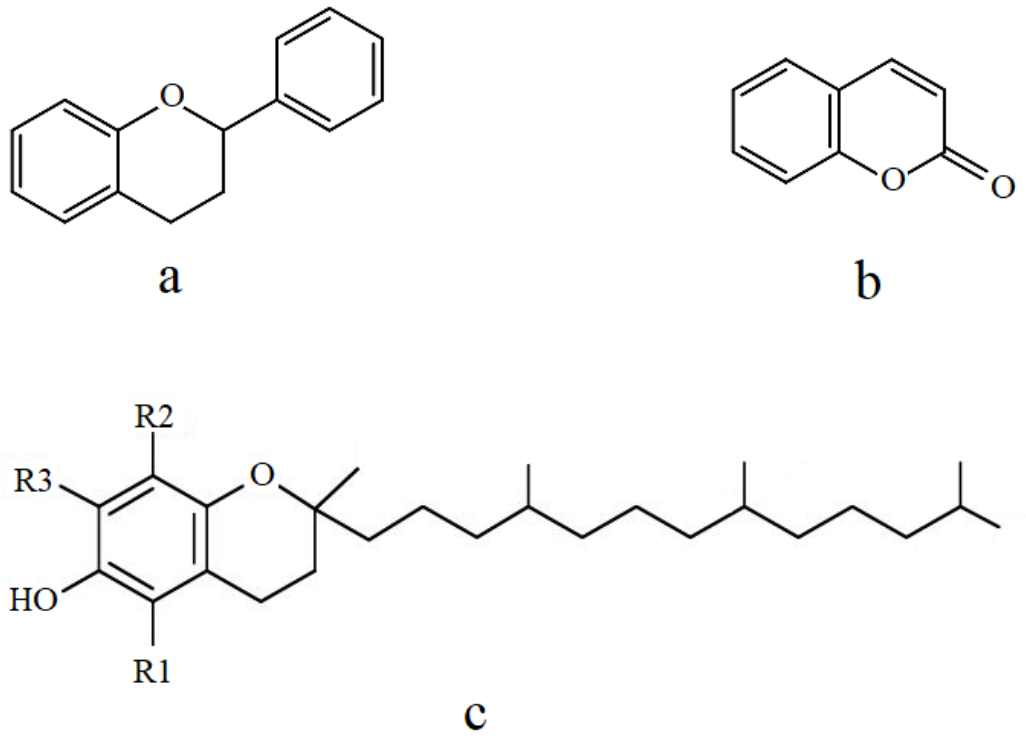
$$AH^- + ROO^{\cdot} \rightarrow RH + A^{\cdot -} \quad (3)$$

$$AH^- + H_2O + H^+ \rightarrow 2H_2O + A \quad (4)$$

Já os antioxidantes secundários retardam a etapa de iniciação da autoxidação, por meio de mecanismos diferentes, como complexação de metais, sequestro de oxigênio, decomposição de hidroperóxidos, absorção da radiação ultravioleta ou desativação de oxigênio singlete, dando origem as espécies não radicais (ANGELO e JORGE, 2007).

Os grupos mais importantes de antioxidantes naturais são os tocoferóis (vitamina E), flavonoides e cumarinas (BAJEROVÁ et al., 2014; CHERNG et al., 2008), suas estruturas básicas podem ser observadas na Figura 2.

Figura 2 – Estrutura química: a) flavonoides; b) cumarinas e c) tocoferóis



Fonte: própria autora

Os antioxidantes sintéticos em sua maioria são fenóis, com impedimento estérico e butilados, e polifenóis. Esses antioxidantes são mais baratos comparados aos naturais e também bastante efetivos em retardar o processo de oxidação (MUKHOPADHYAY, 2006), mas os possíveis potenciais mutagênicos e cancerígenos de alguns desses compostos, vêm estimulando a pesquisa por antioxidantes naturais (CHLUDIL et al., 2008; LU et al., 2002).

Estes compostos são uma resposta que muitos microrganismos, como os fungos, encontram para os mesmos tipos de problemas complexos com os quais plantas e animais devem lidar. O acumulo de espécies reativas de oxigênio pode levar as células a alterações genotóxicas ou citotóxicas ameaçando a sobrevivência desses seres. Diante deste estresse oxidativo, os organismos desenvolvem mecanismos para sua proteção, como a produção de compostos antioxidantes que compõem a primeira linha de defesa o que inclui evitar ou eliminar espécies antes que os danos oxidativos aconteçam (AHMAD, 2012).

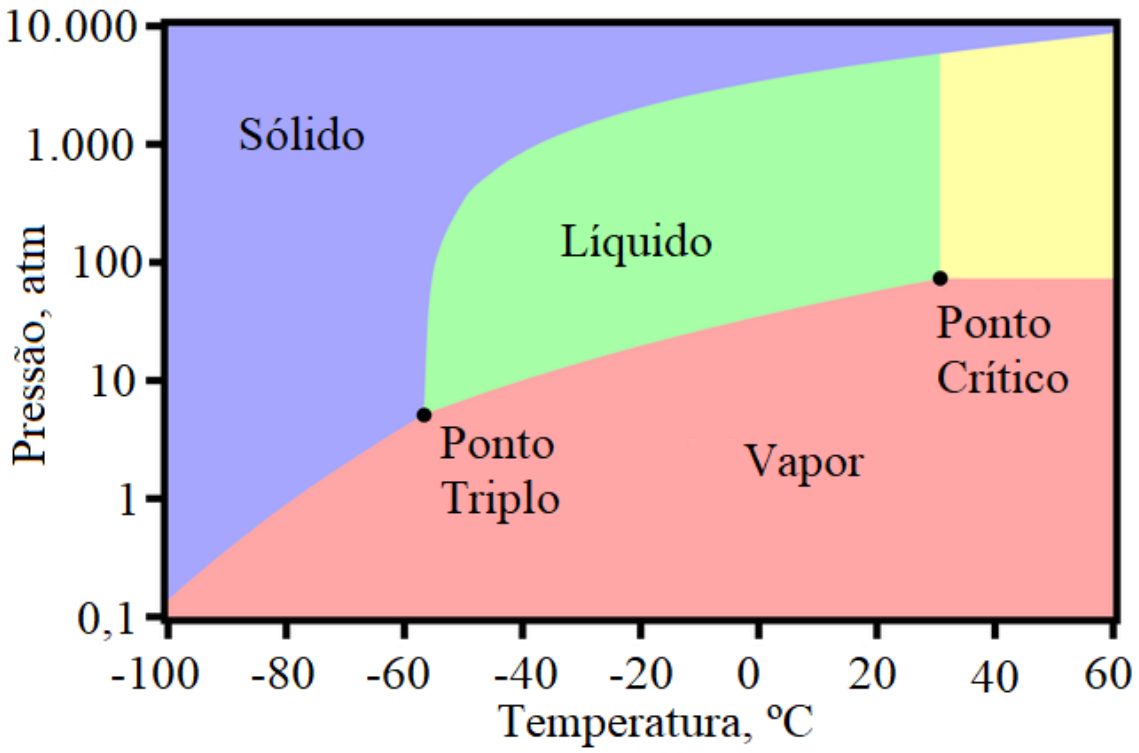
3.3 Extração de compostos

As vantagens das rápidas taxas de crescimento e facilidade de cultivo demonstrado por microrganismos são acreditados para fornecer a produção sem obstáculos de compostos desejáveis e atender a crescente demanda (FOWLER e KOFFAS, 2009). Além disso, extrações de biocompostos utilizando metodologias não convencionais, como extração com fluído supercrítico e extração assistida por ultrassom, são consideradas metodologias ecologicamente adequadasao meio ambiente (MATASSA et al., 2016) e, portanto, são alternativas preferidas para superar sérios problemas ambientais das metodologias convencionais de síntese química (TAN et al., 2018).

3.3.1 Extração com fluido supercrítico

Extração sólido-líquido é uma operação unitária que separa um soluto ou uma mistura de solutos presentes em uma matriz sólida quando a mesma entra em contato com um solvente adequado. Este solvente pode estar na fase líquida ou supercrítica. Um fluido é supercrítico quando sua temperatura e pressão estão acima dos valores críticos (ECKERT et al., 1996). A temperatura e pressão críticas correspondem as condições mais altas as quais o gás não pode ser convertido em líquido pelo aumento da pressão (MAUL, 1999). Para o dióxido de carbono (CO_2) estes valores são 31,15 ºC e 72,8 atm (NATIONAL INSTITUTE OF STANDARTS AND TECHNOLOGY, 2019), na Figura 3 é possível observar o diagrama de fases para o CO_2 e identificar o ponto crítico.

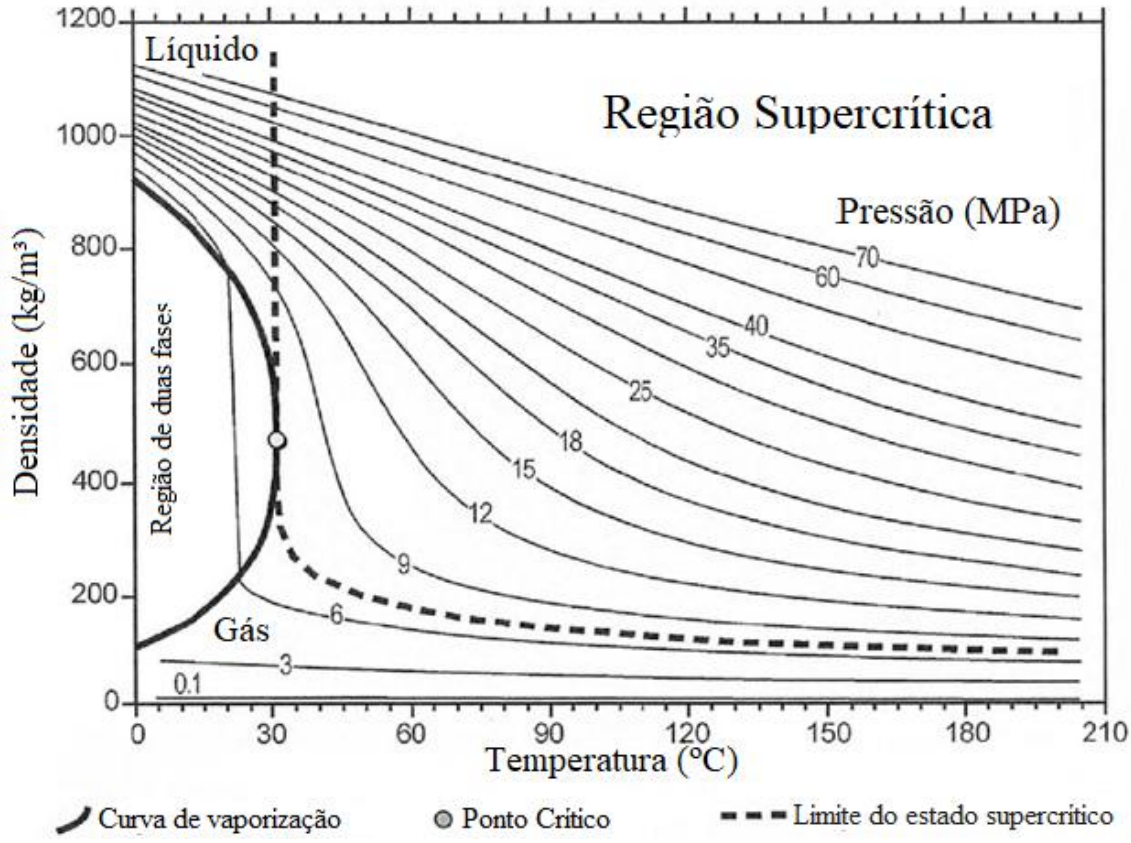
Figura 3 – Diagrama de fases para o CO_2



Fonte: Adaptado de KNEZ et al. (2014)

Fluidos supercríticos apresentam um significante poder solvente, pois apresentam densidade semelhante aos líquidos. Por operações na região crítica, a pressão e a temperatura podem ser usados para regular a densidade, que tem uma relação direta com a potência solvente de um fluido supercrítico (MCHUGH e KRUKONIS, 2013). Na Figura 4 é possível observar que uma pequena alteração nas condições de trabalho próximo do ponto crítico de CO_2 pode resultar em uma mudança significativa na densidade (ONYEBUCHI et al., 2018). As propriedades de viscosidade semelham-se aos gases, enquanto a difusividade é cerca de duas ordens de grandeza maior do que a difusividade típica dos líquidos, o que causa gradiente de densidade significativo na interface soluto/solvente, resultando em taxas de transferência de massa superiores aos solventes convencionais (BRUNNER, 2005).

Figura 4 - Diagrama densidade CO_2



Fonte: Adaptado de ONYEBUCHI et al. (2018)

A extração utilizando fluidos supercríticos tem vantagens imediatas sobre as técnicas tradicionais de extração: é um processo flexível devido a possibilidade de modulação contínua do solvente em relação ao poder de extração e a seletividade, permite a eliminação de solventes orgânicos poluentes e o dispendioso pós-processo de extração para a eliminação do solvente (REVERCHON e DE MARCO, 2006).

O solvente mais utilizado para a extração supercrítica é o dióxido de carbono (SCARPIN et al., 2017), pois é seguro, pode fornecer extratos com melhor seletividade, garante um processo de separação inócuo, tem baixo custo e permite operações supercríticas a pressões relativamente baixas e em temperaturas amenas (DE MELO et al., 2014). O CO_2 supercrítico é um bom solvente para solutos apolares, no entanto sua solubilidade diminui com o peso molecular dos compostos que se quer extrair, como por exemplo, os flavonoides que dificilmente são solúveis em CO_2 puro (MEIRELES, 2008).

Contudo, a solubilidade de compostos orgânicos polares ou sua interação com a matriz pode ser melhorada, aumentando a pressão ou adicionando um modificador polar (co-solvente). O aprimoramento da extração causado por um modificador pode estar relacionado a diferentes

fenômenos de modificação como mudança na polaridade, densidade e viscosidade do fluido de extração; miscibilidade dos modificadores e solventes; solubilidade do soluto; interação entre CO_2 supercrítico e a matriz e a ruptura da ligação entre soluto e a matriz sólida. O efeito do co-solvente resulta em mudanças na solubilidade, propriedades de transporte e resistência entre as partículas na matriz e pode aumentar o rendimento da extração dependendo da pressão e temperatura utilizadas. O aumento da solubilidade na presença de co-solvente pode ser associado a interações entre componentes, particularmente ligações de hidrogênio (LUCIEN e FOSTER, 2000).

Devido à complexidade estrutural e variabilidade dos materiais a serem tratados e da variedade de compostos que podem ser extraídos, o processo de extração utilizando fluido supercrítico está longe de ser considerado exaustivamente estudado, embora aplicações industriais já terem sido desenvolvidas. Além disso, está crescendo o interesse na extração de substâncias com valor agregado como antioxidantes, produtos farmacêuticos e corantes (REVERCHON e DE MARCO, 2006).

Na literatura alguns autores reportam a utilização da técnica de extração com fluido supercrítico para obter compostos com ação antioxidante. Kitzberger et al. (2007) extraiu compostos antioxidantes e antimicrobianos utilizando CO_2 supercrítico a 40 ºC e 200 bar utilizando 15% de etanol e obteve um rendimento de 3,81 %. Mazutti et al. (2012) obteve um rendimento de 1,19 % na extração utilizando fluido supercrítico com CO_2 puro nas condições de 300 bar e 50 ºC e nestas mesmas condições obteve o rendimento em massa de 4,20 % utilizando 10% de etanol como co-solvente para a extração de compostos antioxidantes produzidos por *Agaricus brasiliensis*. Segundo De Souza et al. (2018) é possível obter 6,09 % de rendimento de extrato das folhas de *Arctium lappa* utilizando a pressão de 150 bar e uma temperatura de 50 ºC.

3.3.2 Extração assistida por ultrassom

A maior parte das técnicas de extração consiste na manipulação das propriedades físicas do solvente para reduzir a tensão superficial, aumentar a solubilidade do soluto e promover maior taxa de difusão. Contudo técnicas de extração usando solventes a baixas pressões, como o uso da extração assistida por ultrassom, podem representar uma escolha apropriada para o processamento de muitos sistemas. Os fundamentos desses processos são diferentes dos métodos convencionais, uma vez que a extração ocorre devido a mudanças na estrutura celular causada por ondas sonoras (MEIRELES, 2008).

Segundo a Royal Society of Chemistry (2019) a extração assistida por ultrassom é definida como o processo de transferir uma substância de qualquer matriz para uma fase líquida, assistida por ondas sonoras (> 20KHz em frequência) que se propagam através do meio líquido. O ultrassom, assim como outras ondas, se propaga pelo meio criando compressão e rarefação (MASON et al., 2005). Em uma potência alta o ciclo de rarefação pode exceder as forças de atração das moléculas líquidas e formar bolhas de cavitação a partir de núcleos gasosos no interior do líquido. Estas bolhas distribuídas por todo o líquido crescem até um tamanho crítico se tornando instáveis e violentamente entram em colapso (SHUKLA, 1992). A implosão de bolhas de cavitação leva ao acúmulo de energia em pontos quentes, que produzem ondas de energia de alto cisalhamento e turbulência levando a uma alta transferência de massa (SORIA e VILLAMIEL, 2010).

O ultrassom é utilizado como um método alternativo, barato, reproduzível, simples e de relevância industrial para a extração de biocompostos (SORIA e VILLAMIEL, 2010). Entre seus principais benefícios estão a redução do tempo de extração e processamento, baixa quantidade de energia e solventes utilizados, além da diminuição do uso de operações unitárias (CHEMAT et al., 2017).

O ultrassom é uma onda mecânica e suas características como frequência, comprimento de onda e amplitude pode influenciar a cavitação e, portanto, a extração (PINGRET et al., 2013).

Dal Prá et al. (2015) obteve atividade antioxidante de 13,0 % para o extrato obtido pela extração assistida por ultrassom de *Brassica Oleacea* utilizando um banho ultrassônico de 132 W de potência. Sallet et al. (2019) obteve um rendimento de extrato de 14,46 % utilizando etanol como solvente na EAU da biomassa do fungo *Mortierella isabelina* nas condições de intensidade de ultrassom de 75,11W.cm^{-2} e ciclo de pulso de 0,93. E Ishak et al. (2020) utilizou a uma sonda de ultrassom de 770 W de potencia, com pulso de 10 % para extrair compostos antioxidante da casca verde de Musa acuminata e obteve um rendimento máximo de 14,9 % e atividade antioxidante de 80,8 %.

4 ARTIGO 1 - Antioxidant compounds extracted from *Diaporthe schini* using supercritical CO_2 plus cosolvent

Artigo publicado na Bioprocess and Biosystems Engineering

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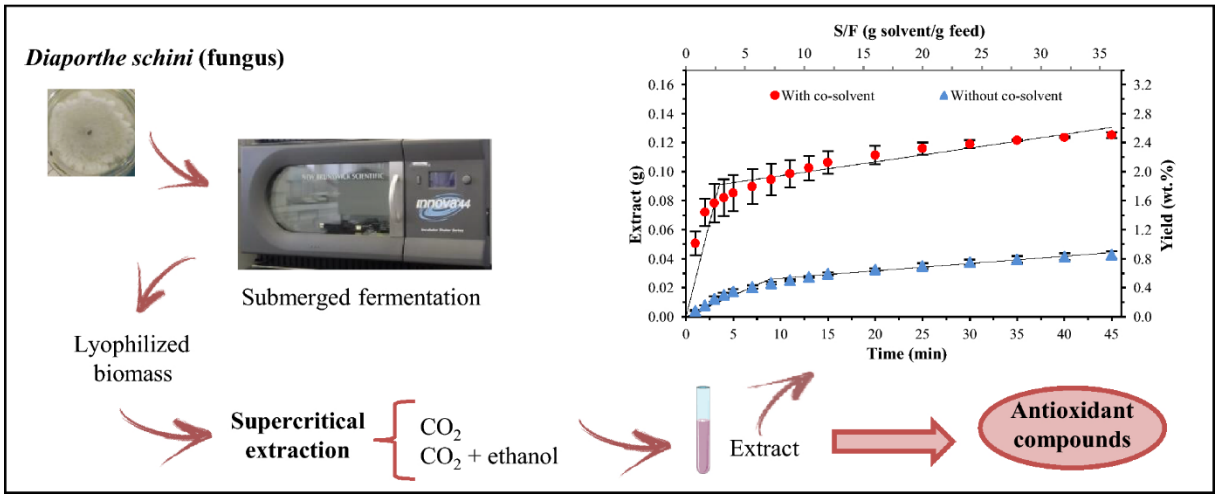
Abstract

Endophytic fungi have been highlight in the production of secondary metabolites with different bioactive properties, such as in the production of the antioxidant compounds. Therefore, the objective of this work was the extraction of the antioxidant compounds from the biomass of *Diaporthe schini* using supercritical carbon dioxide (CO_2) without and with ethanol as cosolvent. The biomass was produced by submerged fermentation and the parameters evaluated in the extraction process were: pressure (150–250 bar), temperature (40–60 ºC) and cosolvent [biomass: cosolvent ratio, 1:0, 1:0.75 and 1:1.5 (w/v)]. Extraction yield, antioxidant activity and chemical composition of the extracts were determined. The highest extraction yield (3.24 wt.%) and the best antioxidant activity against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (96.62 %) were obtained at 40 ºC, 250 bar and biomass:cosolvent ratio of 1:1.5 (w/v). The chemical compounds 1,4-diaza-2,5-dioxo- 3-isobutyl bicyclo[4.3.0]nonane and benzeneethanol identified in GC/MS could be responsible for the antioxidant activity found in this study.

Keywords: Submerged fermentation, *Diaporthe schini*, Supercritical CO_2 extraction, Cosolvent, Antioxidant activity

Graphic abstract



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4.1 Introduction

The Brazilian Pampa biome occupies part of Rio Grande do Sul State and it presents vegetation, climate, and soil with distinct characteristics, which makes it an ecosystem with high plant, animal, and microorganism diversity (LUPATINI et al., 2013; DE SOUZA et al., 2017). A part of this natural ecosystem corresponds to endophytic fungi, which are present in the internal tissues of plants. These fungi do not cause apparent damage to their host, while they produce biologically active secondary metabolites (BILAL et al., 2018). Such products have been a promising source of new compounds and chemical agents, which can be applied in medicine, agriculture, and pharmaceutical industries (ZHANG et al., 2013). Studies have already been carried out in our laboratory regarding the isolation and identification of fungi from the Brazilian Pampa biome for the production of secondary metabolites with herbicidal (DE SOUZA et al., 2017; DANIEL et al., 2018; DE SOUZA et al., 2015), antifungal and antibacterial (DOS REIS et al., 2019), and antioxidant (VALENTE et al., 2018) activities.

Diaporthe species are endophytic fungi that have been used in the production of different compounds with antitumor (CARVALHO et al., 2012; SHARMA et al., 2018), antifungal (EL-GENDEY et al., 2018; TANNEY et al., 2016), antibacterial (LI et al., 2015; SEBASTIANES et al., 2012; TANAPICHATSAKUL et al., 2018), bioherbicidal (DE SOUZA et al., 2017, 2015), and antioxidant (TANAPICHATSAKUL et al., 2018) properties. Antioxidant compounds reduce free radical formation, scavenge reactive oxygen species and upregulate or protect antioxidant defenses (PIETTA, 2000). From the fungi, these compounds

can be produced by fermentation and they need an extraction step after had been obtained from the biomass.

Different methods can be used for the extraction of bioactive compounds, such as solvent extraction (DEMIRCI et al., 2018), precipitation (KUNGEL et al., 2018), ultrasound-assisted extraction (LIN et al., 2018), and supercritical fluid extraction (SFE) (VALENTE et al., 2018; TRENTINI et al., 2019). Usually, these extraction techniques have a higher yield compared to SFE. However, SFE offers advantages such as the selectivity, cleaner extracts, less time extraction and reduced solvent consuming (ŞAHIN et al., 2011). Carbon dioxide (CO_2) is the most commonly used solvent in the SFE (SCAPIN et al., 2017), which can provide extracts with better selectivity, and ensures an innocuous separation process (DE MELO et al.,2014). In addition, a cosolvent, such as ethanol, may be used to facilitate the extraction of compounds (KITZBERGER, 2007; MAZZUTTI, 2012). Several studies using the supercritical CO_2 for the extraction of the antioxidant compounds are found in the literature (KITZBERGER et al., 2007; MAZZUTTI et al., 2012; CONFORTIN et al., 2017; DE SOUZA et al., 2018). However, studies with supercritical fluid extraction from *Diaporthe* species and using ethanol as cosolvent have not been found so far in the main scientific database.

Based on this context, the aim of this work was to extract antioxidant compounds from the biomass of the endophytic fungus *Diaporthe schini* produced by submerged fermentation. Supercritical fluid extraction using pure CO_2 and CO_2 plus ethanol as cosolvent was the technology used. The yield, antioxidant activity and chemical composition of the extracts were evaluated.

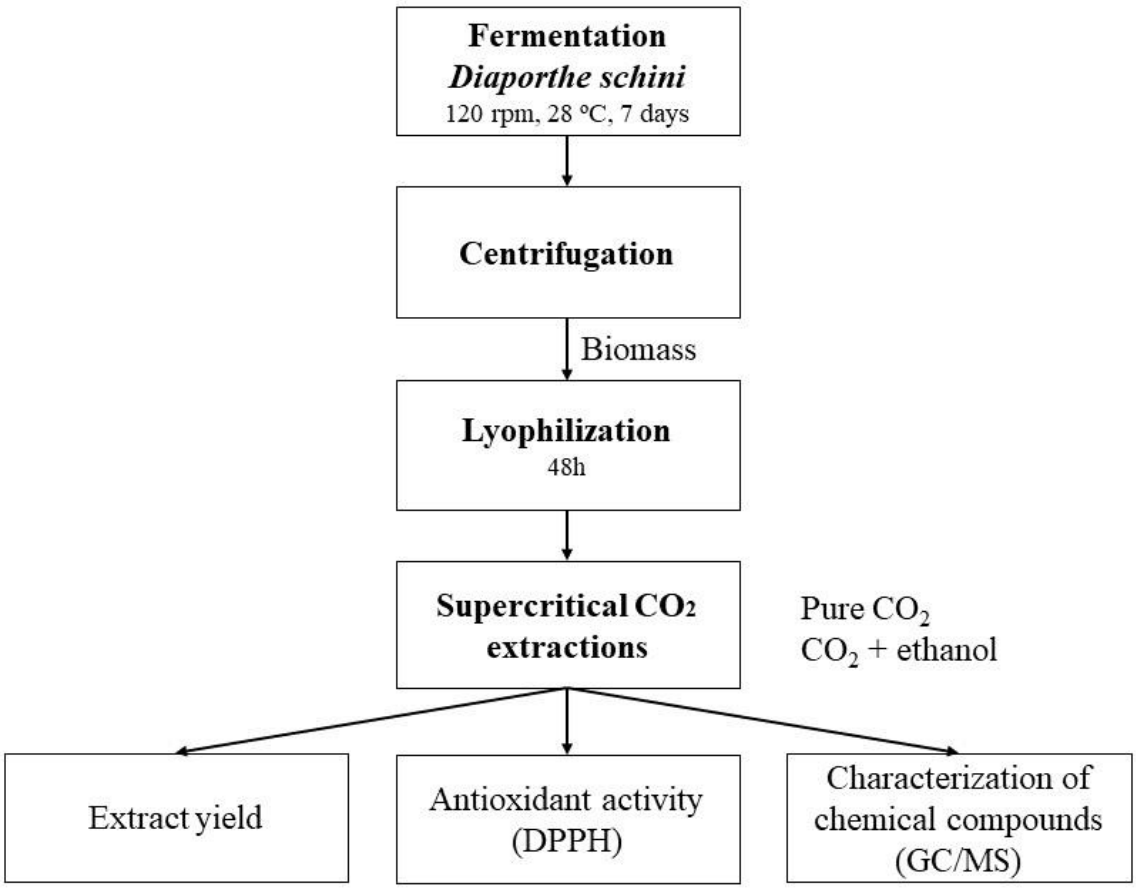
4.2 Material and methods

4.2.1 **Microorganism**

Diaporthe schini was previously isolated by de Souza et al. (2017) from the *Solanum americanum* plant, collected in the Pampa biome (Rio Grande do Sul, Brazil). The identification of the fungus species was carried out according to described by dos Reis et al. (2019).

Figure 1 shows the flow scheme of the production and extraction of the antioxidant compounds from the biomass of *Diaporthe schini* using SFE with pure CO_2 and with CO_2 plus ethanol as cosolvent, as described in the following sections.

Figure 1 - Flow scheme of the production and extraction of the antioxidant compounds from the biomass of *Diaporthe schini* by SFE



Source: own author

4.2.2 **Fermentation**

The fermentation was carried out according to described by dos Reis et al. (2019). *Diaporthe schini* was transferred to Falcon tube containing 10 mL of a medium (200 g L^{-1} potato, 20 g L^{-1} dextrose, 20 g L^{-1} sucrose, 2.0 g L^{-1} ammonium sulfate, 1.0 g L^{-1} ferrous sulfate heptahydrate, 1.0 g L^{-1} manganese sulfate monohydrate, and 0.5 g L^{-1} magnesium sulfate heptahydrate), which was maintained for 24 h at 28 ºC for the pre-inoculum. The pre-inoculum was transferred to an Erlenmeyer containing 90 mL of the same composition medium and maintained at 28 ºC and 120 rpm in an orbital shaker (Inova 44R, New Brunswick) for 2 days. The fermentation was carried out in Erlenmeyer flasks containing 150 mL of medium, in an orbital shaker at 120 rpm and 28 ºC for 7 days. The medium was composed of 10% (v/v) of the inoculum and 90% (v/v) of a medium containing 17.05% (w/v) corn steep liquor (CSL), 20

g L^{-1} sucrose, 2.0 g L^{-1} ammonium sulfate, 1.0 g L^{-1} ferrous sulfate heptahydrate, 1.0 g L^{-1} manganese sulfate monohydrate and 0.5 g L^{-1} magnesium sulfate heptahydrate, and the initial pH was adjusted to 6.0 (DE SOUZA, 2015). A centrifugation at 4000 rpm (Centrifuge 5804R, Eppendorf) for 10 min was performed to separate the biomass from the broth. The fermentation broth (approximately 97 wt.%) was used in a previous work (DOS REIS et al., 2019) and the biomass (approximately 3 wt.%) was used in this work. After the centrifugation, the biomass was lyophilized (L101, Liotop, São Carlos, Brazil) for 48 h, and macerated for the extractions.

4.2.3 Supercritical CO_2 extractions

The supercritical CO_2 extractions were performed in laboratory scale equipment as described in the work of Sallet et al. (2017). Approximately 5 g of dried cells or mixed with the cosolvent (ethanol) were loaded in the extraction vessel for the extraction procedure. CO_2 was pumped in the bed and the pressure and temperature were adjusted according to Table 1. The extraction was started after 20 min, when it reached the equilibrium of pressure and temperature. The extraction time was fixed to 45 min, according to preliminary assays, and the solvent flow rate was 4 g/min. During the kinetic extractions, the extract was collected at intervals of 1 min until the first 5 min, at 2 min from 5 to 15 min of the extraction and at intervals of 5 min until the end of each experimental assay (45 min), totalizing 16 samples for each assay. The variables temperature (40–60 ºC), pressure (150–250 bar) and cosolvent [biomass:cosolvent ratio, 1:0; 1:0.75 and 1:1.5 (w/v)] were defined in preliminary assays. The solvent density (ρ) (Table 1) was obtained from the Chemistry WebBook—National Institute of Standards and Technology (NIST, 2017).

After the extraction, the cosolvent was evaporated at 40 ºC for 2 days and the extract yield was determined by gravimetric method, according to the equation 1.

$$\text{Yield (wt.\%)} = \frac{\text{mass of the extract (g)}}{\text{initial mass of dry biomass (g)}} \cdot 100 \qquad (1)$$

The extract was diluted with 15 mL of ethyl alcohol for the determination of the antioxidant activity (DPPH) and characterization of chemical compounds by gas chromatography coupled to mass spectrometry (GC/MS).

4.2.4 Kinetic extraction curves

The curves were constructed by cumulative mass of the extract as a function of the extraction time, aiming to evaluate the behavior of the extractions over time. The experimental data of kinetic yield were fitted to a spline model with two straight lines, according to Equations (2) and (3) (MEIRELES, 2008):

1) For $t \leq t_{CER}$:

$$Y(t)= b_0+b_1.t \quad (2)$$

2) For $t > t_{CER}$:

$$Y(t)= b_0-b_2.t_{CER}+(b_1+b_2).t \quad (3)$$

where b_0 is the linear coefficient of line 1; b_1 and b_2 are the slopes of lines 1 and 2, respectively; t is the extraction time; t_{CER} is the end of the constant extraction rate (CER) period and Y(t) is the extract yield as a function of time.

From the spline fitting, four derived quantities were obtained: (1) the end of the CER period (t_{CER}); (2) the mass transfer rate for the CER period (M_{CER}); (3) the yield for the CER period (R_{CER}); and (4) the mass ratio of solute in the fluid phase at the extraction vessel outlet for the CER period (Y_{CER}).

4.2.5 **Free radical scavenging activity (DPPH)**

The ability of the extract of scavenging the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was assessed by calculating the percent inhibition of this radical using the methodology proposed by Dal Prá et al. (2015) with modifications. The reaction mixture consisted of a solution containing 1500 μL of sample, 1480 μL of ethanol solution of DPPH (10^{-4} M) and 20 μL of ethanol, which was vigorously stirred and left to stand for 30 min in the dark. A blank assay was performed using 1500 μL of sample and 1500 μL of ethanol and a control was conducted with 1480 μL of ethanol solution of DPPH and 1520 μL of ethanol. The absorbance was measured at 522 nm in a spectrophotometer UV-Vis (Shimadzu IR-Prestige-21). The percent inhibition of the DPPH radical (AA_{DPPH}) was determined according to Equation (4).

$$AA_{DPPH}=\left(\frac{A_{DPPH}-(A-A_B)}{A_{DPPH}}\right).100 \quad (4)$$

where A_{DPPH}, A and A_B correspond to the absorbance values of the control, the sample and the blank, respectively.

4.2.6 Gas chromatography/mass spectrometry (GC/MS)

The samples were analyzed in gas chromatography (GC-2010 Plus, Shimadzu, Kyoto, Japan) and mass spectrometry (GCMS-QP2010 Ultra, Shimadzu, Kyoto, Japan) system, according to the methodology of dos Reis et al. (2019), with some modifications. The helium flow rate was 1.18 mL/min. A volume of 1 μL of sample was injected with a 1:40 split ratio.

4.3 RESULTS AND DISCUSSION

4.3.1 Extraction yield

The extraction yield by SFE from the biomass of *Diaporthe schini* is presented in Table 1. Higher yield was obtained using cosolvent, being the highest result (3.24 wt.%) obtained at 40 ºC, 250 bar, and biomass:ethanol ratio of 1:1.5 (w/v) (Assay 8). Without the use of cosolvent, the best extraction yield (1.34 wt.%) was obtained at highest temperature and pressure (60 ºC and 250 bar, respectively). These results are in agreement with Valente et al. (2018), who obtained high yield (0.86 wt.%) using 60 ºC and 250 bar in the SFE-CO_2 of the antioxidant compounds from the endophytic fungus *Botryosphaeria dothidea*. Likewise, Dal Prá et al. (2016) obtained the maximum yield (4.55 wt.%) using supercritical CO_2 at 60 ºC and 250 bar for the extraction of bioactive compounds from palm (*Elaeis guineensis*). According to Dal Prá et al. (2016), an increase in pressure provides better solvent permeability in the solid matrix.

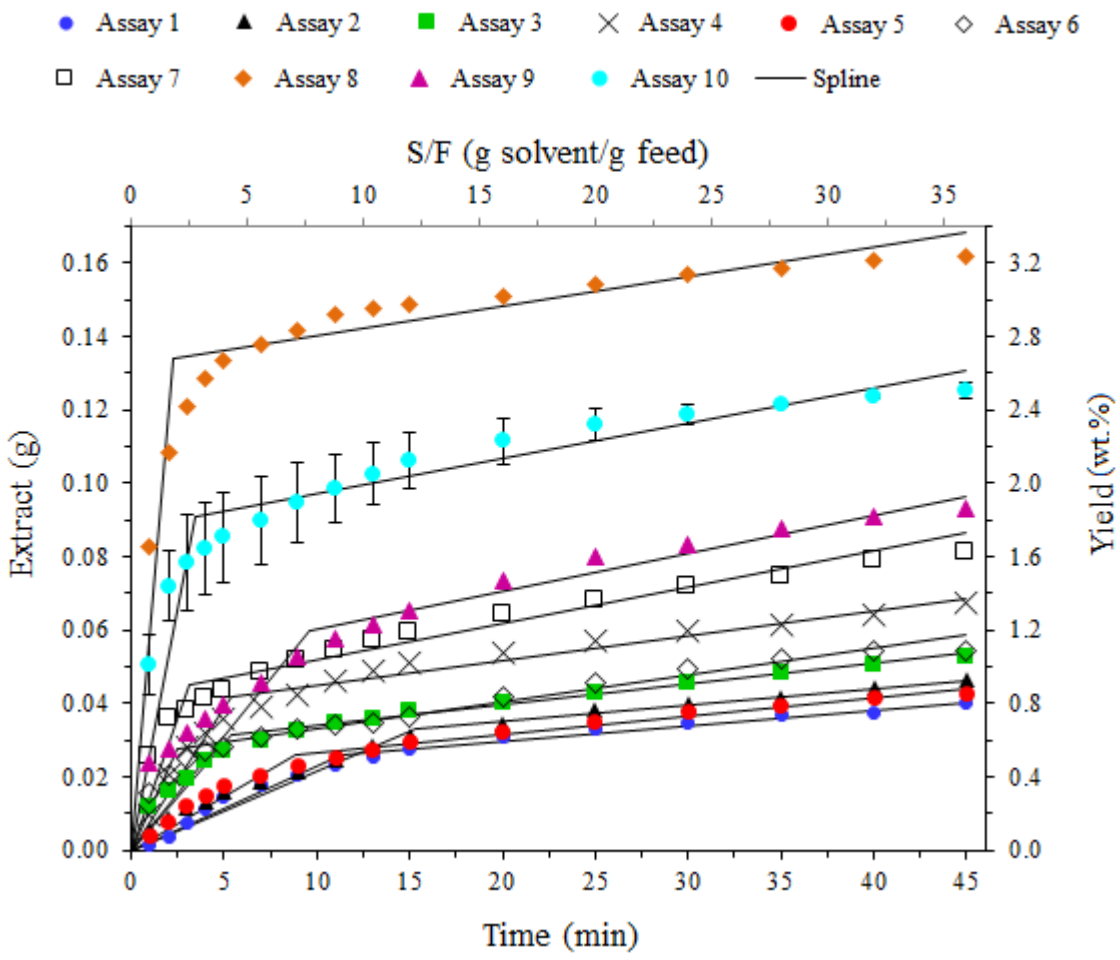
Table 1 - Extraction yield and antioxidant activity (DPPH) of the extracts obtained from the biomass of *Diaporthe schini* using SFE with pure CO_2 and with CO_2 plus ethanol (cosolvent).

Assay	Temperature (ºC)	Pressure (bar)	Biomass:cosolvent (w/v)	ρ (kg/m^3)	Yield (wt.%)	DPPH (%)
1	40	150	1:0	750.50	0.80	23.67
2	60	150	1:0	565.51	0.93	25.67
3	40	250	1:0	887.53	1.05	27.60
4	60	250	1:0	776.88	1.34	41.44
5*	50	200	1:0	765.94	0.85 ± 0.06	24.05 ± 0.10
6	40	150	1:1.5	750.50	1.09	59.89
7	60	150	1:1.5	565.51	1.62	71.47
8	40	250	1:1.5	887.53	3.24	96.62
9	60	250	1:1.5	776.88	1.86	90.88
10*	50	200	1:0.75	765.94	2.50 ± 0.20	92.74 ± 4.70

*Mean ± standard deviation of the triplicate of the central point.

In general, the use of ethanol as cosolvent increased extraction efficiency (Figure 2). Using SFE at 40 ºC and 200 bar, Kitzberger et al. (2007) also observed an increase in the shiitake extraction yield from 0.57 % (w/w) with pure CO_2 to 3.81 % (w/w) with CO_2 plus 15 % of ethanol as cosolvent. According to Mazzutti et al. (2012), obtaining higher extraction yield using cosolvent could be explained by increasing the solubility of polar compounds in the mixture ethanol/CO_2, as compared to the solubility in pure CO_2, which is a non-polar solvent. The best result was obtained at highest pressure (250 bar) and lowest temperature (40 ºC) evaluated. Increasing the pressure from 150 to 250 bar, at a constant temperature, the extraction yield was improved. This behavior could be justified by increase of the supercritical solvent density, which raises the solubility of the CO_2 and thus enhances the extraction rate (GOYENECHE et al., 2018). Similar results were reached by Elgndi et al. (2017), who obtained higher extraction yield for essential oils with the increase of pressure from 100 to 300 bar, at 40 ºC.

Figure 2 - Extracts obtained from the biomass of *Diaporthe schini* by SFE with pure CO_2 (Assays 1-5) and with CO_2 plus ethanol (Assays 6-10)



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4.3.2 Kinetic extraction curves

Kinetic curves for the extraction of compounds from *Diaporthe schini* by supercritical extraction using pure CO_2 and CO_2 with ethanol as cosolvent are presented in Figure 2. The extraction could be summarized in two predominant periods. Initially, in the first minutes of the extraction, it was possible to observe the first period in which there was a higher extraction rate and, in the sequence until the end of the operation, it was possible to notice the second period in which there was a lower extraction rate. Several studies use models for fitting extraction kinetic parameters, among them is the spline model, which is used for the extraction of compounds using pressurized fluids (SALLET et al., 2017; CONFORTIN et al., 2017; ZABOT et al., 2014).

The spline model was adjusted for the data obtained in the extractions and the kinetic parameters can be observed in Table 2. The parameters (t_{CER}, R_{CER}, M_{CER}, and Y_{CER}) describe the moment in which the diffusional contribution begins to be important compared to the convective contribution. Due to the mass transfer rate be constant in this period, it is common fitting the kinetic parameters (SALLET et al., 2017).

Table 2 - Kinetic parameters of the extraction of compounds from the biomass of *Diaporthe schini* by SFE with pure CO_2 and with CO_2 plus ethanol.

Assay	**t_{CER} (min)**	**R_{CER} (wt.%)**	**$M_{CER}\times10^3$ (g/min)**	**$M_{Sol,CER}$ (g)**	**S/F_{CER} (g solvent/g raw material)**	**$Y_{CER}\times10^3$ (g extract/g CO_2)**
1	11.3	0.53	2.27	45.2	9.04	0.58
2	15.3	0.66	1.89	61.2	12.24	0.54
3	5.4	0.62	5.04	21.6	4.32	1.44
4	5.4	0.84	7.24	21.6	4.32	1.95
5*	8.9 ± 1.3	0.52 ± 0.05	2.92 ± 0.30	35.5 ± 5.4	7.09 ± 1.07	0.78 ± 0.07
6	2.5	0.55	10.40	10.0	2.00	2.77
7	3.1	0.90	12.50	12.4	2.48	3.62
8	2.3	2.68	54.20	9.2	1.84	14.59
9	9.6	1.20	4.82	38.4	7.68	1.56
10*	3.5 ± 0.2	1.82 ± 0.30	32.85 ± 4.67	14.0 ± 0.8	2.80 ± 0.17	8.82 ± 0.60

CER: Constant Extraction Rate; t_{CER}: end of the CER period; R_{CER}: extract yield for the CER period; M_{CER}: mass transfer rate for the CER period; $M_{Sol,CER}$: mass of solvent used in the CER period; S/F_{CER}: solvent to feed mass ratio in the CER period; Y_{CER}: mass ratio of solute in the fluid phase at the extraction vessel outlet for the CER period. Assays 1-5: SFE-CO_2; Assays 6-10: SFE-CO_2 + ethanol.
*Mean ± standard deviation of the triplicate of the central point.

For the SFE using pure CO_2 (Assays 1-5), a constant extraction rate was observed in the first 5 to 15 min. During this period, convective mass transfer predominates and most of solute is removed from the biomass. In the best condition (Assay 4), the mass transfer rate for the CER period (M_{CER}) was 7.24 mg/min, the mass ratio of solute in the fluid phase at the extraction vessel outlet (Y_{CER}) was 1.95 mg extract/g CO_2, and the extract yield (R_{CER}) achieved was 0.84 wt.% (Table 2). Valente et al. (2018) obtained a high yield (0.735 wt.%) and the following kinetic parameters: M_{CER} = 1.8 mg/min, Y_{CER} = 0.46 mg extract/g CO_2 and the end of the CER

period (t_{CER}) of 20.2 min, at 60 ºC and 250 bar. Such results were lower than those obtained in the present study, under the same temperature and pressure conditions.

For the extraction process using ethanol as cosolvent (Assays 6-10), the time for the constant extraction rate ranged from 2.3 to 9.6 min. The best results were obtained in Assay 8, being the mass transfer rate for the CER period (M_{CER}) of 54.20 mg/min, the mass ratio of solute in the fluid phase at the extraction vessel outlet (Y_{CER}) of 14.59 mg extract/g CO_2, and the extract yield (R_{CER}) of 2.68 wt.%, highlighting the advantage of using ethanol as cosolvent in relation to extraction with pure CO_2. As higher the amount of solute reached by the solvent, higher will be the mass ratio of solute in the fluid phase at the extraction vessel outlet for the CER period (Y_{CER}) (VALENTE et al., 2018; SALLET et al., 2017).

4.3.3 Antioxidant activity and extracts composition

The antioxidant activity of the extracts obtained from *Diaporthe schini* was determined using DPPH as free radical. Table 1 shows the results using different conditions for supercritical extraction. The extractions using ethanol as cosolvent resulted in higher antioxidant activities. The best result (96.62 %) was obtained in the biomass:ethanol ratio of 1:1.5 (w/v), at 40 ºC and 250 bar (Assay 8). For the extractions with pure CO_2, the highest antioxidant activity (41.44 %) was found at highest temperature and pressure (60 ºC/250 bar) (Assay 4). Kitzberger et al. (2007) obtained a limited antioxidant activity, near to 11 %, in DPPH essays for supercritical shiitake extracts with pure CO_2 from 30 to 50 ºC and from 150 to 300 bar. According to the authors, this behavior can be dependent on the extraction of non-polar components with low antioxidant activity due to the non-polar characteristic of the solvent. However, in SFE at 40 ºC and 200 bar, they observed an antioxidant activity of 72.97 % with 15 % of ethanol as cosolvent. Dal Prá et al. (2015) obtained antioxidant activity against the DPPH radical from 13 % to a maximum of 80 % for a crude and hydrolyzed extract of *Brassica oleracea* var. *capitata*, respectively, obtained by ultrasound-assisted extraction using ethanol and methanol as solvents. Zhao et al. (2006) reported values of DPPH radical scavenging activity ranging from 25.6 % to 90.2 % for barley extracts obtained by sonication using different extraction solvents.

Figure 3 suggests that the antioxidant activity can be directly related to the extraction yield since an increase in the antioxidant effect was observed with increased yield. However, Assay 6 showed higher antioxidant activity than Assay 4, despite the lower yield. This behavior could be explained by the presence of cosolvent, which modifies the polarity of CO_2 (PEREIRA et al., 2013), increasing the activity. In Table 3 some studies found in the literature related to

the extraction of antioxidant compounds from various microorganisms are presented, using different extraction methods. The supercritical CO_2 extraction with addition of cosolvent was more selective for antioxidant compounds (96.62%) compared to solvent extraction (ethyl acetate, ≅ 77%) for *Diaporthe* species.

Figure 3 - Yield and antioxidant activity (DPPH) of the extracts obtained from the biomass of *Diaporthe schini* by SFE with pure CO_2 (Assays 1-5) and with CO_2 plus ethanol (Assays 6-10)

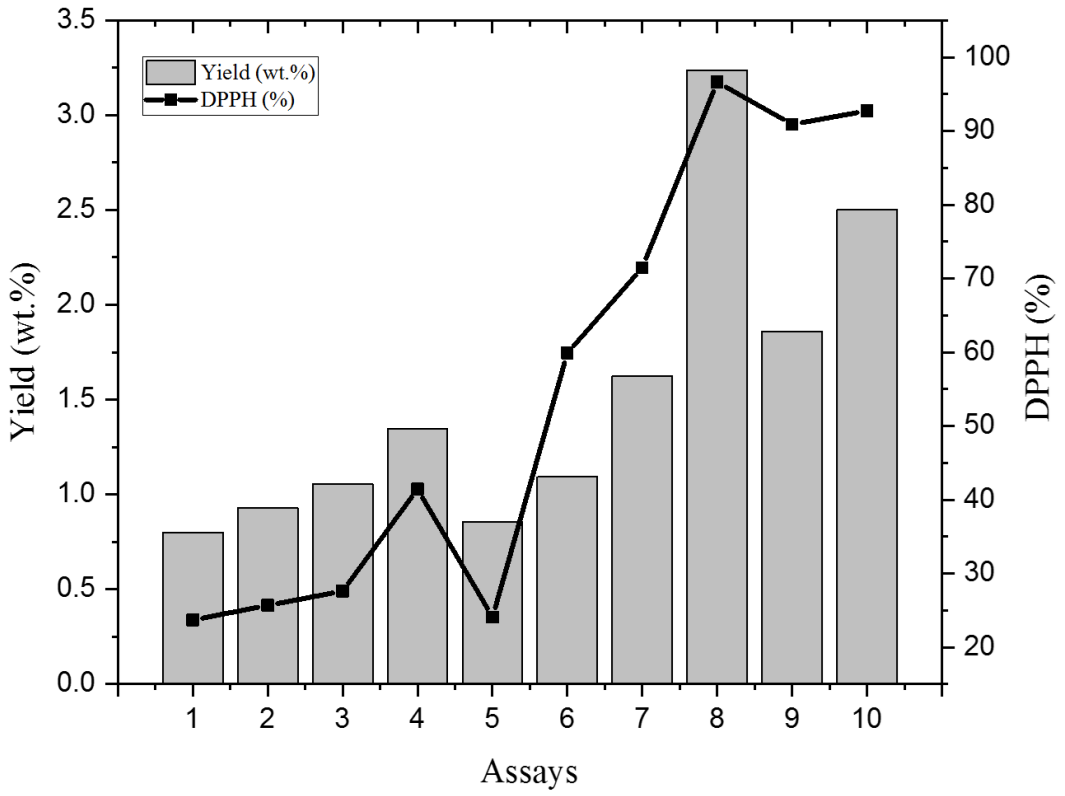

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Table 4 presents the chemical characterization to identify bioactive compounds with antioxidant activity. Thirteen compounds were found in the extracts. The compound 1,4-diaza-2,5-dioxo-3-isobutyl bicycle[4.3.0]nonane was found in all samples and, according to literature, potential antioxidant could be related to this compound (TAKAYA et al., 2007; TAN et al., 2018). Another compound identified in the study was the benzeneethanol, an aromatic compound observed both in the extracts in which the non-polarity of the CO_2 was modified by the addition of cosolvent (Assays 8 and 9), as in the extracts using pure CO_2 (Assays 2, 3, 4 and

5). Kim et al. (2014) attributed antioxidant activity to this compound, which was observed in fractions from brewed Korean rice wines.

Table 3 - Optimized extraction yield and antioxidant activity (DPPH) of extracts obtained from various sources using different extraction methods.

Extraction	**Microorganism**	**Optimized yield (%)**	**Antioxidant activity (DPPH) (%)**	**Reference**
Supercritical CO_2	*Diaporthe schini*	1.34	41.44	This work
Supercritical CO_2 plus ethanol	*Diaporthe schini*	3.24	96.62	This work
Supercritical CO_2	*Agaricus brasiliensis*	1.19	-	(MAZZUTTI et al., 2012)
Supercritical CO_2 plus ethanol	*Agaricus brasiliensis*	4.20	-	(MAZZUTTI et al., 2012)
Aqueous extraction	*Scenedesmus bajacalifornicus*	5.80	71.12	(PATIL and KALIWAL, 2019)
Ultrasound-assisted extraction	*Inonotus hispidus*	33.29	82.31	(HOU et al., 2019)
Ethanol precipitation (60 %)	*Morchella esculenta*	4.18	52.99	(LI et al., 2019)
Ethanol precipitation (95 %)	*Phellinus baumii*	52.09	83.42	(WANG et al., 2019)
Ethyl acetate extraction	*Diaporthe* sp.	-	76.47	(VASUNDHARA et al., 2017)

The chemical compounds mentioned have been evidenced in the scientific literature for their antioxidant properties, suggesting that the antioxidant capacity found in the extracts obtained from the biomass of *Diaporthe schini* by SFE with pure CO_2 and with CO_2 plus ethanol could be attributed to these compounds. Other bioactive compounds identified in this study have biological properties, such as ethyl linoleate, which has anti-inflammatory activity (XIA, 2018), and the compound 9,12-Octadecadienoic acid (Z, Z)-, methyl ester, which has anticancer property (TYAGI and AGARWAL, 2017; WEI et al., 2011), and it was identified only in the extraction with cosolvent (Assay 8)

Table 4 - Compounds identified by GC/MS in the extracts obtained from the biomass of *Diaporthe schini* by SFE with pure CO_2 and with CO_2 plus ethanol.

		Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5***	**Assay 6**	**Assay 7**	**Assay 8**	**Assay 9**	**Assay 10***
Compound	**Formula**	**Area %**									
1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	$C_{11}H_{18}N_2O_2$	5.94	13.88	5.49	25.34	16.16	37.57	40.08	34.46	46.03	37.41
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	-	-	-	-	-	-	-	24.81	-	-
9-Octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$	12.46	-	-	-	-	-	-	-	-	-
9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{21}H_{38}O_4$	-	10.21	-	-	-	-	-	-	-	-
9-Octadecenoic Acid (Z)-, Ethyl ester	$C_{20}H_{38}O_2$	28.73	18.41	-	-	44.89	-	-	-	-	10.08
13-Octadecenal, (Z)-	$C_{18}H_{34}O$	-	53.22	-	-	-	-	-	-	-	-
13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	-	-	-	-	1.23	-	-	-	-	-
Benzeneethanol	$C_8H_{10}O$	-	4.28	5.19	14.01	8.03	-	-	4.63	8.89	-
Di-(9-Octadecenoyl)-Glycerol	$C_{39}H_{72}O_5$	52.87	-	-	-	-	-	-	-	-	-
Ethyl linoleate	$C_{20}H_{36}O_2$	-	-	53.15	33.48	-	27.28	22.14	-	16.50	8.32
Heptadecanoic acid, ethyl ester	$C_{19}H_{38}O_2$	-	-	-	-	4.50	-	-	-	-	-
(R)-(-)-14-Methyl-8-hexadecyn-1-ol	$C_{17}H_{32}O$	-	-	36.17	27.17	25.18	35.15	-	36.10	28.58	44.19
Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy-	$C_{30}H_{52}O_2$	-	-	-	-	-	-	37.78	-	-	-

Assays 1-5: SFE-CO_2; Assays 6-10: SFE-CO_2 + ethanol. *Mean of the triplicate of the central point.

4.4 Conclusion

The extraction by supercritical fluid from the biomass of the endophytic fungus *Diaporthe schini* was evaluated for the first time aiming to obtain antioxidant compounds. The best results for extraction yield (3.24 wt.%) and antioxidant activity against the DPPH radical (96.62 %) were observed using ethanol as cosolvent, in the biomass:ethanol ratio of 1:1.5 (w/v), at lowest temperature (40 ºC) and highest pressure (250 bar) evaluated. The superiority of the extraction using cosolvent was evident through the extraction curves and the application of the spline model to obtain kinetic parameters, being possible to obtain a smaller time for the constant extraction rate and a better extract yield for the CER period. Therefore, supercritical CO_2 extraction using ethanol as cosolvent demonstrated to be a selective and promising technique for the extraction of the antioxidant compounds from *Diaporthe schini*.

Acknowledgments

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5 ARTIGO 2 - Ultrasound-assisted extraction of antioxidant compounds from *Diaporthe schini*

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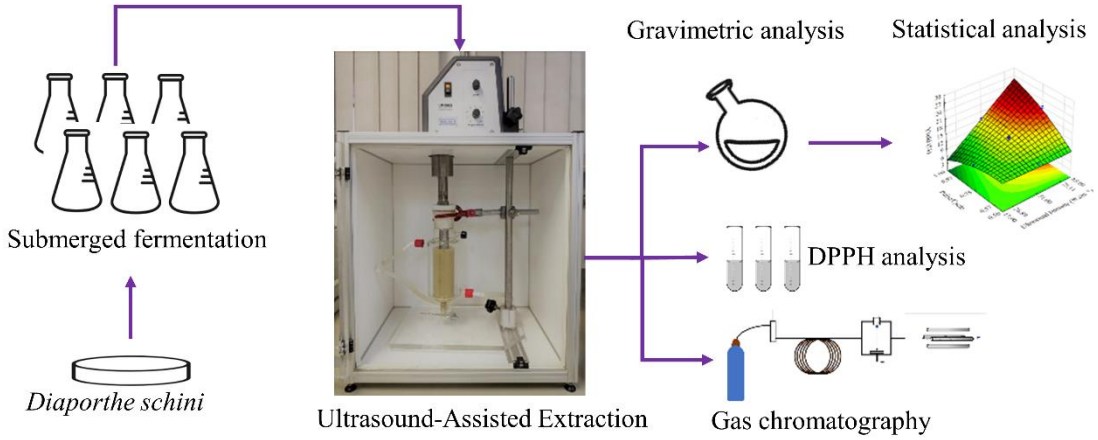
Abstract

Ultrasound-assisted extraction (UAE) of antioxidant compounds from *Diaporthe schini* biomass using ethanol as solvent was performed and the results were compared with those of heat-assisted extraction (HAE; conventional technique). Ultrasound intensity (17-85 $W.cm^{-2}$) and pulse cycle (0.50-1.00) were evaluated according to a Central Composite Rotatable Design (CCRD) to obtain the highest extraction yield were evaluated. Extraction yield of 22.30 ± 0.47% and antioxidant capacity (DPPH) of 91.35 ± 0.27% were achieved in the validation condition (85 $W.cm^{-2}$ and 0.93) of the UAE. The best extraction yield (8.34 ± 0.38 %) and antioxidant activity (91.32 ± 0.98 %) of the HAE were observed at 50 ºC. The GC/MS analysis of the UAE extracts showed the presence of the 9,12 octadecadienoic acid (Z, Z)-, methyl ester, hexadecanoic acid, ethyl ester, ethyl octadec-9-enoate, and 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane compounds, which could be responsible for the antioxidant activity observed. This study was the first attempt to optimize the UAE parameters for the antioxidant compounds extraction from *Diaporthe schini*. UAE improved the extraction yield and reduced the extraction time in relation to the HAE.

Keywords: Ultrasound-assisted extraction, Heat-assisted extraction, *Diaporthe schini*, bioactive compounds, Antioxidant activity.

Graphic abstract



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5.1 Introduction

Researches with antioxidants systems to protect against free radicals has been increased in recent years. These systems include some antioxidants produced in the human body and others from the diet, which are needed to decrease the cumulative effects of oxidative damage (PIETTA, 2000). The production of these antioxidants from microorganisms has been increased, especially from endophytic fungi, which are a promising source of natural products (SORIA and VILLAMIEL, 2010). These microorganisms living in association with plants are the most important way to produce natural products, drug discovery and development processes (NEWMAN and CRAGG, 2016). The secondary metabolites produced by these microorganisms show potential interest in the industry with applicability in medicine, agriculture, food and cosmetics (STROBEL, 2002) because some of them present bioactive compounds with antioxidant activity.

An extraction process is necessary to obtain the bioactive compounds from microorganisms biomass. Different methods have been used for this extraction from fungal cells. Maceration, heat-assisted extraction (HAE) and Soxhlet extraction are conventional methods for the bioactive compounds extraction, however, these techniques are time-consuming and require a large volume of solvent (CHEMAT et al., 2017; WANG and CHEN, 2006; LIN et al., 2018). Currently, innovative methods have been applied in the extraction of compounds, such as supercritical fluid extraction (SFE) and ultrasound-assisted extraction

(UAE). Da Rosa et al. (2020) and Mazzutti et al. (2012) extracted antioxidant compounds from *Diaporthe schini* and *Agaricus brasiliensis* biomass, respectively, using SFE with carbon dioxide, however, the extractions yield was low. On the other hand, the ultrasound extracted a variety of compounds using different solvents (polar or non-polar) and a simpler equipment (CHEMAT et al., 2017).

The UAE has been applied to disruption cells by the implosion of cavitation bubbles leading to the accumulation of energy, which produces high shear and turbulent energy waves improving the mass transfer (SORIA and VILLAMIEL, 2010). This technology offers some advantages in terms of productivity, yield and selectivity, with better processing time, enhanced quality, reduced chemical and physical hazards, and could be considered environmentally friendly (CHEMAT et al., 2017). Several studies have been performed to evaluate the efficiency of UAE for the extraction of antioxidant compounds from biomass of plants and fungi or mushrooms, demonstrating the feasibility and potential advantages of this process (DAL PRÁ et al., 2015; YILDIZ et al., 2015; LIU et al., 2019). To the objective of this work was to extract a high yield of metabolites with antioxidant capacity from *Diaporthe schini* biomass using high-intensity ultrasound-assisted extraction. An experimental design was performed to evaluate the influence of variables (intensity and pulse cycle) in the extraction and the chemical composition of extracts was determined using gas chromatography. A comparative study was performed using heat-assisted extraction at different temperatures.

5.2 Materials and Methods

5.2.1 Microorganism and Fermentation

Diaporthe schini was previously isolated by de Souza et al. (2017). The endophytic fungus was inoculated in potato dextrose agar (PDA) and incubated for 7 days at 28 °C. The fermentation was performed using 150 mL medium composed of: 17.05 % (w/v) corn steep liquor (CSL), 20 g. L^{-1} sucrose, 2.0 g. L^{-1} ammonium sulfate, 1.0 g. L^{-1} ferrous sulfate heptahydrate, 1.0 g. L^{-1} manganese sulfate monohydrate, and 0.5 g. L^{-1} magnesium sulfate heptahydrate) (DOS REIS et al., 2019) in an orbital incubator (Inova 44R, New Brunswick) at 28 °C, 120 rpm for 7 days.

At the end of the fermentation, the biomass and broth were separated by centrifugation at 4000 rpm for 10 min (Centrifuge 5804R, Eppendorf). The cells were lyophilized (L101, Liotop, São Carlos, Brazil) for 48 h, and macerated for the extractions.

5.2.2 Extraction techniques

5.2.2.1 Ultrasound-assisted extraction (UAE)

The extractions were performed using a high-intensity ultrasound processor of 400 W and a frequency of 24 kHz (Hielscher, Model UP 400S, Germany) equipped with a titanium probe (Model H22, Tip 22), with a maximum ultrasound intensity of 85 $W.cm^{-2}$. The experimental apparatus for ultrasound-assisted extraction was described in the work of Sallet et al. (2019).

Extractions were performed using approximately 2.5 g of biomass and 100 mL of ethanol, the temperature was adjusted to 25 °C ± 2 °C by a jacket with circulating water, and the extraction time was fixed in 15 min according to preliminary assays (data not shown). A Central Composite Rotatable Design (CCRD) was performed to evaluate the ultrasound intensity (17–85 $W.cm^{-2}$) and pulse cycle (0.5–1.0) with triplicate in the central point. Table 1 shows the CCRD with real and coded variables (in parenthesis). Afterward, three additional assays were carried out to validate the results of the experimental design. A control extraction assay was conceived without ultrasound with the same conditions (biomass, solvent and time).

5.2.2.2 Heat-assisted extraction (HAE)

Heat-assisted extraction consists of stirring the solid and solvent at a specific temperature (LÓPEZ et al., 2018). The procedure was performed according to the work of López et al. (2018) with some modifications. The biomass (5 g) was placed in an Erlenmeyer flask with 100 mL of ethanol for extraction. A thermostatic water-bath with continuous agitation (Dubnoff Metabolic Bath Reciprocating Shake MA093) was used and the extraction time and agitation were 40 min and 100 rpm, respectively. Triplicate assays in different temperatures (30 °C and 50 °C) were evaluated.

After the extractions, the samples, of both techniques, were centrifuged at 4000 rpm for 10 min, the cell-free fluid was evaporated at 40 °C under vacuum and the solvent was recovered. The extract yield was determined by the gravimetric method, according to Equation (1).

$$\text{Yield (wt.\%)} = \frac{\text{mass of the extract (g)}}{\text{initial mass of dry biomass (g)}} \cdot 100 \quad (1)$$

5.2.3 Antioxidant activities of extracts

The percent inhibition of scavenging the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical (AA_{DPPH}) was determinate according to the methodology proposed by Dal Prá et al. (2015) with some modifications (Equation (2)).

$$AA_{DPPH} = \left(\frac{A_{DPPH} - (A - A_B)}{A_{DPPH}} \right) \cdot 100 \quad (2)$$

Where A_{DPPH} (reaction mixture), A (control), and A_B (blank assay) corresponded to the absorbance values measured at 522 nm in a spectrophotometer UV-vis (Shimadzu IR-Prestige-21). The reaction mixture consisted of a solution containing 1500 μL of the sample, 1480 μL of an ethanol solution of DPPH (10^{-4} M) and 20 μL of ethanol. A blank assay was performed using 1500 μL of sample and 1500 μL of ethanol and control was conducted with 1480 μL of an ethanolic solution of DPPH and 1520 μL of an ethanol, which were vigorously stirred and left to stand for 30 min in the dark.

5.2.4 Gas chromatography/mass spectrometry (GC/MS)

The samples were analyzed in gas chromatography (GC-2010 Plus, Shimadzu, Kyoto, Japan) and mass spectrometry (GCMS-QP2010 Ultra, Shimadzu, Kyoto, Japan) system, according to the methodology described by dos Reis et al. (2019), with some modifications. Helium was the transport gas at a flow rate of 1.18 mL. min^{-1}. A volume of 1 μL of the sample was injected with a 1:40 split ratio.

5.2.5 Statistical analysis

The effects of ultrasound intensity and pulse cycle on extraction yield were evaluated using STATISTICA 10.0® (Statsoft Inc., USA) at 95% confidence level ($p < 0.05$). Tukey test

($p < 0.05$) was used to determine significant differences between extraction techniques evaluated.

5.3 Results and discussion

5.3.1 Ultrasound-assisted extractions

Table 1 shows the experimental conditions of the CCRD and the results of the extraction yield and the antioxidant activity (DPPH) for the ultrasound-assisted extraction. The highest yield 21.14 % (assay 6) was observed with the highest ultrasound intensity (85 $W.cm^{-2}$) and the pulse cycle of 0.75, and the lowest yield was in assay 3 (8.44 %) with a lower intensity (26.89 $W.cm^{-2}$) and a high pulse cycle (0.93). A control assay was performed to compare with the ultrasound-assisted experiments. All assays were higher than the control with a yield of 3.26 %, therefore, the influence of ultrasound could be observed.

Table 1 - Extraction yield and antioxidant activity (DPPH) of the extracts from biomass of *Diaporthe schini* using ultrasound-assisted extraction

Assay	**Ultrasound intensity ($W.cm^{-2}$)**	**Pulse cycle (-)**	**Extraction yield (%)**	**DPPH (%)**
1	26.89 (-1)	0.57 (-1)	10.11	90.67
2	75.11 (1)	0.57 (-1)	11.62	91.04
3	26.89 (-1)	0.93 (1)	8.44	90.88
4	75.11 (1)	0.93 (1)	21.05	91.22
5	17.00 (-1.41)	0.75 (0)	10.43	90.91
6	85.00 (1.41)	0.75 (0)	21.14	90.82
7	51.00 (0)	0.50 (-1.41)	12.33	91.19
8	51.00 (0)	1.00 (1.41)	15.26	90.48
9	51.00 (0)	0.75 (0)	14.19	90.54
10	51.00 (0)	0.75 (0)	15.06	90.54
11	51.00 (0)	0.75 (0)	14.63	90.57
12*	-	-	3.26	56.12

* Extraction without ultrasound (control sample).

Heat-assisted extractions at different temperatures were performed to compare with the efficiency in the ultrasound-assisted extraction. The best extraction yield was 8.34 ± 0.38 % at 50 ºC, however, the extraction yield at 30 and 50 ºC did no presented significance difference

according to Tukey test ($p < 0.05$). The yield extraction using heat-assisted was lower when compared with the extraction results obtained in the experimental design.

Table 2 - Extraction yield and antioxidant activity of heat-assisted extraction and validation condition of ultrasound-assisted extraction from *Diaporthe schini* biomass

Assay	**Ultrasound intensity ($W.cm^{-2}$)**	**Pulse cycle (-)**	**Temperature (ºC)**	**Extraction Yield (%)**	**DPPH (%)**
1 HEA	-	-	30	6.78 ± 0.97^{a}	90.78 ± 0.96^{a}
2 HEA	-	-	50	8.34 ± 0.38^{a}	91.32 ± 0.89^{a}
Validation condition UAE	85.00	0,93	25	22.30 ± 0.47^{b}	91.35 ± 0.27^{b}

Same letters in the same column represent no significant difference at 95% ($p > 0.05$) according Tukey test.

Studies achieved better results for antioxidant compounds extraction using UAE. Zhang et al. (2019a) optimized UAE variables for the extraction of bioactive and antioxidants compounds from *Asparagus officinalis L.* and obtained 71.1 mg/g in better conditions, Zhang et al. (2019b) obtained a yield of 32.27 % in the extraction of papaya seed oil with functional compounds and antioxidant activity, while Hashemi et al. (2018) observed the best extract yield (2.8 % (w/v)) using ultrasound to extract essential oil from *Aloysia citriodora* Palau leaves with antioxidant activity.

Da Rosa et al. (2020) extracted antioxidant compounds from *Diaporthe schini* with a supercritical fluid, with lower yield (3.24 wt.%) than with UAE was observed under the best condition. The best yield in the UAE could be explained by the increase in mass transfer during the extraction process using ultrasound. This effect may be associated with the formation of cavitation bubbles that provide increased energy and cell disruption facilitating solvent penetration (DAL PRÁ et al., 2015). Furthermore, Soria and Villamiel (2010) claim that with mild operating conditions employed in ultrasound-assisted extraction showed no significant changes in the structural/molecular properties and functionality of most bioactive compounds, the relation to important for the heat-sensitive components. Another advantage of UAE technique in conventional extraction is the faster extraction rate and more effective energy use (NAVARRO et al., 2016).

5.3.2 Antioxidant activity

All samples obtained by UAE showed high antioxidant activity against DPPH radical (Table 1), which ranged from 90.54 to 91.22% against DPPH radicals, showing little variation. The use of ultrasound improved the extraction of compounds with antioxidant activity, since the DPPH of the control sample was 56.12%, lower results than all CCRD assays. High was also observed for the HAE, with no significant difference ($p > 0.05$) between the temperatures evaluated (Table 2). These results indicated that it was possible to obtain a similar antioxidant activity with a higher extract yield in a shorter extraction time using UAE. Ismail et al. (2019) achieved a higher antioxidant activity in UAE than HAE from baobab (*Adansonia digitata*) seeds. The same was observed by Caleja et al. (2017) in the extraction of rosmarinic acid, which has antioxidant properties from *Melissa officinalis L.*.

5.3.3 Optimization of the extraction yield

Data from Table 1 were used to evaluate the influence of variables (ultrasound intensity and pulse cycle) on the extraction yield and antioxidant activity. The evaluated variables had no significant influence ($p > 0.05$) on the antioxidant activity (DPPH). However, for the extraction yield, an empirical coded equation (Eq. 3) as functions of ultrasound intensity and pulse cycle was obtained, where Y is the extraction yield, UI is ultrasound intensity, and PC is the pulse cycle.

$$Y\ (\%) = 14.64 + 7.33 \times UI + 2.98 \times PC + 5.55 \times UI \times PC \qquad (3)$$

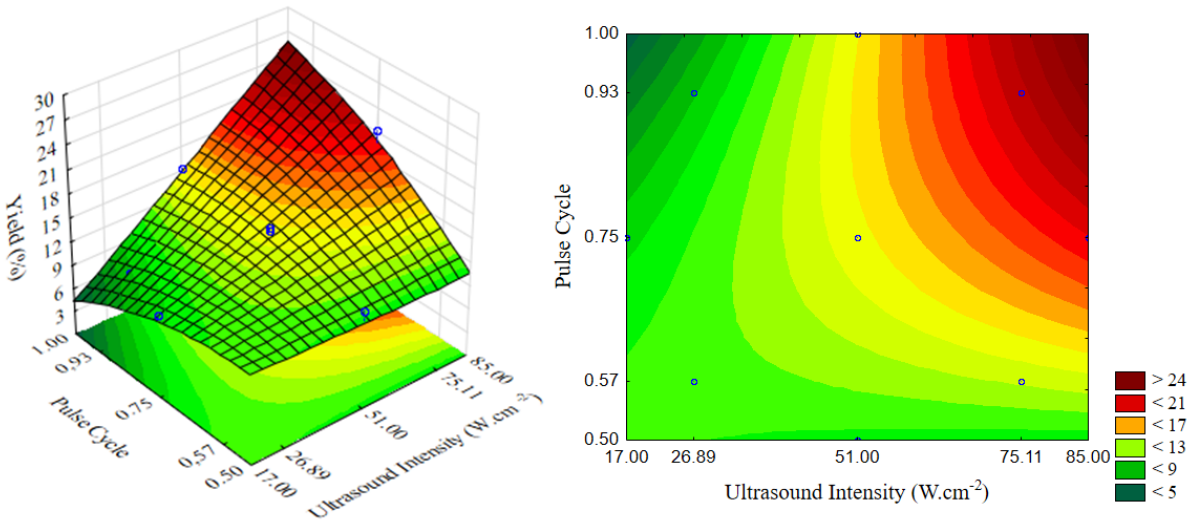
The analysis of variance (ANOVA) was performed to validate the empirical model at the assumed 5% significance (confidence level of 95%), and the results were shown in Table 3. The empirical model was validated since the coefficient determination (R^2) was greater than 90%, indicating a satisfactory correlation between the independent variables and the response, and the F_{calc} value 23.47 was higher than the tabulated value (4.35) (RODRIGUES and IEMMA et al., 2014). Figure 1 shows the response surface and contour diagram for the extraction yield. The significance of each coefficient in equations was estimated according to the level of p-values, thus insignificant parameters ($p < 0.05$) were excluded from the model.

Table 3 - ANOVA for extraction yield from *Diaporthe schini* by ultrasound-assisted extraction

Source of variation	Sum of squares	Degrees of freedom	Mean square	F Test	R^2
Regression	155.58	3	51.86	23.47*	0.91
Residual	15.48	7	2.21		
Total	171.06	10			

*$F_{0.05;3:7}$ = 4.35

Figure 1 - Response surface and contour diagram for extraction yield



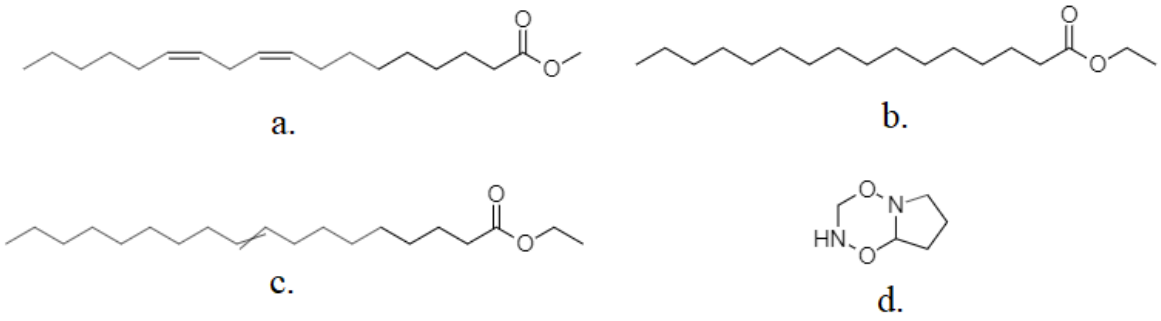
Source: own author

The variables had a positive effect, indicating that the increase in the ultrasoundintensity and pulse cycle resulted in an increase in the extraction yield. A validation condition (triplicate) was performed to confirm the result observed according to Fig. 1. In this condition, the ultrasound intensity of 85 W.cm^{-2} and the pulse cycle of 0.93 were evaluated, the extraction yield obtained was 22.30 ± 0.47% and the antioxidant activity was 91.35 ± 0.27%. According to Tukey test, these results were statistically different ($p < 0.05$) of the results obtained with HAE. New assay was performed using an ultrasound intensity of 85 W.cm^{-2} and a pulse cycle of 1.00, and the result for extraction yield was 16.95 ± 0.49% and DPPH was 90.82 ± 0.23%. According to this result, it can be observed that the maximum pulse cycle (1.00) was not interesting, because despite the ultrasound intensity being constant, the cavitation bubbles may not have imploded at the same frequency, decreasing the mass transfer.

5.3.4 Analysis of Diaporthe schini extracts by gas chromatography

The extracts of the validation assays were analyzed by gas chromatography associated to a mass spectrometer to estimate which compounds were extracted by UAE. Figure 2 shows the molecular structure of the main compounds observed. GC/MS identified fatty acid methyl esters as 9,12-octadecadienoic acid, methyl ester (Fig. 2 a), a long-chain fatty acid ethyl ester as hexadecanoic acid, ethyl ester (Fig. 2 b), an ethyl octadec-9-enoate (Fig. 2 c), and a diaza-compounds such as 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane (Fig. 2 d). The antioxidant activity observed in this study could be associated with these identified compounds and also with the synergy between them. These compounds are of interest in the pharmaceutical and food areas (ELGNDI et al., 2017).

Figure 2 - Molecular structure of the compounds obtained by GC/MS. a) 9,12 Octadecadienoic acid (Z,Z)-, methyl ester; b) Hexadecanoic acid, ethyl ester; c) Ethyl octadec-9-enoate; d) 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane



Source: own author

Hexadecanoic acid, ethyl ester was also identified in essential oil from Oxytropis falcate Bunge with antioxidant characteristics (JIANG, 2009). Valente et al. (2018) and da Rosa et al. (2020) also observed the presence of the compound 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane in extracts of the supercritical CO_2 extraction to obtain high antioxidant capacity from *Botryosphaeria dothidea* and *Diaporthe schini* biomass, respectively.

5.4 Conclusion

Ultrasound-assisted extraction (UAE) from *Diaporthe schini* biomass was more efficient than heat-assisted extraction (HAE). Higher extract yield and antioxidant activity were obtained using ultrasound compared to conventional extraction (HAE). The validated assay was

performed at an ultrasonic intensity of 85 $W.cm^{-2}$ and a pulse cycle of 0.93 resulting in an extraction yield of 22.30 ± 0.47% and an antioxidant activity of 91.35 ± 0.27%, while a heat-assisted extraction the maximum yield was 8.34 ± 0.38% and antioxidant activity was 91.32 ± 0.89%. Results from this study indicated that the use of UAE reduces the extraction time and increased the extraction yield when compared to the HAE technique. The identified compounds identified by GC/MS could be attributed to the antioxidant activity found in the extracts.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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6 DISCUSSÃO

O uso de fungos endofíticos para a produção de metabólitos secundários com valor para a indústria vem sendo uma alternativa para a obtenção de compostos com atividades bioativas como a capacidade antioxidante devido a sua versatilidade e fácil produção, não sendo refém de condições climáticas, como é o caso de plantas. Foi utilizada uma espécie de fungo isolada no bioma Pampa para a produção de compostos antioxidantes a partir da fermentação submersa. Na sequência os compostos foram extraídos das células liofilizadas utilizando dois métodos principais, a extração com fluido supercrítico e a extração assistida por ultrassom. O ponto em comum entre estas metodologias, é o fato de que elas rompem as células, tornando a extração mais rápida em virtude do mecanismo de transferência de massa por convecção.

A extração utilizando fluido supercrítico foi realizada com dióxido de carbono (CO_2) puro e CO_2 com etanol como co-solvente. A principal diferença entre essas estratégias foi o rendimento obtido, sendo possível obter um rendimento de extrato 141,79 % maior e atividade antioxidantes 133,15 % maior utilizando co-solvente em relação ao CO_2 puro. Isso pode estar relacionado ao aumento da solubilidade dos compostos polares na mistura entre etanol e CO_2. Os melhores resultados foram obtidos nas condições de menor temperatura e maior pressão podendo ser atribuído a maior densidade do solvente nestas condições.

Para a extração assistida por ultrassom, os resultados obtidos para rendimento de extrato foram superiores aos resultados obtidos nos experimentos de controle (sem o uso do ultrassom) e utilizando a técnica de extração assistida pelo calor. A condição de validação para o melhor rendimento utilizando a extração assistida por ultrassom foi com a intensidade de ultrassom máxima e o ciclo de pulso elevado, mas não constante. Este resultado está relacionado com a maior produção de bolhas por cavitação liberando uma maior quantidade de energia e consequentemente aumentando a transferência de massa.

A extração assistida pelo calor foi escolhida entre as técnicas convencionais para a comparação por ser simples e envolver mecanismo de transferência de massa semelhante com às técnicas estudadas. A agitação desse sistema faz com que seja possível o aumento da taxa de extração devido à transferência por convecção, indicando resultados de rendimento superiores a extração com CO_2 supercrítico, em todas as condições, e inferiores à extração com ultrassom. O resultado para a atividade antioxidante superou os resultados obtidos com a extração em estado supercrítico sem co-solvente, indicando nesta técnica, a necessidade do uso de um agente modificador de polaridade para a matriz utilizada. Entretanto, esses resultados são inferiores aos obtidos nas condições otimizadas das técnicas não convencionais. Com isso é possível

afirmar que as técnicas não convencionais, quando otimizadas, se mostraram superiores a técnica de extração convencional, indicando a necessidade do estudo dos parâmetros e a otimização do processo, para se obter os melhores resultados operacionais.

Com a extração assistida por ultrassom foi possível obter o melhor rendimento de extrato, sendo 588,24 % maior que o melhor resultado obtido pela técnica de extração com fluido supercrítico, que apesar de não ter sido possível alcançar alto rendimento de extrato foi possível obter maior atividade antioxidante indicando maior seletividade para estes compostos. Contudo por conta do menor tempo de operação e por ser possível manter elevado rendimento de compostos antioxidantes a melhor técnica para ser utilizada neste caso é extração assistida por ultrassom.

7 CONCLUSÃO

A partir dos resultados, pode-se observar que os extratos produzidos a partir da fermentação submersa do fungo *Diaporthe schini* apresentaram alta atividade antioxidante. O uso de CO_2 em estado supercrítico com etanol na razão biomassa: co-solvente de 1:1,5 (m/v), na menor temperatura (40 ºC) e maior pressão (250 bar) avaliados, resultou em maior atividade antioxidante (96,62 %), e rendimento de extrato (3,24 %). Por outro lado, a extração assistida por ultrassom apresentou o maior rendimento de extrato (22,30 ± 0,47 %) e atividade antioxidante de frente ao radical DPPH (91,35 ± 0,27 %), na condição de validação, sendo realizada com intensidade de ultrassom de 85 $W.cm^{-2}$ e ciclo de pulso de 0,93.

Portanto, foi possível verificar que o melhor método para a extração destes compostos foi utilizando o ultrassom, uma vez que foi possível obter uma maior quantidade de extrato em um menor tempo de operação, mantendo elevada a extração de compostos com atividade antioxidante. Os resultados obtidos neste trabalho demonstraram a possibilidade de obtenção de extratos com atividade antioxidante a partir de metodologias não convencionais, visto que compostos relacionados a atividade antioxidante foram identificados por cromatografia gasosa e também os resultados se mostraram superiores a técnica de extração convencional utilizada, extração assistida por calor.

SUGESTÕES PARA TRABALHOS FUTUROS

- Avaliar qual molécula é responsável pela atividade antioxidante, ou se a sinergia entre as moléculas maximiza este efeito.
- Otimizar a fermentação para a máxima obtenção de compostos antioxidantes.
- Integrar o processo de extração utilizando em conjunto fluido supercrítico e ultrassom.
- Aplicar técnicas de cromatografia de coluna com diferentes resinas para purificar os compostos produzidos pelo fungo.

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