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Dissertação

Isolamento de fungo endofítico a partir de *Achyrocline satureioides*: caracterização fitoquímica e análise de efeito antiglioma

Nathalia Stark Pedra

Pelotas, 2018

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica e Bioprospecção do Centro de Ciências Químicas, Farmacêuticas e de Alimentos da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Mestre em Ciências (Bioquímica e Bioprospecção).

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Dissertação aprovada, como requisito parcial, para a obtenção do grau de Mestre em Ciências (Bioquímica e Bioprospecção), Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas.

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“(...)Mas não sou completa, não. Completa lembra realizada. Realizada é acabada. Acabada é o que não se renova a cada instante da vida e do mundo. Eu vivo me completando...mas falta um bocado.”

(Clarice Lispector)

Resumo

PEDRA, Nathalia Stark Isolamento de fungo endofítico a partir de *Achyrocline sattureioides*: caracterização fitoquímica e análise de efeito antiglioma. 2018. Nº 87f. Dissertação (Mestrado) – Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas.

O Glioblastoma multiforme (GBM) é o tumor cerebral primário mais maligno caracterizado por instabilidade genômica e infiltração difusa, promovendo assim, um ambiente oxidativo, proliferação celular e difícil prognóstico para os pacientes. Os fungos endofíticos residem no interior dos tecidos saudáveis de plantas e representam uma importante fonte de compostos antitumorais. *Achyrocline satureioides*, uma planta medicinal popularmente conhecida como "marcela", possui interessantes propriedades farmacológicas. Uma vez que não há evidências a cerca da composição de fungos endofíticos de *A. satureioides*, o objetivo deste estudo foi isolar um fungo endofítico, obter extratos brutos e fracionados, caracterizar quimicamente as frações mais efetivas, investigar seu efeito antiglioma e supressão do estresse oxidativo no ambiente tumoral. Os metabólitos produzidos pelo endófito isolado foram extraídos com os solventes orgânicos diclorometano e acetato de etila, resultando nos extratos brutos eDCM e eAcoEt, os quais foram submetidos a um fracionamento guiado. O perfil citotóxico dos extratos brutos (24-72 h) e fracionados (48 h) foi determinado mediante ensaio de MTT e Sulforodamida B (SRB) sobre linhagens de glioma C6 e U87MG. Ensaio clonogênico, análise do ciclo/morte celular e parâmetros de estresse oxidativo foram avaliados sobre linhagem C6 exposta aos extratos brutos, fração mais efetiva de cada extrato e composto 1 por 48 h. Os dados foram analisados por ANOVA seguido de post-hoc de Tukey e considerados significativos para $P<0.05$. No presente estudo os extratos eDCM e AcoEt do fungo endofítico isolado apresentaram citotoxicidade significativa com IC_{50} de 1.23-2.66 µg/mL e 29.92-87.06 µg/mL, respectivamente após 48 h, com consequente aumento do sistema antioxidante, como o conteúdo sulfidril (SH), enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). As frações $F1_{DCM}$, $F2_{DCM}$, $F3_{DCM}$ e $F4_{DCM}$ (obtidas de eDCM), bem como, $F3_{AcoEt}$ e $F4_{AcoEt}$ (obtidas de eAcoEt) exibiram efeitos citotóxicos. Observamos ainda que $F3_{DCM}$ e $F3_{AcoEt}$ apresentaram atividade antiproliferativa significativa com IC_{50} de 1.09 e 21.43 µg/mL, respectivamente. A $F3_{AcoEt}$ induziu um acúmulo de células em apoptose tardia no estágio G2/M. Por outro lado, a $F3_{DCM}$ reduziu significativamente a formação de colônicas em 90%, exibiu aumento da defesa antioxidante e diminuição na produção de espécies reativas de oxigênio (EROs). Nenhuma alteração foi observada em cultivo primário de astrócitos expostos aos extratos brutos e fracionados. Assim, considerando a atividade antiglioma seletiva, a fração $F3_{DCM}$ foi caracterizada por cromatografia líquida de ultra-eficiência e ressonância nuclear magnética. Com base nestes dados, identificamos a Sch-642305, lactona produzida por fungos endofíticos, a qual inibiu 80% da viabilidade celular das células de glioma e promoveu parada no ciclo celular em G2/M induzindo apoptose. A Sch-642305 aumentou a atividade das enzimas SOD e CAT, conteúdo SH e suprimiu a produção de EROs. Além disso, foi possível isolar um composto proveniente da fração $F4_{DCM}$, identificado como 5-metil-meleína, entretanto estudos são necessários a fim de elucidar seus efeitos antiglioma. Estes resultados indicam que os compostos produzidos pelo fungo endofítico isolado a partir de *A. satureioides* representam uma fonte promissora para a busca por novos agentes terapêuticos contra o GBM.

Palavras-chave: *Achyrocline satureioides*, marcela, fungo endofítico, glioma, apoptose, estresse oxidativo

Abstract

PEDRA, Nathalia Stark. Isolation of endophytic fungus from Achyrocline satureioides: phytochemical characterization and analysis of antiglioma effect. 2018. Nº 87f. Dissertação (Mestrado) – Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas.

Glioblastoma multiforme (GBM) is the most malignant primary brain cancer characterized by genomic instability and diffuse infiltration, promoting an oxidative environment, cell proliferation and poor prognosis for patients. Endophytic fungi reside inside the healthy tissues of plants and represent an important source of antitumor compounds. *Achyrocline satureioides* is a medicinal plant popularly known as “marcela”, which has interesting pharmacological properties. Since there are no evidences reporting of the endophytic fungi composition of *A. satureioides*, the aim of this study was to isolate an endophytic fungus, obtain crude and fractionated extracts, characterize effectiveness fractions and investigate its antiglioma effects and suppression of oxidative stress in the tumor microenvironment. Metabolites produced by the endophyte isolated were extracted with the organic solvents dichloromethane and ethyl acetate yielding eDCM and eEtAc crude extracts which were submitted to a guided fractionation. The cytotoxic profile of the crude (24-72 h) and fractionated (48 h) extracts was determined by MTT and Sulforodamide B (SRB) assays on C6 and U87MG glioma lines. Clonogenic assay, cycle/cell death analysis and oxidative stress parameters were evaluated on C6 line exposed to crude extracts, the most effective fraction of each extract and compound 1 for 48 h. The data were analyzed by ANOVA followed by Tukey post-hoc and considered significant for $P<0.05$. In the present study, the eDCM and AcoEt extracts isolated from endophytic fungus showed significant cytotoxicity with IC_{50} of 1.23-2.66 µg/mL and 29.92-87.06 µg/mL, respectively, after 48 h, with a consequent increase in the antioxidant system, such as sulfhydryl content (SH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes. $F1_{DCM}$, $F2_{DCM}$, $F3_{DCM}$ e $F4_{DCM}$ (obtain from eDCM) and $F3_{EtAc}$ e $F4_{EtAc}$ (obtain from eEtAc) exibinhit significant antiproliferative activity with IC_{50} 1.09 and 21.43 µg/mL, respectively. $F3_{EtAc}$ induced accumulation of cells in late apoptosis at the G2/M stage. On the other hand, $F3_{DCM}$ significantly reduced colony formation by 90%, exhibited increased antioxidant defense and decreased production of reactive oxygen species (ROS). No changes were observed in primary culture of astrocytes exposed to crude and fractionated extracts. Thus, considering the selective antiglioma activity, the $F3_{DCM}$ was characterized by ultra-efficient liquid chromatography and nuclear magnetic resonance. Based on these data, we identified Sch-642305, a lactone produced by endophytic fungi, which inhibited 80% of the cell viability of glioma cells and promoted cell cycle arrest in G2/M inducing apoptosis. Sch-642305 increased the SOD and CAT activity, SH content and suppressed ROS production. In addition, it was possible to isolate a compound from the $F4_{DCM}$ fraction, identified as 5-methyl-melein, however studies are needed to elucidate its antiglioma effects. These results indicate that the compounds produced by the endophytic fungus isolated from *A. satureioides* represent a promising source for the search for new therapeutic agents against GBM.

Keywords: *Achyrocline satureioides*; Marcela, endophytic fungus, glioma, apoptosis, oxidative stress

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Lista de Abreviaturas e Siglas

µg	Micrograma
AcoEt	Acetato de etila
CAT	Catalase
DCM	Diclorometano
EGFR	Receptor do fator de crescimento epidermal
eDCM	Extrato obtido com o solvente orgânico diclorometano
eAcoEt	Extrato obtido com o solvente orgânico acetato de etila
EROs	Espécies reativas de oxigênio
GBM	Glioblastoma multiforme
GPx	Glutationa peroxidase
H_2O	Água
H_2O_2	Peróxido de hidrogênio
IC_{50}	*Half maximal inhibitory concentration*
IDH1	Isocitrato desidrogenase-1
mL	Mililitro
MGMT	O_6-metilguanina metiltransferase
MITC	5-(3-metiltriazen-1-il)-imidazol-4-carboximida
MTT	Brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio
$O_2^{\bullet -}$	Ânion superóxido
$OH^{\bullet}$	Radical hidroxil
OMS	Organização Mundial da Saúde
PTEN	Proteína fosfatase homóloga à tensina
SH	Sulfidril
SOD	Superóxido dismutase
SRB	Sulforofamina B
TMZ	Temolozomida
TP53	Proteína tumoral p53
VEGF	Fator de crescimento endotelial vascular

Sumário

1. Introdução

Gliomas são tumores que apresentam características gênicas e fenotípicas similares as células gliais, sendo o tipo mais comum de tumor cerebral primário (OSTROM et al., 2014). Conforme evidenciado por Louis e colaboradores (2016), o sistema de classificação da Organização Mundial da Saúde (OMS) utiliza 4 graus para descrever os gliomas de acordo com a malignidade, características histológicas, alterações fenotípicas e genotípicas.

Segundo a OMS, o glioblastoma multiforme (GBM) é classificado como glioma de grau IV, de acordo com sua malignidade e morfologia (LOUIS et al., 2016). O tratamento padrão atualmente empregado, composto por ressecção cirúrgica seguido de radioterapia e quimioterapia com o agente alquilante temozolomida, fornece aos pacientes um período de sobrevida de apenas 12 a 14 meses após o diagnóstico (RAMIREZ et al., 2013).

Gliomas de alto grau apresentam múltiplas alterações genéticas e elevado estresse oxidativo, sugerindo assim que o microambiente oxidativo favorece características invasivas a esta neoplasia (TRACHOOTHAM; ALEXANDRE; HUANG, 2009). O estresse oxidativo ocorre quando há um desbalanço entre a produção de espécies reativas, como as espécies reativas de oxigênio (EROs), e o sistema de defesa antioxidante (RINALDI et al., 2016). A produção de EROs está envolvida com danos celulares, necrose e apoptose celular devido a oxidação e nitração de proteínas, lipídeos e proteínas, acarretando no mal funcionamento celular (DEL BÓ et al., 2015). Neste sentido, torna-se necessária a busca de novas modalidades terapêuticas capazes de inibir o desenvolvimento do tumor, reduzindo danos oxidativos, aumentando assim, a sobrevida dos pacientes.

Produtos naturais, em especial compostos provenientes de plantas, têm desempenhado importante papel no desenvolvimento de fármacos clinicamente úteis (CHANDRA, 2012). Popularmente conhecida como marcela, a *Achyrocline* spp. apresenta diversas propriedades farmacológicas. O gênero conta com cerca de 40 espécies distribuídas em regiões tropicais e subtropicais da América Latina (RETTA et al., 2012). Diversos aspectos farmacológicos são atribuídos às espécies de *Achyrocline satureioides* devido suas propriedades anti-inflamatórias (DE SOUZA et al., 2007), antioxidante (SALGUEIRO et al., 2016), entre outras. Além disso, Ruffa e colaboradores (2002) revelaram

potente efeito citotóxico contra a linhagem de carcinoma hepatocelular humano (Hep-G2). Demais estudos revelam interessantes efeitos anti-neoplásicos sobre diversas linhagens tumorais exercidos por compostos provenientes de espécies do gênero *Achyrocline* sp. (THOMAS et al., 2012, CARINI; KLAMT; BASSANI, 2014).

Além das plantas, os micro-organismos constituem outra fonte promissora para a busca de compostos bioativos, dos quais derivam diversos produtos com propriedades farmacológicas conhecidas. Strobel e Long (1998) revelam que micro-organismos endofíticos, em especial fungos, associados às plantas podem oferecer mais compostos químicos com efeitos terapêuticos do que a própria planta.

Os fungos endofíticos ou endófitos vivem no interior de tecidos saudáveis dos vegetais e são importantes componentes do micro-ecossistema da planta. Ao longo da evolução, os endófitos e suas plantas hospedeiras têm estabelecido uma relação mutualística, a qual pode ter influenciado significativamente a formação de produtos metabólicos, afetando a qualidade e a quantidade de compostos derivados a partir de plantas medicinais (JIA et al., 2016). Desta forma, muitos fungos endofíticos são capazes de sintetizar uma variedade de metabólitos bioativos que podem ser utilizados como agentes terapêuticos contra diversas doenças (STROBEL, 2003; KHARWAR et al., 2011). Segundo Chen e colaboradores (2014) tais micro-organismos têm sido reconhecidos como um importante recurso de bioativos naturais, especialmente na terapia anticâncer.

Neste contexto, considerando o arsenal terapêutico insuficiente para o tratamento de neoplasias malignas como o GBM e o potencial terapêutico de bioativos provenientes de espécie de *A. satureioides,* a busca por novos compostos naturais e potentes metabólitos antitumorais a partir de fontes inexploradas como os endófitos encorajam pesquisas neste campo.

2. Objetivos

Objetivo geral

Isolar um fungo endofítico a partir da planta medicinal *Achyrocline satureioides* e avaliar seu potencial antiglioma

Objetivos específicos

- Isolar e identificar fungo endofítico a partir de caules sadios de *Achyrocline satureioides*
    - Obter extratos a partir dos metabólitos secundários produzidos pelo fungo endofítico presentes no meio de cultivo líquido, utilizando os solventes orgânicos diclorometano e acetato de etila.
    - Realizar o fracionamento dos extratos orgânicos brutos e identificar componentes químicos
    - Determinar a atividade citotóxica dos extratos brutos, fracionados e Sch-642305 sobre linhagens de glioma (C6 e U87) e cultura primária de astrócitos
    - Elucidar os mecanismos envolvidos na modulação redox do microambiente tumoral de linhagem de glioma C6 exposta aos extratos brutos e fracionados, mediante marcadores de estresse oxidativo

3. Revisão bibliográfica

Gliomas

Os gliomas consistem na forma mais comum de tumor cerebral primário (WEN; REARDON, 2016). Tais tumores apresentam representam cerca de 40% a 60% entre os tumores do sistema nervoso central (SNC) (INCA, 2017) com uma incidência anual de 0.6 a 3.7 a cada 100.000 pessoas dependendo do país (CHERNOV et al., 2017).

Os gliomas podem apresentar características fenotípicas e moleculares de diferentes tipos de células gliais, como astrócitos, oligodendrócitos e células ependimárias. Neste contexto, a OMS distingue estes tumores de acordo com o grau de malignidade (I-IV) (ILKANIZADEH et al., 2014). Neste sentido, gliomas de grau I (astrocitomas pilocíticos) e II (astrocitomas de baixo grau) são classificados como tumores de baixo grau de malignidade, uma vez que apresentam proliferação celular reduzida e limitada capacidade de propagar-se para o parênquima cerebral. Enquanto que tumores de grau III (astrocitomas anaplásicos) e IV (GBM) possuem elevada taxa de proliferação, pleomorfismo e desdiferenciação celular (LOUIS et al., 2007, 2016). Entretanto, o GBM apresenta recursos mais avançados de malignidade, incluindo proliferação, invasão, angiogênese e necrose (FURNARI et al., 2007).

O Glioblastoma multiforme (GBM)

O GBM é o mais frequente e agressivo tumor primário do sistema nervoso central (LEMÉE; CLAVREUL; MENEI, 2015). Tal tumor é caracterizado por uma população heterogênea de células, altamente infiltrativa, angiogênica e resistente à quimioterapia (WEN; KESARI, 2008; RAMIREZ et al., 2013). Devido a estas características, pacientes acometidos por este tipo de tumor apresentam uma sobrevida média de um ano (STUPP; MASON; VAN DEN BENT, 2005, HUSE; HOLLAND, 2010). De acordo com Stupp e colaboradores (2007), há duas razões estabelecidas para descrever o mau prognóstico associado ao GBM. Em primeiro lugar, apesar destes tumores raramente desencadearem mestástases, as células tumorais infiltram-se extensivamente no parênquima cerebral circundante, limitando assim, a possibilidade de ressecção cirúrgica. Em segundo lugar, a barreira hematoencefálica representa

um obstáculo para a entrega adequada de agentes quimioterápicos para os tumores cerebrais devido o local de infiltração do tumor, onde as células malignas estão intercaladas no parênquima cerebral normal.

O GBM pode ser classificado em dois subtipos principais com base em diferenças biológicas e genéticas (FURNARI et al., 2007, OHGAKI; KLEIHUES, 2007), o GBM primário e o secundário. O GBM primário ocorre via *de novo*, sem evidências de perda de malignidade do precursor, enquanto que o GBM secundário desenvolve-se inicialmente a partir de um tumor de grau II ou III, entretanto, a maioria dos GBM (~90%) corresponde a GBM primário (OHGAKI; KLEIHUES, 2013, WILSON; KARAJANNIS; HARTER, 2014).

Conforme revisado por Wilson, Karajannis e Harter (2014), as alterações genéticas típicas para o GBM primário consistem na super expressão do receptor do fator de crescimento epidermal (EGFR), mutações na proteína fosfatase e homóloga à tensina (PTEN) e perda da heterozigose do cromossomo 10. Ainda segundo os autores, as alterações genéticas comumente encontradas no GBM secundário incluem mutações na proteína tumoral p53 (TP53), perda da heterozigose do cromossomo 19 e mutações na isocitrato desidrogenase-1 (IDH1).

Além das alterações em vias que controlam a proliferação, morte e diferenciação das células tumorais, estudos têm mostrado que a presença de um microambiente favorável é elemento essencial para a progressão do câncer (HANAHAN; WEINBERG, 2011). Neste contexto, a elevada taxa metabólica e a produção aumentada de espécies reativas, desempenham um importante papel como mediadores químicos na regulação da transdução de sinal e protegendo as células malignas da apoptose, favorecendo a formação de um ambiente oxidativo e instabilidade genética em pacientes acometidos pelo GBM (SALAZAR-RAMIRO et al., 2016).

Sistema redox no desenvolvimento dos gliomas

Células cancerígenas são caracterizadas por elevado estresse oxidativo (CAIRNS; HARRIS; MAK et al., 2011). Este estresse oxidativo é caracterizado por um aumento na produção de radicais livres e outras espécies reativas, e redução no sistema de defesa antioxidante do organismo (FOGARASI et al., 2016). As EROs são frequentemente produzidas como consequência de

reações metabólicas (SOSA et al., 2013) e são definidas como pequenas espécies contendo oxigênio, as quais incluem o ânion superóxido ($O_2^{\bullet -}$), radical hidroxil ($OH^{\bullet}$) e peróxido de hidrogênio (H_2O_2) (SCHIEBER; CHANDEL, 2014). No GBM, altas quantidades de espécies reativas de oxigênio dentro das células podem reagir com diversas macromoléculas, incluindo DNA cromossomal e mitocondrial, acarretando em danos e mau funcionamento de enzimas de reparo do DNA (SALAZAR-RAMIRO, et al., 2016). Neste contexto, a manipulação dos níveis de EROs mediante modulação do sistema redox torna-se uma alternativa promissora para o desenvolvimento de agentes seletivos para células tumorais, sem causar toxicidade sobre células normais (TRACHOOTHAM; ALEXANDRE; HUANG, 2009).

Durante o estresse oxidativo prolongado ocorrem alterações na atividade das enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). Tais enzimas atuam prevenindo ou reduzindo os danos teciduais causados pelos radicais livres (MARTÍNEZ-MARTOS et al., 2014). A SOD catalisa a dismutação de $O_2^{\bullet -}$ formando H_2O_2, o qual pode ser convertido à água (H_2O) e oxigênio molecular pela ação da enzima CAT, enquanto a GPx reduz o H_2O_2 à H_2O e outros peróxido lipídicos (DEL BÓ et al., 2015, IGHODARO; AKINLOYE, 2017). Deste modo, a modulação do sistema redox mostra-se uma ferramenta interessante para a busca de novos alvos terapêuticos.

Tratamento padrão dos gliomas

De acordo com Sathornsumetee e Rich (2008), o tratamento dos gliomas envolve três modalidades: a ressecção cirúrgica, a radioterapia e a quimioterapia. No entanto, os gliomas malignos não podem ser removidos cirurgicamente devido a sua natureza infiltrativa. Desta forma, a radioterapia consiste atualmente no pilar do tratamento para gliomas malignos (WEN; KESARI, 2008).

O quimioterápico atualmente utilizado para o tratamento do GBM, a temolozomida (TMZ), é um agente alquilante que ultrapassa a barreira hematoencefálica agindo, dessa forma, no ambiente cerebral (SATHORNSUMETEE; RICH, 2008) provocando danos ao DNA e apoptose celular, uma vez que é convertido em sua forma citotóxica, o 5-(3-metiltriazen-

1-il)-imidazol-4-carboximida (MITC). O primeiro alvo citotóxico do TMZ é a O6-metilguanina, cujo grupo metil pode ser removido pela O6-metilguanina metiltransferase (MGMT), uma proteína de reparo do DNA responsável pela remoção de grupos metil na posição O6 da guanina (KAINA et al, 2007, ZHANG; STEVENS; BRADSHAW, 2012). Segundo Villalva e colaboradores (2011), em alguns pacientes a expressão de MGMT tem sido decrescida ou silenciada devido a metilação de regiões promotoras do gene de MGMT, inibindo a remoção de grupos metil na posição O6 da guanina. Desta forma, a metilação da região promotora de MGMT contribui para a resistência ao TMZ em pacientes com GBM.

Neste sentido, o arsenal terapêutico disponível para o tratamento da doença ainda apresenta resultados insatisfatórios, sendo necessária a busca de novas modalidades terapêuticas capazes de inibir o desenvolvimento tumoral e, com isso, aumentar a sobrevida dos pacientes.

***Achyrocline satureioides* Lam. DC.**

Produtos naturais, em especial compostos provenientes de plantas, têm desempenhado importante papel no desenvolvimento de fármacos clinicamente úteis (CHANDRA, 2012).

O gênero *Achyrocline* compreende espécies de plantas pertencentes à família Asteraceae. Há cerca de 40 espécies de *Achyrocline* spp. distribuídas em regiões tropicais e subtropicais das Américas Central e Sul (DEBLE, 2007; RETTA, 2012). Na América do Sul são encontradas *A. alata*, *A. tomentosa*, *A. flaccida*e *A. satureoides* (SOUZA, 2002, ZAMPIERON, 2010), as quais apresentam elevada importância química e biológica.

A *Achyrocline satureioides* Lam. DC. popularmente conhecida como “macela” consiste em uma importante espécie medicinal. Nativa do sul e sudeste da América do Sul, a *A. satureioides* é comum no Brasil (Minas Gerais a Rio Grande do Sul), Uruguai e Argentina (LORENZO et al., 2000, RETTA et al., 2012).

Aspectos farmacológicos de *Achyrocline satureioides*

Na medicina popular têm-se diversas atribuições às espécies pertencentes ao gênero *Achyrocline* spp. Extratos de folhas e caules de *A.*

satureioides são utilizados para o tratamento de distúrbios gastrointestinais, epilepsia, reumatismo, irregularidades menstruais, cólicas, dores articulares e musculares (LORENZI; MATOS, 2002, BARATA et al., 2009), além de conduzir ao alívio de sintomas de úlceras gástricas (SANTIN et al., 2010). Demais estudos revelam que *A. satureoides* detém atividades imunomodulatória (CONSENTINO et al., 2008), antimicrobiana (CASERO et al., 2014) e antiherpes (BIDONE et al., 2015), bem como propriedades anti-hiperglicêmicas (HENG et al., 2010) e potentes atividades anti-inflamatória (DE SOUZA et al., 2007) e antioxidante (SALGUEIRO et al., 2016).

Dados da literatura revelam importantes efeitos antitumorais atribuídos às espécies pertencentes ao gênero *Achyrocline* spp. Ruffa e colaboradores (2002) revelaram atividade citotóxica de extratos de *A. satureioides* sobre o carcinoma hepatocelular humano (Hep-G2), enquanto que Cosentino e colaboradores (2008) demonstraram que *A. satureioides* não apresenta atividade citotóxica para as células mononucleares do sangue periférico humano e leucócitos polimorfonucleares, evidenciando assim seu potencial efeito antitumoral, uma vez que apresenta baixa toxicidade para células humanas normais. Além disso, Walker (2013) revelou potente efeito antitumoral de *Achyrocline* spp. sobre a linhagem de câncer de mama BT-474.

Micro-organismos endofíticos

Fungos endofíticos compreendem micro-organismos que infectam internamente tecidos de plantas sem causar qualquer sintoma perceptível de doença, e vivem em associação mutualística com plantas durante todo ou parte todo seu ciclo de vida (PORRAS-ALFARO; BAYMAN, 2011). Neste sentido, os micro-organismos diferenciam-se dos fitopatógenos, que são prejudiciais e causadores de doenças, e dos epífitos, que habitam a superfície vegetal (AZEVEDO, 1998, STROBEL; DAISY, 2003). Tais micro-organismos são encontrados em todos os tipos de plantas, como vegetais superiores, gramíneas, algas e plantas herbáceas (NISA et al., 2015).

Os fungos endofíticos evoluíram a partir de dois modos de transmissão: vertical e horizontal. Conforme Rodriguez e colaboradores (2009), a transferência vertical ocorre entre a planta materna e a sua descendente

através de sementes, já a transmissão horizontal ocorre por meio de fissuras entre plantas de um mesmo ambiente.

Os fungos endofíticos também promovem o crescimento da planta hospedeira e a formação de metabólitos secundários relacionados com a defesa do vegetal (CHANDRA et al., 2010). Neste sentido, a produção de substâncias bioativas por endófitos está diretamente relacionada com a evolução independente destes micro-organismos, os quais podem ter incorporado a informação genética a partir de plantas superiores, permitindo-lhes uma melhor adaptação à planta hospedeira (STROBEL, 2003, JIA et al., 2016). Assim, uma vez que interações metabólicas entre endófitos e hospedeiro favorecem a síntese de metabólitos, Kusari e Spiteller (2011) revelam que, devido ao mutualismo, plantas e fungos endofíticos podem produzir metabólitos secundários de interesse medicinais (Figura 1).

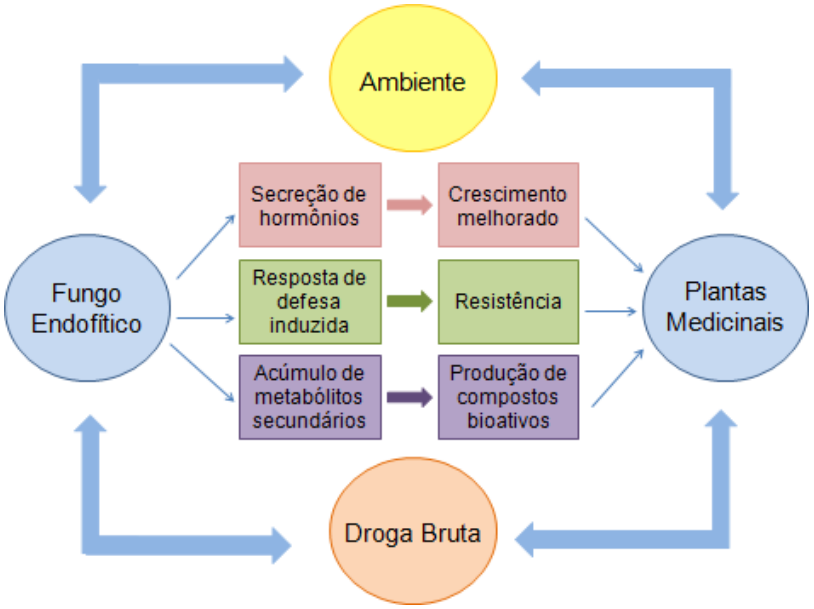

Figura 1. Relações benéficas estabelecidas entre endófito e planta, incluindo melhoramento no crescimento e resistência a estresses bióticos e abióticos de suas plantas hospedeiras, bem como promovendo o acúmulo de metabólitos secundários (Fonte: JIA et al., 2016 com modificações).

Potencial de compostos produzidos por fungos endofíticos

Muitos fungos endofíticos têm o potencial de sintetizar uma variedade de metabólitos bioativos que podem ser utilizados diretamente ou indiretamente,

como agentes terapêuticos contra diversas doenças (STROBEL, 2003; KHARWAR et al., 2011).

Segundo Strobel (2003), plantas medicinais que possuem, por exemplo, uma ação antimicrobiana poderiam ser hospedeiras de fungos endofíticos, que por sua vez poderiam apresentar essa propriedade, sugerindo assim, que o princípio ativo antimicrobiano pode ser produzido pelo micro-organismo e não propriamente pelo vegetal, ou então que tais efeitos terapêuticos somente são constatados devido à associação existente entre a planta e o seu hospedeiro. Neste contexto, micro-organismos associados às plantas podem oferecer mais materiais com efeitos terapêuticos do que a própria planta (STROBEL; LONG, 1998), gerando novos compostos farmacologicamente ativos (PINTO, 2003).

Um amplo espectro de atividades biológicas tem sido atribuído aos endófitos devido suas propriedades antimicrobiana (GARCÍA et al., 2012), antioxidante (YADAV; YADAV; YADAV, 2014), antinociceptiva e anti-inflamatória (BARROS et al., 2011), entre outras. Além disso, tais micro-organismos têm sido reconhecidos como um importante recurso de bioativos naturais, especialmente na terapia anticâncer (CHEN et al., 2016).

Stierle, Strobel e Stierle (1993) descreveram a produção do diterpenóide “Taxol” (ou Paclitaxel), um importante fármaco quimioterápico, pelo fungo endofítico *Taxomyces andreanae*, o qual foi isolado das entrecascas da planta *Taxus brevifolia*, apenas adequando-se as condições de cultivo. Além do taxol, a produção de outros bioativos naturais de interesse farmacêutico tem sido caracterizada por fungos endofíticos obtidos a partir de diversas espécies vegetais (WANG et al., 2014).

O alcalóide camptotecina, primeiramente isolado a partir da planta *Camptotheca cuminata*, é um importante agente antineoplásico (KUSARI, ZUHLKE; SPITELLER, 2009). Estudos demonstram que fungos endofíticos como *Entrophospora infrequens* (AMNA et al. 2006) e *Fusarium solani* (KUSARI; ZÜHLKE; SPITELLER, 2009), isolados a partir de *C. cuminata*, produzem compostos análogos à camptotecina.

A vimblastina e a vincristina são dois compostos amplamente utilizados no tratamento do linfoma e da leucemia, respectivamente. Estes alcalóides são obtidos naturalmente a partir da planta *Catharanthus roseus* ou *Vinca rósea*. *Alternaria* sp (GUO; LI; ZHANG, 1998) e *Fusarium oxysporum* (ZHANG et al.,

2000) representaram o marco inicial da produção da vimblastina e vincristina por micro-organismos hospedeiros de *C. roseus*. Recentemente, Kuriakose, Palem e Jayabaskaran (2016) reportaram o isolamento de um novo endófito, o fungo *Eutypella* spp., capaz de sintetizar a vincristina a partir *C. roseus*, apresentando potente atividade citotóxica sobre a linhagem de carcinoma escamoso humano (A431).

Tan, Qi e Ni (2015) demonstraram que extrato de fungo endofítico isolado a partir de *Prunella vulgaris* L. é capaz de inibir câncer gástrico, tanto *in vitro* quanto *in vivo*. Segundo os autores, os endófitos isolados atuam aumentando a expressão da proteína pró-apoptótica Bax e suprimindo a expressão do fator pró-angiogênico VEGF (fator de crescimento endotelial vascular), reduzindo assim, a proliferação celular.

Neste contexto, conforme evidenciado por Lacava, Andreote e Azevedo (2008), devido o potencial biotecnológico dos endófitos, muitas pesquisas têm focado na busca por novas espécies, bem como novos produtos, como resultado do metabolismo secundário destes micro-organismos. Os autores revelam ainda, que “a busca por novos metabólitos deve concentrar-se em organismos que habitam novos ecossistemas, ou ainda nichos pouco explorados”. Assim, estes micro-organismos servem como uma fonte renovável, reprodutível e inesgotável de novas estruturas com elevado potencial farmacêutico.

4. Manuscrito

Endophytic fungus isolated from *Achyrocline satureioides* exhibits selective antiglioma activity – the role of Sch-642305.

Nathalia Stark Pedra*[1], Kennia de Cássia Araújo Galdino[1], Daniel Schuch da Silva[1], Priscila Treptow Ramos[1], Natália Pontes Bona[1], Mayara Ssandrielly Pereira Soares[1], Juliana Hoffstater Azambuja[2], Kirley Marques Canuto[3], Edy Sousa de Brito[3], Paulo Riceli Vasconcelos Ribeiro[3], Ana Sheila de Queiroz Souza[3], Wilson Cunico[1], Francieli Moro Stefanello[1], Roselia Maria Spanevello[1], Elizandra Braganhol*[1, 2].

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4. Manuscrito

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Endophytic fungus isolated from *Achyrocline satureioides* exhibits selective antiglioma activity – the role of Sch-642305

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Abstract

Glioblastoma multiforme (GBM) is the most devastating primary brain tumor. Endophytes represent an interesting source of natural metabolites. These microorganisms reside in the tissues of living plants and act to improve their growth. Evidence revealed that several medicinal plants are colonized by endophytic fungi producer of antitumor metabolites. *Achyrocline satureioides* is a Brazilian traditional medicinal plant characterized by its properties against gastrointestinal disturbances, anticancer and antioxidant effects. However, there are no reports describing the endophytic composition of *A. satureioides*. The present study proposes the isolation of endophytic fungus from *A. satureioides*, extract preparation, phytochemical caracterization and evaluation of its antiglioma potential. Our data showed that eDCM and eEtAc crude extracts of endophyte exhibited significantly glioma cytotoxicity with lowest IC_{50} of 2 µg/mL and 56 µg/mL, respectively after 72 h of exposure. In addition, both crude extracts induced cell death by apoptosis with modulation of redox status. In order to bioprospect anticancer metabolites, endophytic fungus extracts were subjected to guided fractionation and purification yielded five fractions of each extract. Six of ten fractions showed selective antiproliferative activity against glioma cells after 48 h of treatment, with IC_{50} values ranged from 0.95-128.4 µg/mL. We observed that $F3_{DCM}$ (from eDCM) and $F3_{EtAc}$ (from eEtAc) fractions promoted significantly cytotoxic with IC_{50} of 1.0 µg/mL and 27.05 µg/mL, respectively, against C6 glioma cells. $F3_{EtAc}$ fraction induced late apoptosis and arrest in G2/M stage, while $F3_{DCM}$ promoted apoptosis with arrest in Sub-G1 phase. Moreover, $F3_{DCM}$ increased antioxidant defense with consequently decrease of ROS production. In addition, when tested in astrocyte cultures, $F3_{DCM}$ showed no cytotoxic activity revealing selective effect. Based on promising potential of $F3_{DCM}$, we identified the production of Sch-642305, a known lactone, which showed antiproliferative properties with lowest IC_{50} of 1 µg/mL. Sch-642305 promoted arrest on cell cycle in G2/M inducing apoptosis. Furthermore, this lactone modulated redox status increasing superoxide dismutase and catalase activities and enhancing sulfhydryl content, consequently suppressing reactive species of oxygen generation. Taken together, these results indicate that metabolites produced by endophytic fungus isolated from *A. satureioides* have therapeutic potential to become a source of natural antiglioma agent.

Keywords: Endophytic fungus, *Achyrocline satureioides*, glioblastoma, lactone, antineoplasic agent, antioxidant.

1. Introduction

Glioblastoma multiforme (GBM), a grade IV glioma, is the most malignant type of brain cancer characterized by high cell heterogeneity, diffuse brain infiltration, necrosis, high rate of cell proliferation, and resistance to current treatments (Ramirez et al., 2013; Piccirillo et al., 2015). Despite aggressive, multimodal therapy consisting of surgery, radiation, and chemotherapy, the outcome of patients with GBM remains poor (Eder; Kalman, 2015) with median overall survival time of approximately 15-17 months (Gilbert et al., 2014). The maintenance of redox homeostasis is crucial for normal cell physiology and reactive oxygen species (ROS) are known to regulate several cellular events, including cell growth, differentiation, apoptosis, metabolism and others (Hecht et al., 2016). Several hallmarks of cancer associated with neoplastic growth promote increased ROS levels inducing elevated oxidative stress (Ciccarese; Ciminale, 2017). This cellular redox imbalance has been found in GBM. High quantities of ROS into the cells can react with several macromolecules, including chromosomal and mitochondrial DNA, leading to damage and malfunction of DNA repair enzymes (Salazar-Ramiro et al., 2016). Therefore, new treatments able to modulate this redox status may be a feasible therapeutic approach against GBM.

Natural products have been exploited extensively to new pharmaceuticals development to treat several diseases. *Achyrocline satureioides* Lam. (DC) (Asteraceae) popularly known as “marcela” have received particular attention for their pharmacological activities (Retta et al., 2012). This plant is native medicinal herb in South America, used in Brazilian folk medicine as an analgesic, sedative, anti-inflammatory and mainly to treat gastrointestinal disorders (Silva et al., 2016; Yamane et al., 2016). Plants are continuously involved in crosstalk with endophytic microorganisms leading to the selection of specific functional traits (Kusari et al., 2014). Indeed, endophytic fungi produce a variety of bioactive metabolites that may directly or indirectly be used as therapeutic agents (Strobel; Long. 1998; Kusari; Spiteller, 2012; Kusari et al., 2012). These microorganisms have also been found to produce the same important natural products synthesized by the host plant, such as alkaloids, phenols, coumarins, steroids, terpenoids, peptides and others with anticancer properties (Chen et al., 2014). Although the chemical constituents and the biological properties of genus *Achyrocline* have been extensively studied (Carini et al., 2014; Salgueiro et al., 2016; Moresco et al., 2017), there are no evidence about the endophytic fungi associated with this genus and the possible therapeutic activities of these microorganisms. Additionally, considering the role of redox status in GBM aggressiveness and how this imbalance contribute to gliomagenesis (Salazar-Ramiro et al., 2016), it becomes important the investigation of new therapeutic agents that modulate redox status. Therefore, in present study we evaluated the selective antiglioma activity of crude organic and fractionated extracts of endophytic fungus from *A. satureioides* and their effects in the modulation of redox environment on GBM through evaluation of oxidative stress biomarkers. Additionally, phytochemical characterization was performed and the lactone Sch-642305 was identified as one of the bioactive molecules with promising antiglioma activity produced by endophytic fungus from *A. satureioides.*

2. Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM); fungizone; penicillin/streptomycin; 0.5% trypsin/EDTA solution and fetal bovine serum (FBS) were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). **4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid** (HEPES); sodium bicarbonate ($NaHCO_3$), Dimethylsulphoxide (DMSO); 3(4, 5-dimethyl)-2,5diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid and hydrogen peroxide were purchased from Synth® (Brazil). All other chemicals and solvents used were of analytical grade. Agar and dextrose was provided by Dinâmica (Dinâmica Química Ltda, Diadema, SP, BR).

Collection of plant tissue and Isolation of endophytic fungi

Stems of *Achyrocline satureioides* (Lam.) D.C. were collected at Transbrasiliana Highway (Rio Grande do Sul, Brazil; geographic coordinates: 31°44'34.7"S and 54°09'19.2"W) and it was identified by Dra. Raquel Ludke from the Botany Department (Biology Institute, UFPel), and a voucher specimen was deposited under the code PEL N° 21 079. Surface sterilization of healthy stems was performed according Bertozzo and Machado (2010), with some modifications. Briefly, tissue material was thoroughly washed using distilled water, sterilized with 70% ethanol for 30 s and 2% sodium hypochlorite for 30 min, then rinsed with sterile distilled water for three times to accomplish surface sterilization. Next, samples were cut into 6-8 pieces (6-10 mm in size), placed on water-agar medium and incubated at 25±2ºC under controlled light conditions (Thelga; Dom Bosco, MG, BR). Following 7 days of culture, hyphal tips of fungi that emerged was periodically picked on petri plates containing 1.7% PDA (potato-dextrose-agar) medium for purification and maintained at same conditions described above. Stock cultures were stored at 25±2ºC and maintained in the culture collection of NeuroCan Laboratory (UFPel).

Morphological identification of endophytic fungus

Isolated fungi were observed and identified at the genus level by culture and microscopic characters of asexual/sexual spores. Briefly, endophytic fungus was seeded in 500 μL of PDA medium distributed on a slide held inside petri dish containing a filter paper soaked in sterile distilled water to maintain the moisture of the system for 20 days at 25°C. After that, the endophytic was stained with cotton blue to identify it morphologically under light microscopy. The identification was based on published descriptions.

Preparation of crude extracts

The endophytic strain was cultivated on 1.7% PDA medium at 25±2ºC under controlled light conditions. Then plugs of mycelium (about 8 mm diameter) from the edges of 7-day-old cultures were cut and inoculated aseptically into a 250 mL Erlenmeyer flask containing 100 mL of 1.7% potato-dextrose-broth (PDB) medium (1 plug per 100 mL of medium), and incubated at 25°C for 25 days. Therefore, the mycelium was separated

from the liquid culture medium by filtration and the secondary metabolism compounds released into the liquid culture medium by the endophytic fungus were extracted by using organic solvents dichloromethane (DCM) and ethyl acetate (EtAc) at 1:2 ratio. After that, all extracts were evaporated in a rotary evaporator under reduced pressure (Rota-evaporador MA120 - Marconi).

Fractionation of crude extracts

Solid phase extraction (SPE) were performed Supelclean (C18, 500 mg) reverse phase cartridges using approximately 20 mg of sample dissolved in 200 µL of methanol (MeOH). Cartridge use was preceded by activation of the adsorbent with 5 mL of MeOH, followed by conditioning with 5 mL of milli-Q water. After that, the sample was applied to the cartridge and eluted sequentially with 5 mL of the following eluents: H_2O (F1); H_2O/MeOH 25% (F2), H_2O/MeOH 50% (F3), H_2O/MeOH 75% (F4) and finally MeOH (F5). This procedure was repeated twice for each sample. Collected fractions were dried in a SpeedVac (Thermo-Fisher) vacuum centrifuge at 40°C for 24 h. Fractions obtained from DCM extract (eDCM) were named as $F1_{DCM}$, $F2_{DCM}$, $F3_{DCM}$, $F4_{DCM}$ and $F5_{DCM}$, while fractions from EtAc extract (eEtAc) were named as $F1_{EtAc}$, $F2_{EtAc}$, $F3_{EtAc}$, $F4_{EtAc}$ and $F5_{EtAc}$.

Ultra-efficiency liquid chromatography- mass spectrometry (LC-MS)

Ultra-efficiency chromatographic analyzes were performed on a chromatograph coupled to two mass spectrometers in series (UPLC-QTOF Waters Acquity / Xevo) with an electrospray ionization interface (ESI). Separations were performed on a Waters Acquity BEH C18 column (150 mm × 2.1 mm, 1.7 µm). Mobile phase was composed by H_2O (A) and acetonitrile (B), each containing formic acid (0.1% v/v). Elution gradient ranged from 2 - 95%, at a flow rate of 500 µL min^{-1}. Samples were pre-filtered on 0.22 µm PTFE filters. The fractions were analyzed in the positive (PI) and negative (NI) ionization modes in a range of 100-1200 Da. ESI conditions were defined as follows: capillary voltage 2800 V, cone voltage 40 V, source temperature 120°C, dissolution temperature 330°C, cone gas flow of 20 L h^{-1}, gas desolvation flow 600 L h^{-1}, and MCP (microchannel plate voltage)-detector at 1900 V.

The identification of compounds was done through the following parameters: (1) molecular formula deduced from the exact mass (4 decimal places), considering a mass error of 5 parts per million (ppm); (2) the isotopic ion pattern (i-fit) and (3) the ion fragmentation pattern compared to literature data.

Nuclear Magnetic Resonance Spectrometry (NMR)

Hydrogen and Carbon NMR (^1H and ^{13}C), uni- and two-dimensional, were performed in an Agilent spectrometer DD2 (14,1 T), equipped with a 5 mm reverse detection probe, operating at the frequencies of 1H and 13C at 599.56 and 150.77 MHz, respectively. Samples were dissolved in 0.6 mL of deuterated chloroform ($CDCl_3$, Cambridge Isotope Laboratories) and analyzed in 5 mm glass tubes. The chemical displacements (δ) were expressed in ppm and referenced by the hydrogen signal of the non-deuterated residual

molecules of the deuterated solvent (δH 7.26 for $CHCl_3$ and δH 3.31 for MeOH) and the central carbon peak of the deuterated solvent (δC 77.23 for CHCl 3 and δC 49.15 for MeOH). Analyzes were performed at 26ºC. In one-dimensional ^{1}H and ^{13}C experiments the following values were established for the acquisition parameters, respectively: spectral widths of 16 and 252 ppm, acquisition times of 1.7 and 0.865 s, pulse widths of 45º of 4.15 μs and 3.20 μs (58 dB), number of transients of 16 and 32K, and relaxation time of 1 s. The one-dimensional experiments were acquired with 32768 points and processed with 65356 points. The two-dimensional homonuclear (COSY) and heteronuclear correlation spectra were field-graded, employing a number of transients of 16 and 32, respectively. In the COSY, 897 x 128 points were used for the acquisition data matrix and 4096 x 4096 points for the processing, while for the HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) experiments 1142 x 256 points on acquisition and 4096 x 2048 points in processing.

General cell culture procedures

Rat C6 and human U87MG glioblastoma cell lines were obtained from American Type Cell Collection (Rockville, Maryland, USA). Cells were grown in culture flasks and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (pH 7.4) containing 1% DMEM (Gibco BRL), 8.4 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% fungizone, 0.5 U/mL penicillin/streptomycin and supplemented with 10% (v/v) FBS. Cells were kept at 37°C in a humidified atmosphere with 5% CO_2. Astrocyte cultures were prepared as previously described by Da Frota Jr. et al. (2009). Briefly, cortex of newborn Wistar rats (1-3 days old) were removed and dissociated mechanically in Ca^{+2} and Mg^{+2} -free balanced salt solution (CMF) (pH 7.4) containing 137 mM NaCl, 5.36 mM KCl, 0.27 mM Na_2HPO_4, 1.1 mM KH_2PO_4, and 6.1 mM glucose. Dissociated tissue was subjected to centrifugation at 1000 *g* for 5 min. Thereafter, the pellet was suspended in DMEM (pH 7.6) supplemented with 10% FBS. Then, cells ($5x10^4$) were seeded in poly-L-lysine-coated 96-well plates. Cultures were allowed to grow to confluence by 20-25 days and the medium was replaced every 4 days. All procedures used in the present study followed the "Principles of Laboratory Animal Care" of the National Institutes of Health and were approved by the Ethical Committee of UFPel (CEEA 4755).

Cell culture treatment

Dried crude organic or fractionated extracts were dissolved in DMSO at stock concentration of 10 mg/mL and further diluted in DMEM/10% FBS to obtain a concentration range from 0.625 μg/mL to 200 μg/mL. Glioblatoma cell lines C6 and U87MG were seeded at $5x10^3$ cells (96-well plates) for cytotoxicity experiments and allowed to grown for 24h. Astrocyte cultures were prepared as described above. Cell cultures were treated for 24, 48 or 72 h. To clonogenic assay, the C6 glioma cells were seeded in 6-well plates ($3x10^2$ cells) and treated with crude extracts and fractions at concentrations close to inhibitory concentration 50% (IC_{50}) following 48 h of exposure. To cell cycle and apoptosis/necrosis analysis, C6 glioma cells were seeded in 6-well plates ($1x10^5$ cells/well) and treated with eDCM and eEtAc crude extracts and $F3_{EtAc}$

fraction at concentrations close to IC_{50}, while compound 1 was evaluated at 1 µg/mL after 48 h of exposure. In addition, oxidative stress biomarkers was determined in C6 glioma cells seeded in 6-well plates ($3x10^5$ cells) exposure to eDCM and eEtAc crude extracts, $F3_{DCM}$, $F3_{EtAc}$ and compound 1 following 48 h of treatment. Cells exposed to DMSO (0.05% final concentration) were considered control.

Cytotoxicity study

Cell viability assay

Cell viability was evaluated by determination of the soluble 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) reduction by cell dehydrogenases (Mosmman et al., 1983). This method is based on the ability of viable cells to reduce MTT and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/mL) was added to the incubation medium in the wells at a final concentration of 0.5 mg/mL. Glioma cells and astrocytes were left for 90 min at 37°C in a humidified 5% CO_2 atmosphere. The medium was then removed and precipitate was eluted with DMSO. The optical density of each well was measured at 492 nm in a microplate reader (SpectraMAX 190). Results were expressed as percentage of control.

Cell proliferation assay

Sulforhodamine B (SRB) colorimetric assay was used for cell density and cytotoxicity determination, based on staining of total cell protein content with SRB dye (Pauwells et al., 2003). Briefly, cultures were washed and fixed with 50% trichloroacetic acid (w/v) for 30 min (4°C); cells were washed 5 times with dH_2O, stained with 0.4% SRB (w/v) for 45 min (RT) and washed 5 times with 1% acetic acid (v/v). Finally, SRB complexes were eluded in 10mM Tris buffer following by 15 min shaking. Absorbance was measured at 540 nm in a microplate reader (SpectraMAX 190). Results were expressed as percentage of control.

Clonogenic assay

Clonogenic assay is an *in vitro* cell survival method based on the capability of a single cell to grow into a colony, which can be used to determine the effectiveness of cytotoxic agents (Franken et al., 2006). Following 48 h treatment, C6 cells ($3x10^2$ cell/well) were seeded in 6-well plates and cultured for additional 10 days in absence of treatment. Then, cells were fixed with ice-cold methanol (100%) and stained with crystal violet 1% (w/v) to visualize colonies. Colonies were counted using microscope (40x) and length of colonies were determined by software ImageJ 1.51j8 (National Institutes of Health, USA).

Cell cycle analyses

Following 48 h of treatment, the medium and the cells were harvested and centrifuged (10 min; 1000 *g*). Supernatant was removed and cell pellet was washed once with PBS and fixed with 70% EtOH. After 2 h, cells were washed and incubated with staining solution (Triton X-100 1%, 2 mg/mL RNase, 2 mg/mL propidium iodide (PI) in PBS). After 30 min, data were collected using FACS Calibur Flow Cytometer (BD

Bioscience, Mountain View, CA, USA). Results were expressed as percentage of control.

Cell death analyses

Apoptotic or necrotic cells were quantified using annexin V-FITC- PI) double staining kit. Following 48 h of treatment, the medium and the cells were harvested and centrifuged (10 min at 2000 *g*). Cell pellet was washed twice with PBS and it was incubated (5 min, RT) with a biding buffer containing FITC-conjugated annexin V and PI, following manufacture instructions. Apoptotic and/or necrotic cells were quantified using FACS Calibur Flow Cytometer (BD Bioscience, Mountain View, CA, USA). Cells were classified as follows: viable cells ($Annexin^-/PI^-$), early apoptotic ($Annexin^+/PI^-$), late apoptotic ($Annexin^+/PI^+$) or necrotic cells ($Annexin^-/PI^+$). Results were expressed as percentage of control.

Oxidative stress parameters

Determination of reactive oxygen species (ROS)

Intracellular generation of ROS was determined by DCFH assay as described by Dos Santos and colleagues (2017). This method is based on the oxidation of 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to dichlorofluorescin (DCFH) by intracellular esterases, which is trapped within the cell. Thereby, DCF-DA reacts with ROS emitting fluorescence. In brief, following 48 h of treatment, cultures were incubated with 1 µM DCFH-DA for 30 minand fluorescence was measured at 488/525 nm in a microplate reader (SpectraMax M3). ROS production was reported as percentage of control.

Superoxide dismutase (SOD) activity

SOD activity was assayed as described by Misra and Fridovich (1972). This assay is based on the inhibition of superoxide-dependent adrenaline auto-oxidation to adenochrome. This reaction is intermediate by superoxide, which is scavenged by SOD. The absorbance was measured at 480 nm in a microplate reader (SpectraMax M3) and the results were expressed as percentage of control.

Catalase (CAT) activity

CAT activity was measured as described by Aebi (1984). This assay is based on the decomposition of 30 mM hydrogen peroxide (H_2O_2) in 50mM potassium phosphate buffer (pH 7.0) continuously monitored at 240 nm for 180 s at 37°C. CAT activity was reported as percentage of control.

Glutathione peroxidase (GPx) activity

GPx activity was measured using a commercial kit (RANSEL®; Randox Lab, Antrim, UK). This assay is based on oxidation of GPx catalyses glutathiose (GSH) by cumene hydroperoxide. In presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with concomitant

oxidation of NADPH to $NADP^+$. NADPH disappearance was measured at 340 nm and GPx activity was reported as percentage of control.

Total sulfhydryl content quantification

Total sulfhydryl content was determined according to Aksenov and Markesbery (2001). This process is based on the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, whose reaction form an oxidized disulfide generating a yellow derivative (TNB). The reaction was started by the addition of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). The absorbance was measured at 412 nm in a microplate reader (SpectraMax M3) and the results were expressed as percentage of control.

Statistical analysis

Statistical analysis was carried using GraphPad Prism 5 software. Data were expressed as mean ± standard error (SEM) and were subjected to analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparisons. Differences between mean values were considered significant when $P<0.05$.

3. Results

Isolation and identification of endophytic fungus

Here, endophytic fungus was isolated from stems of *A. satureioides* and named as MF31b11. Endophyte exhibited filamentous colonies with cottony aspect and regular edge, which color ranges from white to brown. Upon microculture analysis, septate conidiophores and cylindrical phialides were observed at microscope. However, structural characterization of the conidia and phialides difficulties did not allow morphological identification of the endophyte.

Endophytic fungus extracts selectively decrease glioma cell proliferation and viability

In order to evaluate whether MF31b11 exhibits antitumor activity, in the first set of experiments the liquid culture of isolated fungus was submitted to extraction with DCM and EtAc, which were chosen to isolate molecules with differential chemical properties. The resultant crude extracts (eDCM and eEtAc) were used to determine the citotoxicity of secondary metabolites produced by MF31b11. Rat C6 and human U87MG glioma cell lines were exposed to increasing concentrations of crude extracts for 24, 48 or 72 h. Extract concentrations applied were determined in previous experiments (data not shown) and ranged from 0.625-10 µg/mL and 12.5-200 µg/mL for eDCM and eEtAc, respectively. As shown in Figure 1, eDCM and eEtAc inhibited ~90% C6 (panels A and C) and U87MG (panels B and D) glioma cell proliferation following 72 h of treatment and the effect was time and concentration-dependent, as determined by SRB assay. Additionally, eDCM exhibited stronger antitumor activity when compared to eEtAc in both glioma cell lines as indicated by IC_{50} values, which ranged from 0.45-6.22 µg/mL and 33.59-129.2 µg/mL for eDCM and eEtAc, respectively (Fig. 1). The effect of crude extracts on GBM cell viability was also evaluated by MTT assay (Fig. 2). Results show that both extracts decreased glioma viability in a time-dependent manner and eDCM

was more cytotoxic than eEtAc (Fig. 2, panels A-D), confirming the previous data (Fig. 1). Notably, eDCM and eEtAc did not alter normal astrocyte cell culture viability (Fig. 2, panels E and F), indicating selective effect against tumor cells.

3.3 Fractionated extracts exhibit selective antiglioma effect

To better investigate which molecule(s) were involved in eDCM and eEtAc antiglioma activity, crude extracts were fractioned as described in material and methods and its citotoxicity was determined in C6 and U87MG glioma cells by SRB and MTT assays as above. From 10 obtained fractions, 6 identified as $F1_{DCM}$, $F2_{DCM}$, $F3_{DCM}$, $F4_{DCM}$, $F3_{EtAc}$ and $F4_{EtAc}$ showed significant cytotoxic activity against both glioma cell lines (Fig. 3). The variability of antitumor effect could be observed by IC_{50} values, which ranged from 0.95 to 128.4 µg/mL and may be related to differential chemical properties exhibited by these fractions, as expected. Citotoxicity analyses were also performed using MTT assay at the same experimental conditions (Fig. 4). In a general way, cell viability results and IC_{50} values obtained were in according to antitumor potential exhibited by these fractions. Interestingly, although the antiglioma effect exerted by fractions, no alteration was observed in primary astrocyte cell viability after 48 h of exposure (Fig. 4; panel G), reinforcing selective antiglioma activity.

Extracts and fractions of endophytic fungus decrease glioma cell colony formation

Clonogenic assay was employed to determine the reproductive cell death and effectiveness of cytotoxic agents (Franken et al., 2006). C6 cells were treated with crude extract or fractions at concentration corresponding to IC_{50} and colony length and formation was determined as described in material and methods. eDCM and eEtAc crude extracts, $F2_{DCM}$, $F3_{DCM}$ and $F3_{EtAc}$ fractions decreased in 83%, 76%, 88%, 99% and 58%, respectively, C6 glioma colony formation when compared to control (Fig. 5; panels A-K). Colony length was also decreased by 59%, 46%, 37%, 59%, 52%, 49% and 33% following exposure to eDCM, eEtAc, $F1_{DCM}$, $F3_{DCM}$, $F4_{DCM}$, $F3_{EtAc}$ and $F4_{EtAc}$, respectively (Fig. 5; panels L and M). Taken together, data obtained from clonogenic, proliferation and cell viability analyses point $F3_{DCM}$ as the most effective antiglioma fraction when compared to the others. Therefore, further experiments of phytochemical analysis of this fraction were performed.

Structural elucidation of compound 1

^{1}H-NMR (MeOD) of molecule isolated from $F3_{DCM}$ (Table 1) exhibited signals in (1H, d, H-3) and 5.96 (1H, dd, *J* = 10 Hz, H-2), which were characteristics of oleofinic hydrogens. Signals in δ 5.05 (1H, m, H-11), 4.22 (1H, t, *J* = Hz, H-4) were related to hydrogens of oxygenated carbon. Moreover, eight signals in the region of δ 2.83 -1.28 were attributed to alkyl hydrogen. The ^{13}C NMR spectrum (Table 1) showed fourteen spectral lines, of which one was consistent with ketone carbonyl (δ 202.5, C-1) and one with ester carbonyl (δ 173.8, C-12). Signals in δ 149.5 (C-12) and δ 130.7 (C-2) were associated to olefinic carbons, while signals in δ 74.8 (C-11) and δ 67.2 (C-4) were

attributed to oxygenated sp^3 carbons. The eight remaining signals were related to alkyl carbons (Table 1).

The edited NMR-^{1}H-^{13}CHSQC of compound 1revealed the correlations of the hydrogenated carbons and allowed to differentiate the CH and CH_3 signals from CH_2. Thus, 6 methylene carbons, 5 methylene carbons, 1 methyl carbon and 2 non-hydrogenated carbons (carbonyls) were identified. The couplings observed in the COZY (Table 1) confirmed the vicinal and geminal hydrogens of the alicyclic carbon chain.

The high resolution mass spectrum (Fig. 6) exhibited ion $[M+H]^+$ with *m/z* 251.1885 and $[M-H]^-$ with m/z 253.1435 consistent with the molecular formula $C_{14}H_{120}O_{14}$ and therefore corresponding to a structure with a hydrogen-deficiency index (HDI) of 5, suggesting an hydroquinone bicyclic lactonic.

NMR-^{1}H-^{13}CHMBC spectrum (Table 1) allowed the characterization of the hydroquinone through the following correlations to two and three bonds: signals of olefinic hydrogens in δ_H 7.03 and 5.96 (H-3 and 2) with the carbonyl at δ_C 200.3 (C-1) and the carbinolic carbon in δ_C 67.2 (C-4). Lactone was characterized by the coupling of aliphatic hydrogen in δ_H 2.68 (H-11) and hydrogen bonded to oxygenated carbon at 5.05 (H-11) with the carbonyl in δ_C 173.8 (C-12). The hydroquinone and lactone ring junctions were confirmed by the coupling of the methinic hydrogens at δ_H 2.83 (H-5) and 2.66 (H-6) with the methilene carbons at δ_C 39.9 (C-13) and 24.3 (C-7), respectively, as well as in the carbonylacetone at δ_C 200.3 (C-1). Finally, comparison of ^{1}H and ^{13}C NMR data with those in the literature confirmed the chemical structure of compound 1 (Fig. 6, panel C) (Adelin et al., 2011a), which was characterized as (4S,8aR,12S,12aR)-12-hydroxy-4-methyil-4,5,6,7,8,8a,12,12a-octahydro-1H-3-benzo-xecine-2,9-dione (IUPAC name), known in the literature as Sch-642305.

Sch-642305 promoted antiproliferative effect and decreased cell glioma colony formation

Sch-642305 showed antiproliferative activity against C6 glioma exhibiting IC_{50} values of 1.1 and 3.4 µg/mL (5 and 15.5 µM) following 48 h of exposure, as determined by SRB and MTT assays, respectively (Fig. 7; panels A and B). However, Sch-642305 not alter normal astrocyte cell culture viability (Fig. 7, panel C), suggesting selective effect against C6 glioma cells. Additionally, Sch-642305 (1 µg/mL or 5 µM) decreased C6 glioma colony formation and length by 70% and 58%, respectively (Fig. 7, panels D-H). Moreover, the cytotoxic activity of $F3_{DCM}$ fraction without the presence of the lactone, named as 'supernatant $F3_{DCM}$' (SN $F3_{DCM}$) was also investigated. SN $F3_{DCM}$ exhibited antiproliferative effect against C6 glioma cells with IC_{50} of 2.0 µg/mL for SRB assay and 5.6 µg/mL for MTT test, following 48 h of treatment. Futhermore, SN $F3_{DCM}$ (1 µg/mL) reduced C6 glioma colony formation and length at 45% and 37%, respectively when compared to control. On the other hand, the exposure of C6 glioma cells to the association of the lactone with the SN $F3_{DCM}$ showed values of IC_{50} of 1.6 and 3.5 µg/mL when determined by SRB and MTT assays, respectively (Fig. 7, panels A and B). In this context, these results indicate that Sch-642305 contributed significantly to

antitumor activity exhibited by $F3_{DCM}$ fraction. Therefore, it becomes interesting to elucidate the mechanisms involved in this effect.

The effect of extracts and fractions of endophytic fungus on cell cycle distribution and cell death

To better understand the antiproliferative effect mediated by crude extracts as well as the most effective fraction of eEtAc ($F3_{EtAc}$) and Sch-642305 compound from $F3_{DCM}$, cell cycle and cell death analyses were performed in C6 glioma following 48 h of treatment. Analysis of cell cycle distribution evidenced that eDCM (2.5 µg/mL), eEtAc (50 µg/mL) and $F3_{EtAc}$ (25 µg/mL) lead to accumulation of cells in the G2/M phase of the cell cycle (12, 1, 14%, respectively) and the formation of sub-G1 apoptotic cells (18, 34, and 10%, respectively; Fig. 8), which are in accordance to apoptosis and late apoptosis rates observed in these cells by annexin V-PI staining (33/17%; 32/18%; 17/30%, respectively; Fig. 9). The isolated molecule, Sch-642305 (1 µg/mL) induced a cell blockage in G2/M phase (7%; Fig. 8, panels E and F) and ~10% of apoptosis/late apoptosis in C6 cells (Fig. 9, panels E and F). These results suggest that the induction of C6 cell cycle blockage and apoptosis by extracts, fractions and Sch-642305 account at least in part for its antiglioma activity.

Extracts and fractions of endophytic fungus alters oxidative stress parameters in glioma cells

Since alterations in ROS parameters are related to cancer progression (Ciccarese; Ciminale, 2017), redox potential of eDCM and eEtAc crude extracts and its respective more effective fractions was further evaluated in glioma cells. Notably, crude extracts increased in a concentration-dependent fashion antioxidant enzyme activities, namely SOD, CAT and GPx by 155%, 200% and 55%, respectively, after treatment with eDCM (10 µg/mL) and by 485%, 270% and 255%, respectively, following treatment with eEtAc (200 µg/mL) when compared to control (Fig.10, panels A-F). Additionally, eDCM and eEtAc induced an increase in total sulfhidryl (SH) content by 160% (10 µg/mL) and 80% (200 µg/mL), respectively (Fig. 10, panels G-H). In accordance to antioxidant activity of both extracts, ROS content was also decreased in a concentration-dependent manner when compared to control (Fig. 10, panels I-J).

$F3_{DCM}$ fraction accounts for significant antioxidant activity exhibited by eDCM crude extract. As shown in Figure 11, $F3_{DCM}$ increased by 300, 500, 240 and 300% SOD, CAT, Gpx activities and SH content, respectively, when compared to control (Fig. 11, panels A, C, E and G). In parallel, ROS content was decreased in a concentration-dependent manner (Fig. 11, panel I). By other hand, $F3_{EtAc}$ promoted a modest effect on redox parameters when compared to eEtAc, increasing in 60, 270 and 86% SOD and CAT activities and ROS content, respectively, when compared to control (Fig. 11, panels B, D and J).

Finally, data revealed a promising redox potential of Sch-642305, which was isolated from $F3_{DCM}$ and accounts for ~30, 50 and 90% of SOD, CAT and ROS scavenger activities, respectively, exhibited by such fraction (Fig. 12, panels A, B and D). However, no statistical difference was observed on GPx activity (Fig. 12, panel C).

These results show that bioactive metabolites produced by endophytic fungus modulate redox status of glioma cells which in turn, may affect cell cycle and death pathways, resulting in inhibition of glioma progression.

4. Discussion

Natural products have played an important role in the development of therapeutic agents. Recent scientific reports have revealed hundreds of secondary metabolites produced from symbiotic microorganisms, especially endophytic fungi, which stand out their pharmacologic properties, especially antitumor activity (Koul et al., 2016; Uesugi et al., 2016; Wang et al., 2017). These microorganisms have been recognized as promising source of bioactive compounds that may act to inhibit or regulate the proliferation and cell cycle, being valuable in anticancer drugs discovery (Chandra, 2012; Koul et al., 2016; Fatima et al., 2016). Although several medicinal plant species are recognize to harbor many endophytic fungi, there are no reports describing the endophytic composition of *A. satureioides*.

In the present study, we described the isolation of endophytic fungus from *A. satureioides* and explored the cytotoxic activity against glioma cells lines. Endophyte isolated was named as MF31b11. Since an endophyte can produce a complex mixture of compounds, it becomes interesting to extract bioactive compounds using organic solvents in increasing order of polarity (Seidel, 2012; Zin et al., 2017). Overall, microbial metabolites released into the liquid culture medium by the endophytic fungus are often extracted with DCM and EtAc (Seidel, 2012; Yadav et al., 2014; Jinfeng et al., 2017). In this study, DCM and EtAc were employed as solvents of lower and higher polarity, respectively, to obtain the secondary metabolite compounds produced by MF31b11.

Initially, we report antiglioma activity of DCM and EtAc crude extracts of endophyte isolated. The eDCM showed very promising antitumor activity with lowest IC_{50} of 4.0 µg/mL against rat C6 and human U87MG glioblastoma cell lines. This effect suggest that metabolites with antiglioma properties, produced by the endophytic fungus isolated, present low polarity. In contrast, both extracts did not promote citotoxicity to primary astrocyte cultures. Recently, endophytic fungi have been shown to produce a plethora of new compounds (Li et al., 2014; Jia et al., 2016). These bioactive metabolites are interesting to development of new medicines. Therefore, to understand the selective antiglioma effect of both crude extracts, we performed a fractionation. $F3_{EtAc}$ and $F3_{DCM}$ fractions showed interesting cytotoxic effects with lower IC_{50} values 2 µg/mL and 71 µg/mL, respectively. In addition, fractions induced significant inhibition of cell growth by clonogenic test, such as crude extracts. Clonogenic cell survival assay determines cytostatic effects of a cytotoxic agent, by measuring the proliferative ability of a single cell to form a clone and produce a viable colony (Franken et al., 2006; Sumantran, 2011), therefore the size and the number of colonies represent indicators of the cell reproductive death (Miyashita et al., 2017).

Cell division, differentiation and death are controlled by several mechanisms ensuring tissue homeostasis (Hanahan; Weinberg, 2011; Wiman; Zhivotovsky, 2017). Cell cycle involves regulation of DNA structure checkpoints, which arrest the cell cycle at the

different phases in response to DNA damage or incomplete replication (Bertoli et al., 2013). Thus, deregulation of the cell cycle induces aberrant cell proliferation characteristic of cancer and loss of cell cycle checkpoint control promotes genetic instability (Williams; Stoeber, 2011). In this context, the cell cycle machinery represents an alternative target for diagnostic and therapeutic interventions (Williams; Stoeber, 2011). In present study, the cell cycle analysis of cells treated with eDCM (IC_{50} 2.5 µg/mL) and eEtAc (IC_{50} 50 µg/mL) crude extracts exhibited significant increase of apoptotic cell population on sub-G1 phase and decrease of cells on G1 stage. Apoptosis is characterized by several morphological changes, which include cell shrinkage, chromatin condensation, and nuclear fragmentation (Walker et al., 2010). Moreover, this process of programmed cell death has been recognized as one of the major causes that mediate inhibition of cell proliferation and may be therapeutically exploited for cancer treatment (Bai; Wang, 2014, Wang et al., 2017). Several studies have been reported that accumulation of cells in sub-G1 phase indicates cell death by apoptosis characterized by DNA fragmentation (Ahmed et al., 2013, Vessoni et al., 2016, Agrawal et al., 2017). By other hand, $F3_{EtAc}$ fraction induced late apoptosis on glioma cells with increase of cells on G2/M phase and a corresponding decrease of cells in G1 stage. The G2/M checkpoint is a known target for cell cycle inhibition (Newell et al., 2017). Evidences suggest that numerous anticancer metabolites from endophytic fungi induce apoptotic death and cell cycle arrest at the G2/M phase (Wang et al., 2015, Pathania et al., 2015, Wang et al., 2017).

Based on promising activity of $F3_{DCM}$ fraction the molecule identified by LC-MS analysis as Sch-642305, a 10-membered macrolide which showed anti-proliferative properties against C6 glioma cells, with lower IC50 of 1 µg/mL (5 µM). This molecule induced cell death mainly by apoptosis, accumulation of cells on G2/M stage, promoting alterations on a reproductive ability cells by inhibiting colony formation. Sch-642305 was first isolated from *Penicillium verrucosum* as a bacterial DNA primase inhibitor (Chu et al., 2003). Moreover, this lactone is described as the major compound produced by the endophytic fungus *Phomopsis* sp. with interesting cytotoxic activity against human colorectal carcinoma (HCT-116), human breast adenocarcinoma (MDA-MB-231) and human myelogenous leukemia (K562) (Adelin et al., 2011a and 2011b). However, there are no reports about the elucidation of its antiglioma activity and its antiproliferative mechanisms.

Several hallmarks of cancer, such as genomic instability, resistance to apoptosis, uncontrolled proliferation and angiogenesis are promoted by the increased ROS levels commonly found in tumor cells (Ciccarese; Ciminale, 2017, Morry et al., 2017). GBM is characterized by high quantities of ROS into the cells, as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), favoring oxidative environment, cell damage and invasiveness of glioma cells (Feng et al., 2016, Salazar-Ramiro et al., 2016). We found that Sch-642305 inhibited oxidative stress, evidenced by increased SOD and CAT enzymes activity and suppression of ROS production. High levels of antioxidant enzymes are required to remove high levels of free radicals to protect against damage to brain tissues (Martínez-Martos et al., 2014). SOD is the first line of enzymatic antioxidant defense which metabolizes $O_2^{\bullet-}$ to H_2O_2 and water (Zhou et al., 2011;

Schieber; Chandel, 2014). However, H_2O_2 when accumulated into the cells and tissue is highly toxic. In order to prevent this phenomenon, CAT acts in the detoxification of H_2O_2 and consequently reducing the damage induced by free radicals (Ighodaro; Akinloye, 2017). In addition, Sch-642305 promoted increase of sulfhydryl content on glioma cells. This group of thiols consist an important non-enzymatic defense system which playing a critical role in oxidative stress, apoptosis, detoxification and cellular signal transmission, and in enzymatic activities (Ergin et al., 2016; Şimşek et al., 2016). These results suggest that antiglioma effect of lactone isolated is due to reduction of ROS levels while increasing antioxidant defense.

Furthermore, eDCM and eEtAc also reduce oxidative damages against glioma cells due to increase of SOD, CAT and GPx antioxidant enzymes consequently, decrease of ROS production. GPx is an important intracellular enzyme that breakdown H_2O_2 to water, playing a crucial role of inhibiting lipid peroxidation process, thereby protecting cells from oxidative stress (Ighodaro; Akinloye, 2017). Thus, these enzymes work in conjunction and therefore could be useful to determine the antioxidant status of several compounds (Lowe, 2014; Del Bó et al., 2015). Similar these results, $F3_{DCM}$ and $F3_{EtAc}$ fractions also showed antioxidant activity against glioma cells. Our study also reveals that the synergism between Sch-642305 and others compounds present in $F3_{DCM}$ fraction induces changes in oxidative parameters like increases enzymatic and non-enzymatic antioxidant defense as well as suppress ROS generation. However, it is important to highlight that the Sch-642305 played an important role to antiproliferative activity and modulation of redox status against glioma cells exhibited by $F3_{DCM}$ fraction.

5. Conclusion

The current study is the first reporting about isolation of endophytic fungus from *A. satureioides*. Crude extracts showed cytotoxic activity against glioma cells by inducing apoptosis. Furthermore, $F3_{DCM}$ and $F3_{EtAc}$ fractions exhibited significant selective antiglioma activity and modulated cell redox status. In addition, our data describe structural identification and cytotoxicity of Sch-642305 from $F3_{DCM}$ fraction, which showed promising chemotherapeutic potential. Sch-642305 promoted cell death by apoptosis, enhanced antioxidant defense system and suppressed ROS production. Hence, our study highlights the role of Sch-642305 as a possible therapy for gliomas as well as the importance of endophyte in novel anti-cancer drug discovery, encouraging research in this field.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

7. Author contribution

NP: Wrote the paper, endophytic fungus manipulation, cell culture, cytotoxicity and oxidative stress experiments.

KG: Manipulation of endophytic fungus and biomass production.

DS, WC: Production of organic crude extracts.

PTR: Cell culture and cytotoxicity experiments of crude and fractionated extracts.
NB: Cell culture and cytotoxicity experiments of fractions, cell cycle, cell death and clonogenic analysis.
JA: Cell culture and cell cycle analysis.
KC: Chemical analysis of extracts and molecule elucidation.
AS, ED, PVR: Chemical analysis and production of fractionated extracts.
MS, RS, FS: Oxidative stress experiments.
EB: Advisor, writing, cell culture and cytotoxicity experiments.

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10. Legends to Figures

Figure 1. Comparative effect of eDCM and eEtAc on glioma cell proliferation. C6 (left panel) and U87MG (right panel) glioma cell lines were exposed to increasing concentrations of eDCM **(A, B)** and eEtAc **(C, D)**. Cell proliferation was determined by SRB assay following 24, 48 or 72 h of treatment, as indicated. Values represent the mean ± SEM from at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). *, **, ***Significantly different from control cells ($P<0.05$, $P<0.01$, and $P<0.001$, respectively).

Figure 2. Comparative effect of eDCM and eEtAc on glioma cell viability. C6 (left panel) and U87MG (right panel) glioma cell lines were exposed to increasing concentrations of eDCM **(A, B)** and eEtAc **(C, D)** for 24, 48 or 72 h of treatment, as indicated. In addition, primary astrocyte cultures were exposed to higher concentrations of crude extracts for 48 h **(E)** and 72 h **(F)**. Cell viability was determined by MTT assay. The values represent the mean ± SEM from at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). *, ***Significantly different from control cells (P<0.05 and P<0.001, respectively).

Figure 3. Comparative effect of fractionated extracts from eDCM and eEtAc on glioma cell proliferation. Glioma cell lines were exposed to increasing concentrations of $F1_{DCM}$, $F2_{DCM}$, $F3_{DCM}$, $F4_{DCM}$, $F3_{EtAc}$ and $F4_{EtAc}$ fractions **(A-F, respectively)**. Cell proliferation was determined by SRB assay following 48 h of treatment. The values represent the mean ± SEM from at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). [a,b] Significantly different from C6 and U87MG control cells, respectively (P<0.001).

Figure 4. Comparative effect of fractionated extracts from eDCM and eEtAc on glioma and astrocyte cell viability. Glioma cell lines were exposed to increasing concentrations of $F1_{DCM}$, $F2_{DCM}$, $F3_{DCM}$, $F4_{DCM}$, $F3_{EtAc}$ and $F4_{EtAc}$ fractions **(A-F, respectively)**. In addition, primary astrocyte cultures **(G)** were exposed to the higher concentration of $F1_{DCM}$ (10 µg/mL), $F2_{DCM}$ (75 µg/mL), $F3_{DCM}$ (10 µg/mL), $F4_{DCM}$ (10 µg/mL), $F3_{EtAc}$ (100 µg/mL) and $F4_{EtAc}$ (100 µg/mL) fractions. Cell viability was determined by MTT test after 48 h of treatment. The values represent the mean ± SEM from at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). [a,b] Significantly different from C6 and U87 control cells, respectively (P<0.001).

Figure 5. Analysis of crude organic and fractionated extracts effect on C6 glioma colony formation. Glioma cells were exposed to eDCM (2.5 µg/mL), eEtAc (50 µg/mL) crude extracts and $F1_{DCM}$ (7.5 µg/mL), $F2_{DCM}$ (25 µg/mL), $F3_{DCM}$ (1 µg/mL), $F4_{DCM}$ (10 µg/mL), $F3_{EtAc}$ (25 µg/mL) and $F4_{EtAc}$ (100 µg/mL) fractions at concentrations close to IC_{50} value **(A-I)**. Colony number **(J, K)** and colony length **(L, M)** were determined by clonogenic assay following 48 h of treatment. Values represent the mean ± SEM from at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). ***Significant different from control cells (P<0.001).

Figure 6. High resolution mass spectrum of compound 1, obtained in negative **(A)** and positive **(B)** ionization modes. **(C)** Structure of characterized molecule Sch-642305.

Figure 7. Analysis of Sch-642305, SN $F3_{DCM}$ and association SN $F3_{DCM}$+Sch-642305 effect cytotoxicity (C6 and astrocyte) and colony formation (C6 glioma). Cytotoxic activity of C6 glioma cells was determined by SRB **(A)** and MTT assays **(B)**. Cell viability of primary astrocyte culture was measured by MTT **(C).** Colony number and colony length were determined by clonogenic assay following 48 h of treatment **(D-H)**. Values represent the mean ± SEM from at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). **, ***Significantly different from control cells (P<0.01 and P<0.001, respectively). [a], [b], [c], [d] Significantly different from $F3_{DCM}$, Sch-642305, SN $F3_{DCM}$ and SN $F3_{DCM}$ + Sch-642305 control cells, respectively (P<0.001).

Figure 8. Analysis of crude extracts, fractions and Sch-642305 effect on C6 glioma cell cycle. C6 glioma cells exposed to eDCM (1 µg/mL), eEtAc (50 µg/mL), $F3_{EtAc}$ (25 µg/mL) and Sch-642305 (1 µg/mL). Cells were treated for 48 h and subsequently analyzed by flow cytometry using PI staining to determine the distribution of cells on cell cycle. The percentage of cells on sub-G1, G1, S and G2/M phases were shown on histogram as H1, H2, H3 and H4, respectively **(A-E)** and in the graph **(F)**.

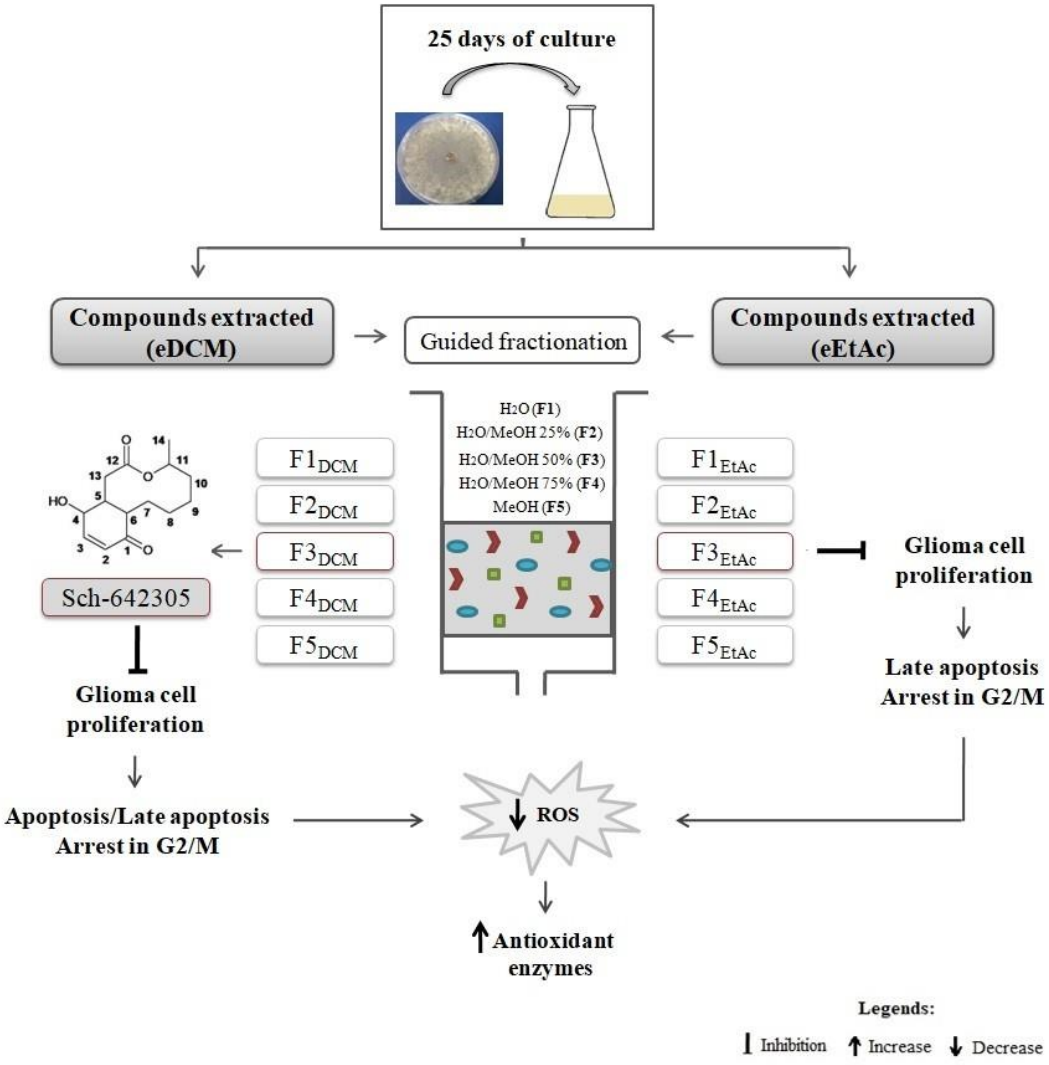
Figure 9. Analysis of crude extracts, fractions and Sch-642305 effect on C6 glioma cell death. C6 glioma was exposed to eDCM (1 µg/mL), eEtAc (50 µg/mL), $F3_{EtAc}$ (25 µg/mL) and Sch-642305 (1 µg/mL). Cells were treated for 48 h and subsequently analyzed by flow cytometry using annexin V/PI double staining to determine cell death by apoptosis, late apoptosis or necrosis. **(A-E)** The percentage of viable cells is represented by the lower left quadrant; the percentage of apoptosis is represented by the lower right, the percentage of late apoptosis is represented by the upper right and the percentage of necrosis is represented by the upper left; **(F)** representative graph of cell death analysis.

Figure 10. Analysis of oxidative stress parameters in C6 glioma cells exposed to eDCM (left panel) and eEtAc (right panel) crude extracts. Analyses were performed after 48 h of treatment. **(A, B)** Superoxide dismutase (SOD) activity; **(C, D)** catalase (CAT) activity; **(E, F)** glutathione peroxidase (GPx) activity; **(G, H)** total sulfhydryl content and **(I, J)** ROS production. Values represent mean ± SEM of at least three independent experiments. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). *, **, ***Significantly different from control cells (P<0.05, P<0.01 and P<0.001, respectively).

Figure 11. Analysis of oxidative stress parameters in C6 glioma cells exposed to $F3_{DCM}$ (left panel) and $F3_{EtAc}$ (right panel) fractions. Analyses were performed after 48 h of treatment. **(A, B)** Superoxide dismutase (SOD) activity; **(C, D)** catalase (CAT) activity; **(E, F)** glutathione peroxidase (GPx) activity; **(G, H)** total sulfhydryl content and **(I, J)** ROS production. Values represent mean ± SEM of at least three independent experiments. Data were analyzed by ANOVA followed by *post-hoc* comparisons

(Tukey test). *, **, ***Significantly different from control cells (P<0.05, P<0.01 and P<0.001, respectively).

Figure 12. Analysis of oxidative stress parameters in C6 glioma cells exposed to Sch-642305 after 48 h of treatment. **(A)** Superoxide dismutase (SOD) activity; **(B)** catalase (CAT) activity; **(C)** glutathione peroxidase (GPx) activity **(D)** total sulfhydryl content and **(E)** ROS production. Values represent mean ± SEM of at least three independent experiments. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). *, **, ***Significantly different from control cells (P<0.05, P<0.01 and P<0.001, respectively).



Graph abstract

Table 1. ^{1}H and ^{13}C NMR data (uni and bidimensional) of Sch-642305 (MeOD).

#C	**δC 1**	**δH 1**	**HMBC**	**COSY**
C=O	202.5		7.03; 2.66	
C=O	173.8		2.68; 2.54	
=CH	149.5	7.03 (1H. dd)	4.22	5.96; 4.22
=CH	130.7	5.96 (1H.d. 10Hz))	4.22	7.03
CH	74.8	5.05 (1H. m)	2.54; 1.84	1.28
CH	67.2	4.22 (t	7.03; 5.96; 2.54; 1.28; 1.40	7.03
CH	47.8	2.66	5.96; 4.22; 2.68; 2.54; *2.17; 1.09*	
CH_2	39.9	2.68; 2.54	2.83	2.83
CH	37.9	2.83 (t	7.03; 2.68; 2.54; 2.17	2.68; 2.54
CH_2	30.5	1.40 (m	1.28	2.07
CH_2	24.3	2.17 (m; 1.09 (t)	2.66; 1.40; 1.09	2.16; 1.56
CH_2	24.2	1.84; 1.24	5.05	1.84; 1.24
CH_2	22.8	1.56; 1.34	2.66; 1.84; 1.40; 1.09	1.09
CH_3	18.7	1.28 (6H. d)		

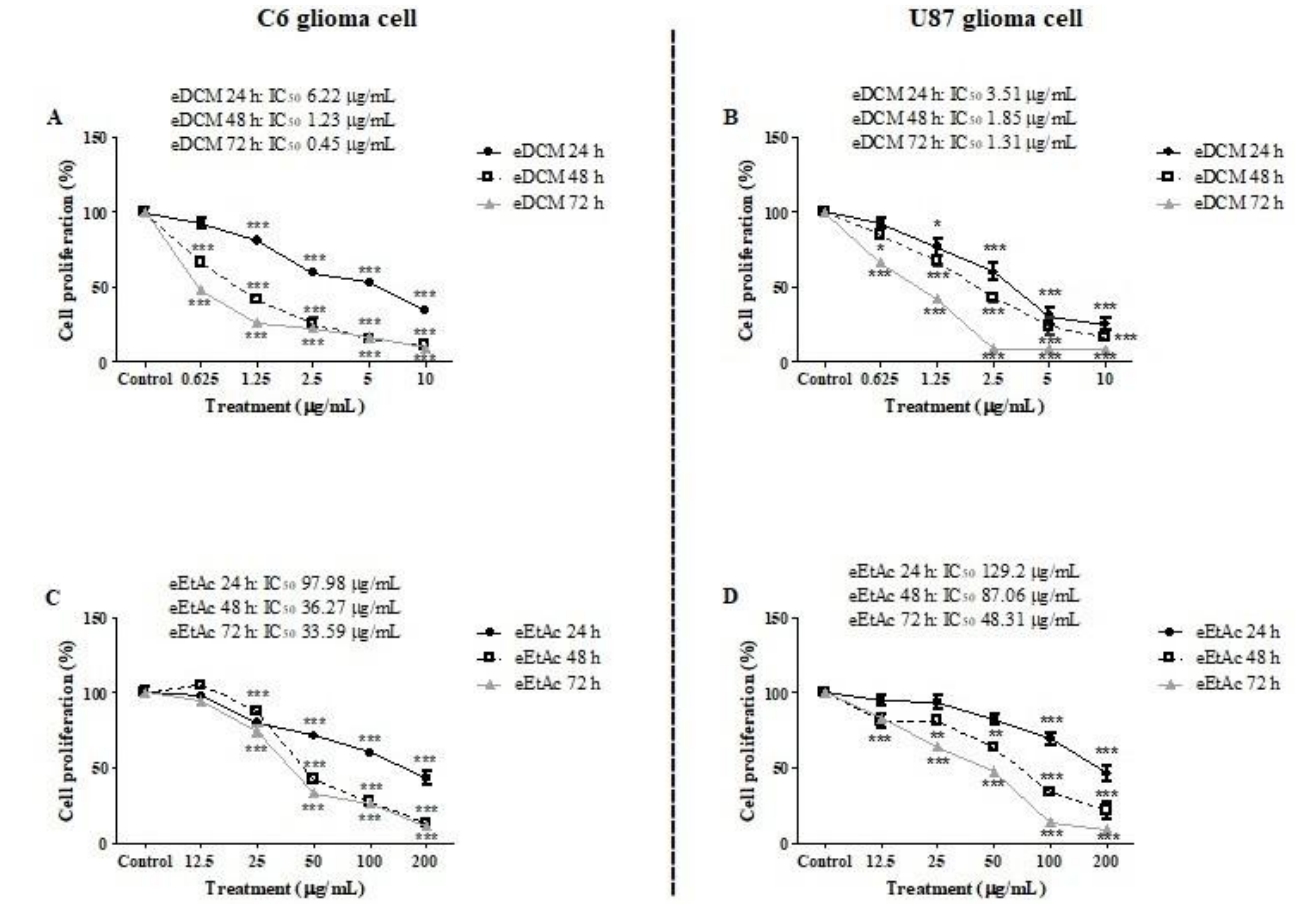

C6 glioma cell
U87 glioma cell
A
eDCM 24 h: IC50 6.22 µg/mL
eDCM 48 h: IC50 1.23 µg/mL
eDCM 72 h: IC50 0.45 µg/mL
B
eDCM 24 h: IC50 3.51 µg/mL
eDCM 48 h: IC50 1.85 µg/mL
eDCM 72 h: IC50 1.31 µg/mL
C
eEtAc 24 h: IC50 97.98 µg/mL
eEtAc 48 h: IC50 36.27 µg/mL
eEtAc 72 h: IC50 33.59 µg/mL
D
eEtAc 24 h: IC50 129.2 µg/mL
eEtAc 48 h: IC50 87.06 µg/mL
eEtAc 72 h: IC50 48.31 µg/mL
Cell proliferation (%)
Treatment (µg/mL)
Control
0.625
1.25
2.5
5
10
12.5
25
50
100
200
eDCM 24 h
eDCM 48 h
eDCM 72 h
eEtAc 24 h
eEtAc 48 h
eEtAc 72 h

Figure 1

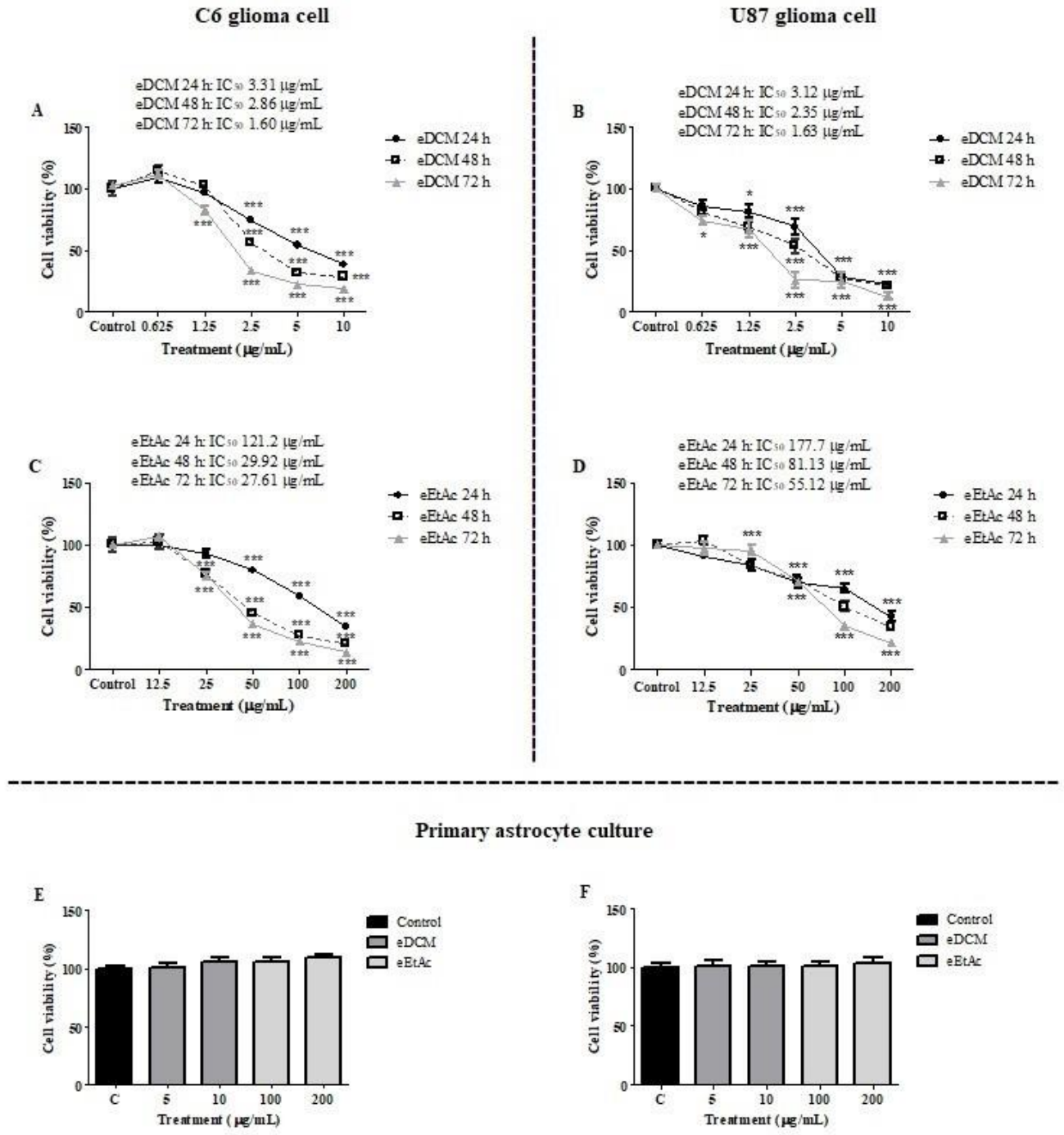

C6 glioma cell
U87 glioma cell
A
eDCM 24 h: IC50 3.31 µg/mL
eDCM 48 h: IC50 2.86 µg/mL
eDCM 72 h: IC50 1.60 µg/mL
B
eDCM 24 h: IC50 3.12 µg/mL
eDCM 48 h: IC50 2.35 µg/mL
eDCM 72 h: IC50 1.63 µg/mL
C
eEtAc 24 h: IC50 121.2 µg/mL
eEtAc 48 h: IC50 29.92 µg/mL
eEtAc 72 h: IC50 27.61 µg/mL
D
eEtAc 24 h: IC50 177.7 µg/mL
eEtAc 48 h: IC50 81.13 µg/mL
eEtAc 72 h: IC50 55.12 µg/mL
eDCM 24 h
eDCM 48 h
eDCM 72 h
eEtAc 24 h
eEtAc 48 h
eEtAc 72 h
Cell viability (%)
Control 0.625 1.25 2.5 5 10
Control 12.5 25 50 100 200
Treatment (µg/mL)
Primary astrocyte culture
E
F
Control
eDCM
eEtAc
C 5 10 100 200
Treatment (µg/mL)

Figure 2

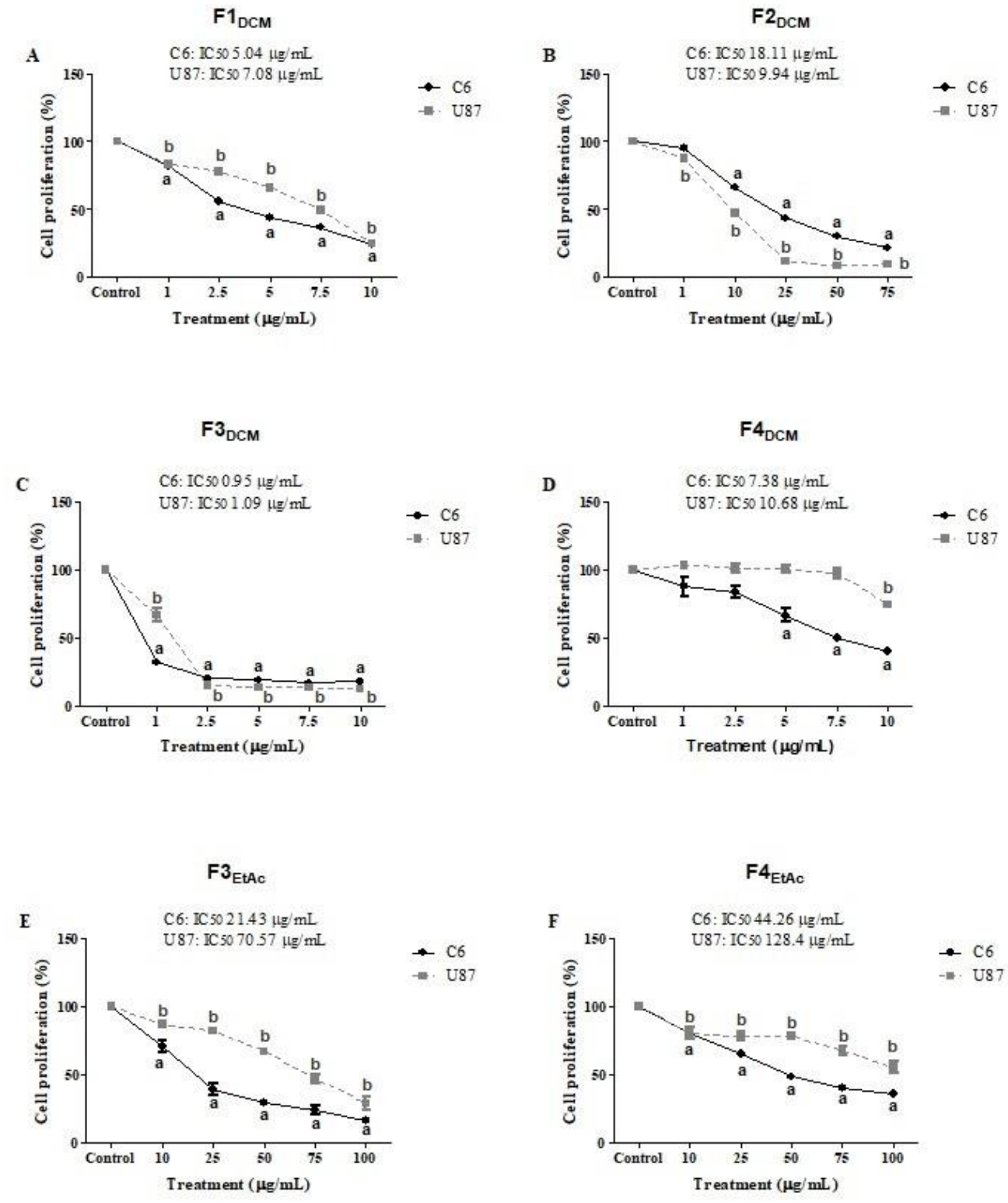

F1DCM
A
C6: IC50 5.04 µg/mL
U87: IC50 7.08 µg/mL
C6
U87
Cell proliferation (%)
Control 1 2.5 5 7.5 10
Treatment (µg/mL)
F2DCM
B
C6: IC50 18.11 µg/mL
U87: IC50 9.94 µg/mL
C6
U87
Cell proliferation (%)
Control 1 10 25 50 75
Treatment (µg/mL)
F3DCM
C
C6: IC50 0.95 µg/mL
U87: IC50 1.09 µg/mL
C6
U87
Cell proliferation (%)
Control 1 2.5 5 7.5 10
Treatment (µg/mL)
F4DCM
D
C6: IC50 7.38 µg/mL
U87: IC50 10.68 µg/mL
C6
U87
Cell proliferation (%)
Control 1 2.5 5 7.5 10
Treatment (µg/mL)
F3EtAc
E
C6: IC50 21.43 µg/mL
U87: IC50 70.57 µg/mL
C6
U87
Cell proliferation (%)
Control 10 25 50 75 100
Treatment (µg/mL)
F4EtAc
F
C6: IC50 44.26 µg/mL
U87: IC50 128.4 µg/mL
C6
U87
Cell proliferation (%)
Control 10 25 50 75 100
Treatment (µg/mL)

Figure 3

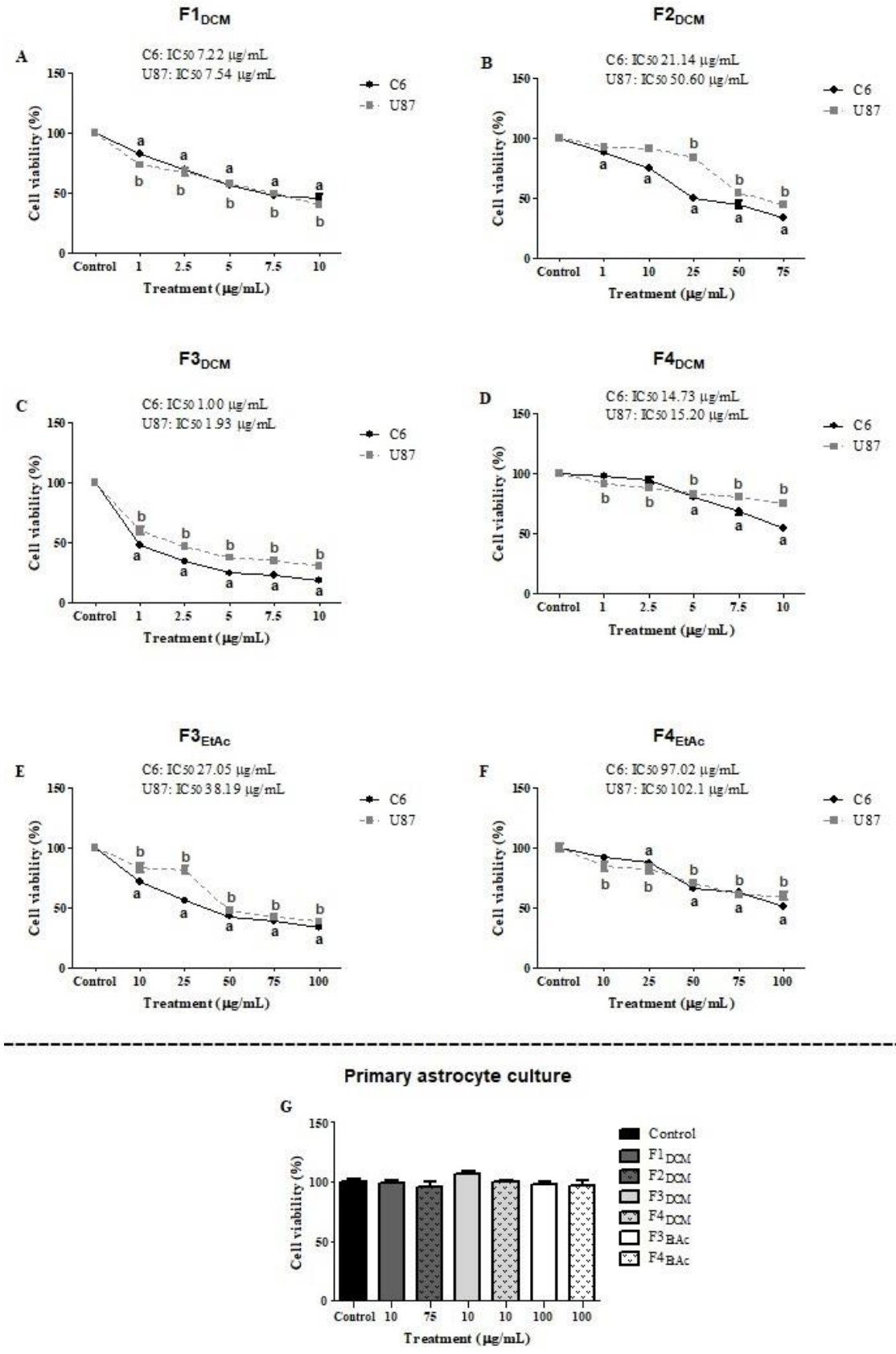

F1DCM
A
C6: IC50 7.22 µg/mL
U87: IC50 7.54 µg/mL
C6
U87
Cell viability (%)
Control 1 2.5 5 7.5 10
Treatment (µg/mL)
F2DCM
B
C6: IC50 21.14 µg/mL
U87: IC50 50.60 µg/mL
Control 1 10 25 50 75
F3DCM
C
C6: IC50 1.00 µg/mL
U87: IC50 1.93 µg/mL
F4DCM
D
C6: IC50 14.73 µg/mL
U87: IC50 15.20 µg/mL
F3EtAc
E
C6: IC50 27.05 µg/mL
U87: IC50 38.19 µg/mL
Control 10 25 50 75 100
F4EtAc
F
C6: IC50 97.02 µg/mL
U87: IC50 102.1 µg/mL
Primary astrocyte culture
G
Control
F1DCM
F2DCM
F3DCM
F4DCM
F3BAc
F4BAc
Control 10 75 10 10 100 100

Figure 4

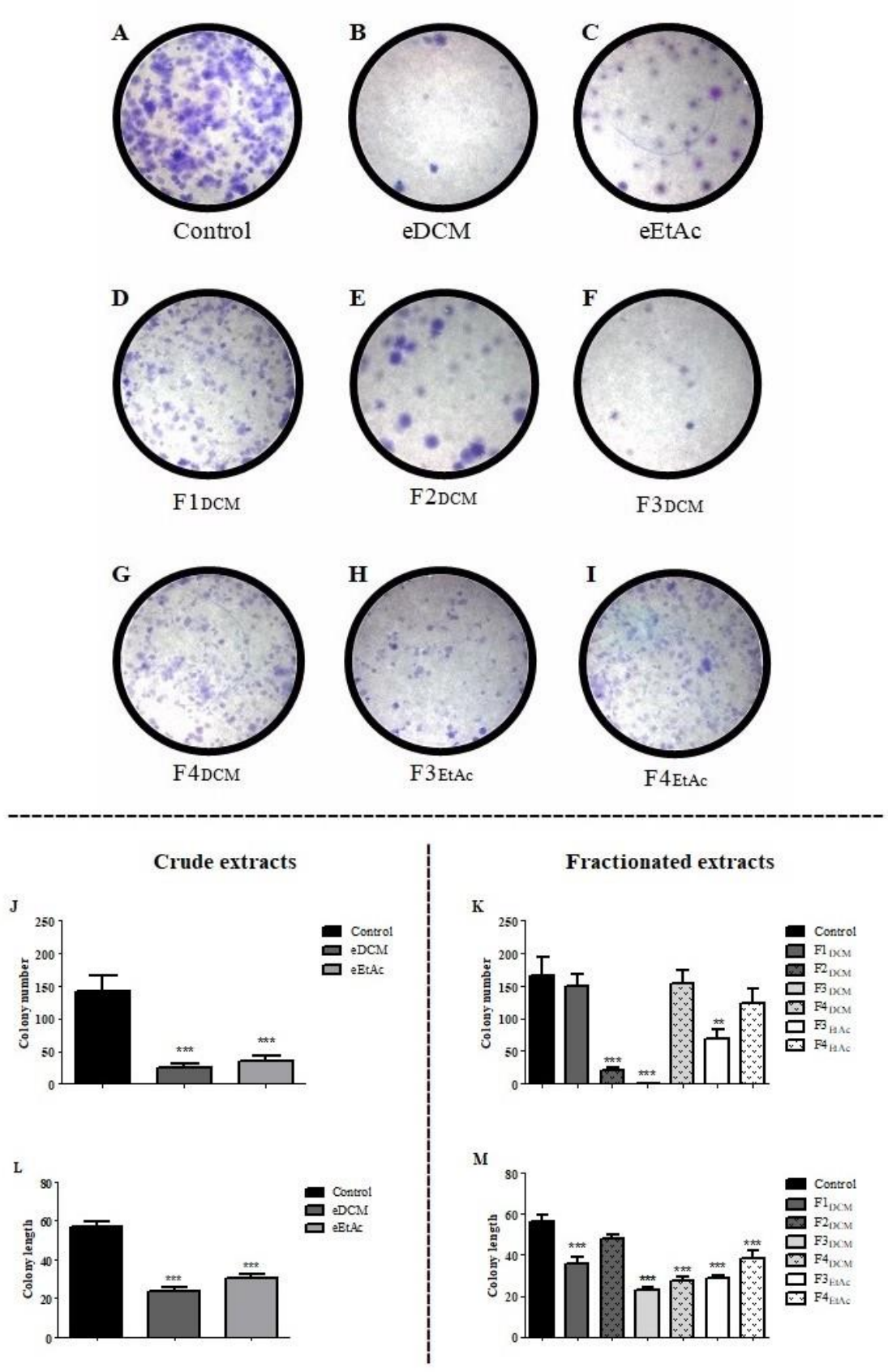
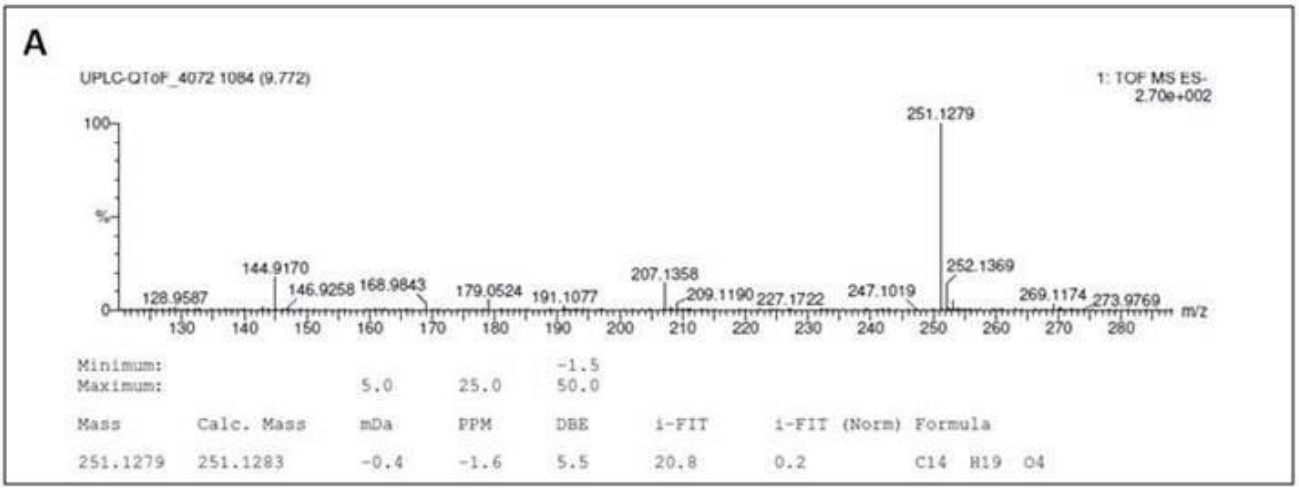
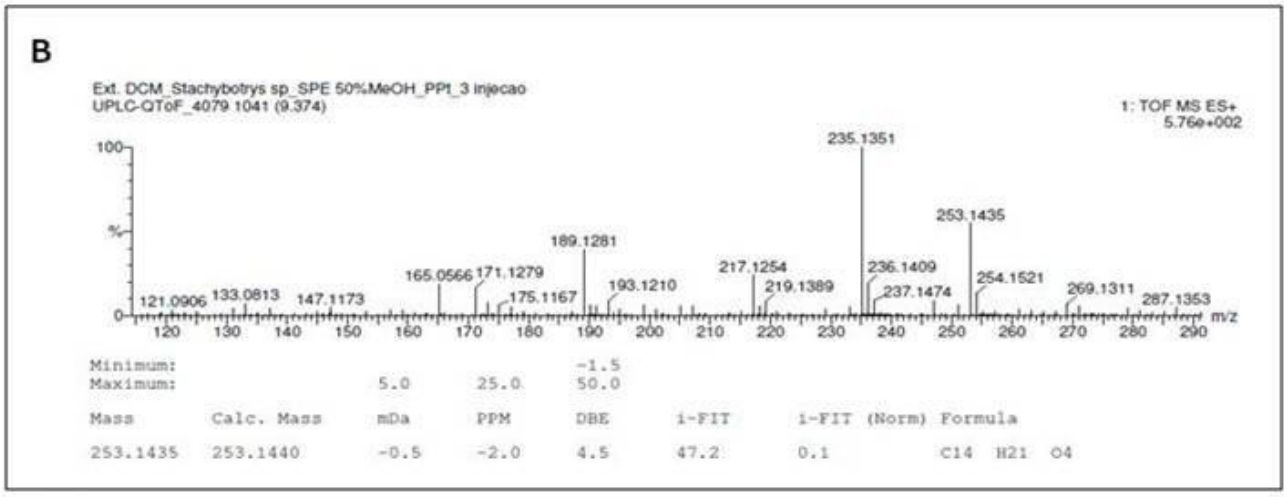

A
B
C
Control
eDCM
eEtAc
D
E
F
F1DCM
F2DCM
F3DCM
G
H
I
F4DCM
F3EtAc
F4EtAc
Crude extracts
Fractionated extracts
J
K
Colony number
Control
eDCM
eEtAc
F1DCM
F2DCM
F3DCM
F4DCM
F3EtAc
F4EtAc
L
M
Colony length

Figure 5


A
UPLC-QToF_4072 1084 (9.772)
1: TOF MS ES-
2.70e+002
251.1279
144.9170
146.9258
168.9843
179.0524
191.1077
207.1358
209.1190
227.1722
247.1019
252.1369
269.1174
273.9769
128.9587
m/z
Minimum: -1.5
Maximum: 5.0 25.0 50.0
Mass Calc. Mass mDa PPM DBE i-FIT i-FIT (Norm) Formula
251.1279 251.1283 -0.4 -1.6 5.5 20.8 0.2 C14 H19 O4


B
Ext. DCM_Stachybotrys sp_SPE 50%MeOH_PPI_3 injecao
UPLC-QToF_4079 1041 (9.374)
1: TOF MS ES+
5.76e+002
235.1351
253.1435
189.1281
165.0566
171.1279
175.1167
193.1210
217.1254
219.1389
236.1409
237.1474
254.1521
269.1311
287.1353
121.0906
133.0813
147.1173
m/z
Minimum: -1.5
Maximum: 5.0 25.0 50.0
Mass Calc. Mass mDa PPM DBE i-FIT i-FIT (Norm) Formula
253.1435 253.1440 -0.5 -2.0 4.5 47.2 0.1 C14 H21 O4

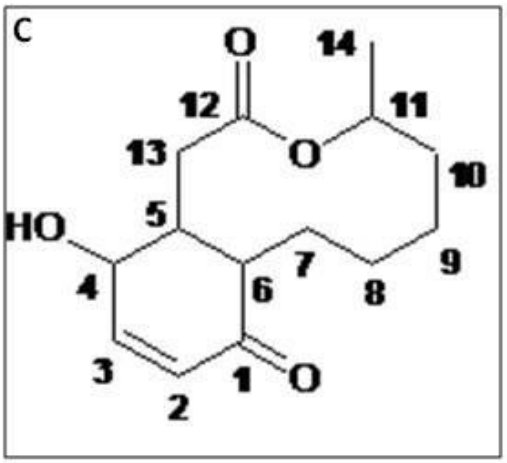

C
O
14
12
11
13
O
10
HO
5
7
9
4
6
8
3
1
O
2

Figure 6

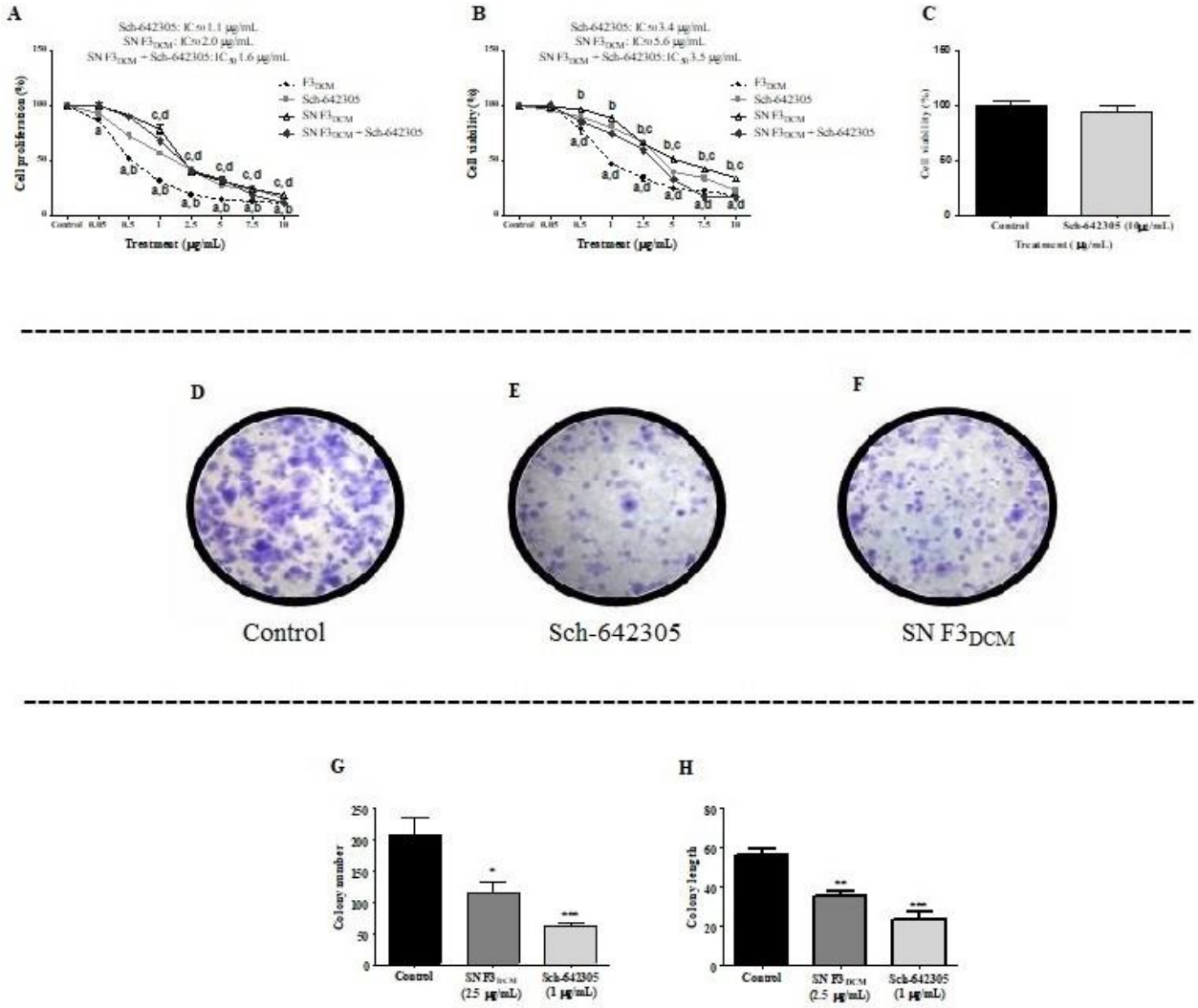

A
B
C
Cell proliferation (%)
Cell viability (%)
Treatment (μg/mL)
Control
Sch-642305
Sch-642305 (1 μg/mL)
D
E
F
Control
Sch-642305
SN F3DCM
G
H
Colony number
Colony length
Control
SN F3DCM (2.5 μg/mL)
Sch-642305 (1 μg/mL)

Figure 7

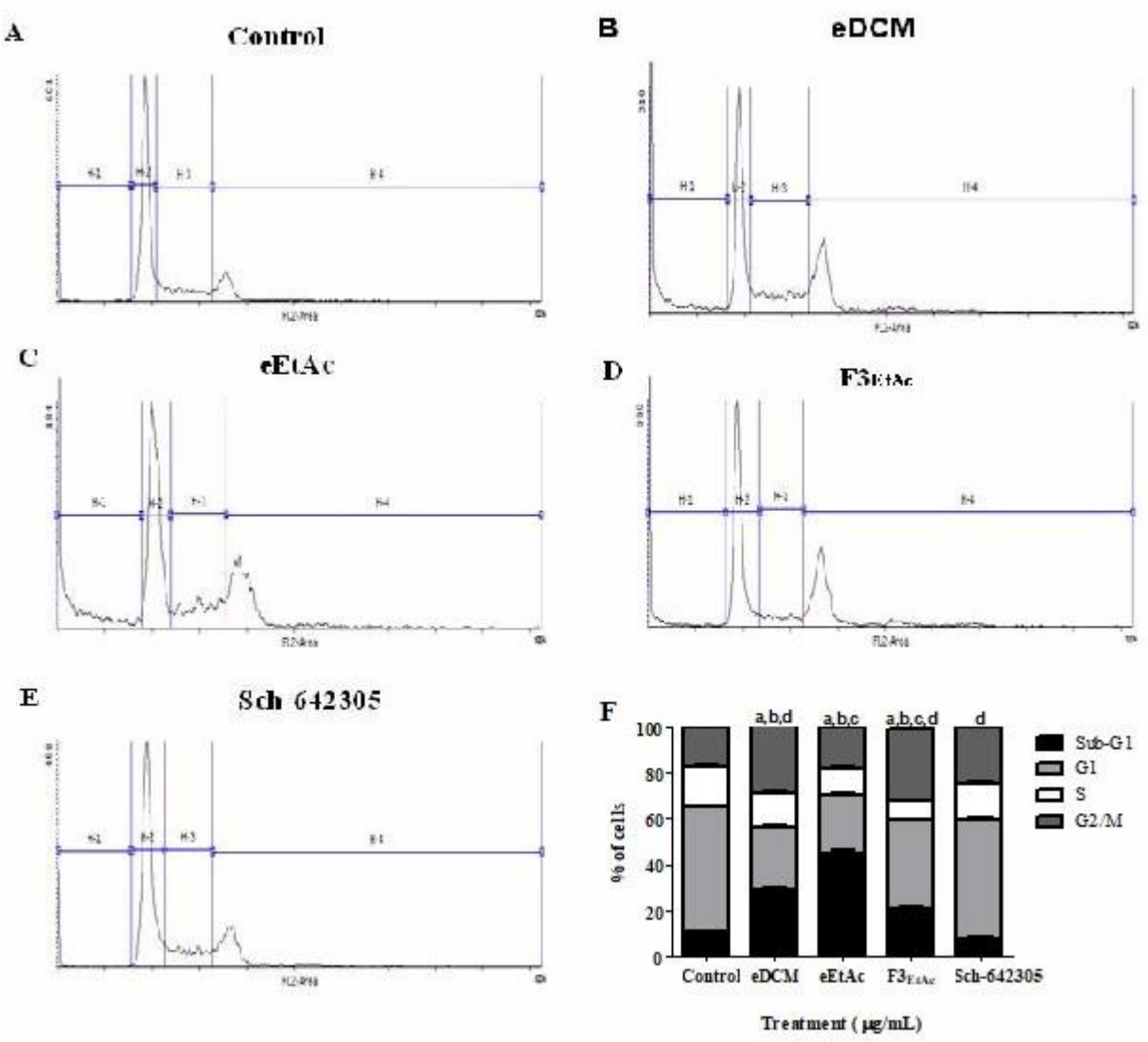

A
Control
B
eDCM
C
eEtAc
D
E
Sch 642305
F
a,b,d
a,b,c
a,b,c,d
d
100
80
60
40
20
0
% of cells
Sub-G1
G1
S
G2/M
Control
eDCM
eEtAc
Sch-642305
Treatment (µg/mL)

Figure 8

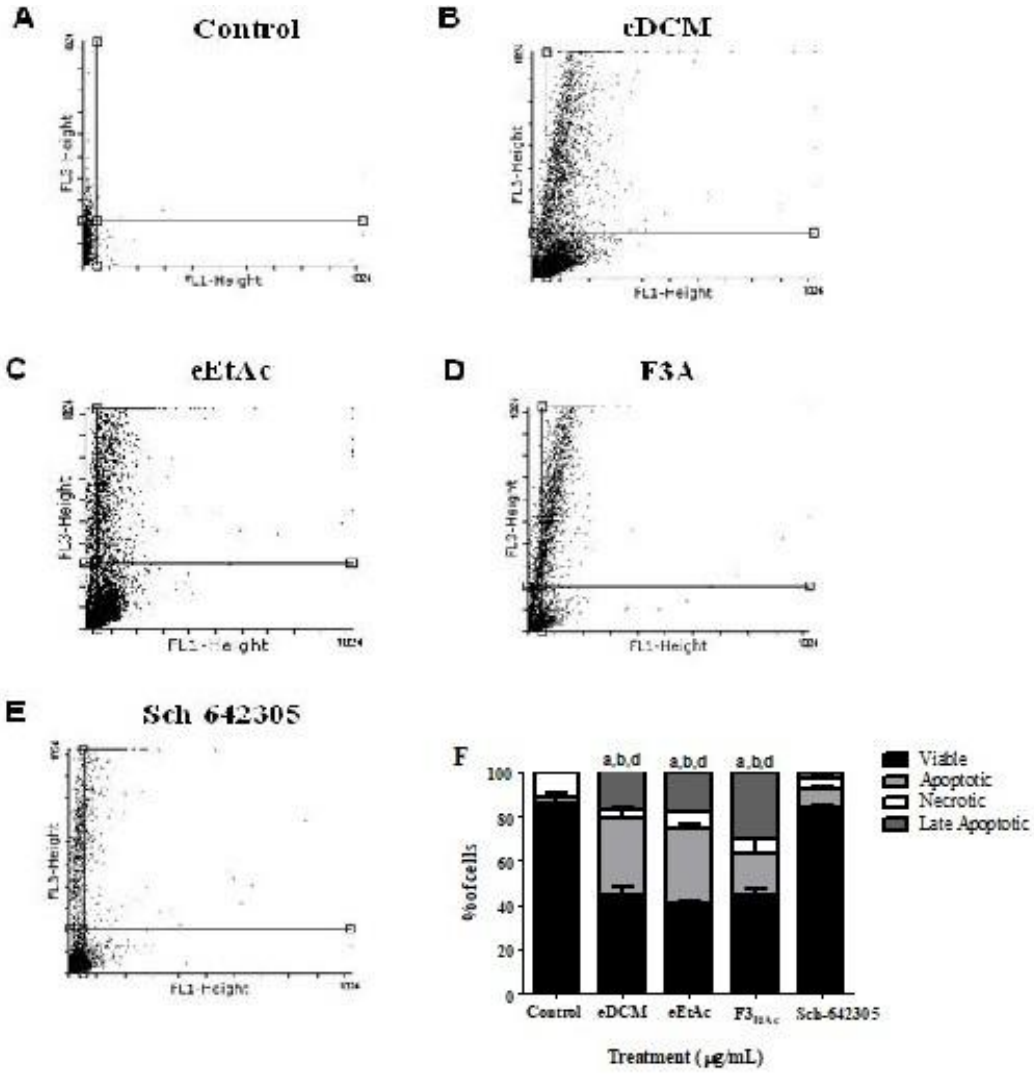

A
Control
B
cDCM
C
eEtAc
D
F3A
E
Sch 642305
F
a,b,d
a,b,d
a,b,d
Viable
Apoptotic
Necrotic
Late Apoptotic
%ofcells
100
80
60
40
20
0
Control
eDCM
eEtAc
F3EtAc
Sch-642305
Treatment (µg/mL)

Figure 9

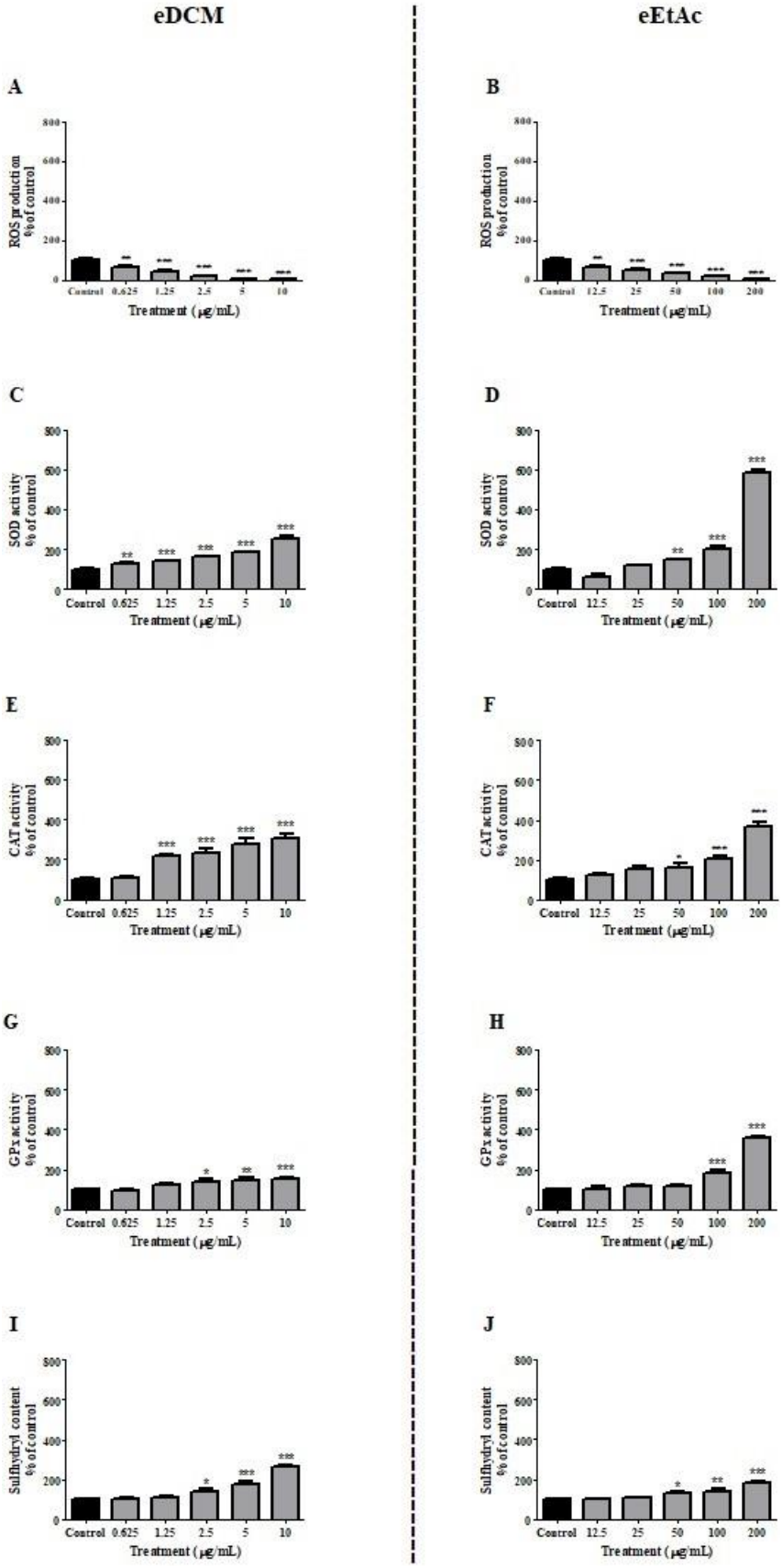

eDCM
eEtAc
A
B
C
D
E
F
G
H
I
J
ROS production % of control
SOD activity % of control
CAT activity % of control
GPx activity % of control
Sulfhydryl content % of control
Control 0.625 1.25 2.5 5 10
Control 12.5 25 50 100 200
Treatment (μg/mL)

Figure 10

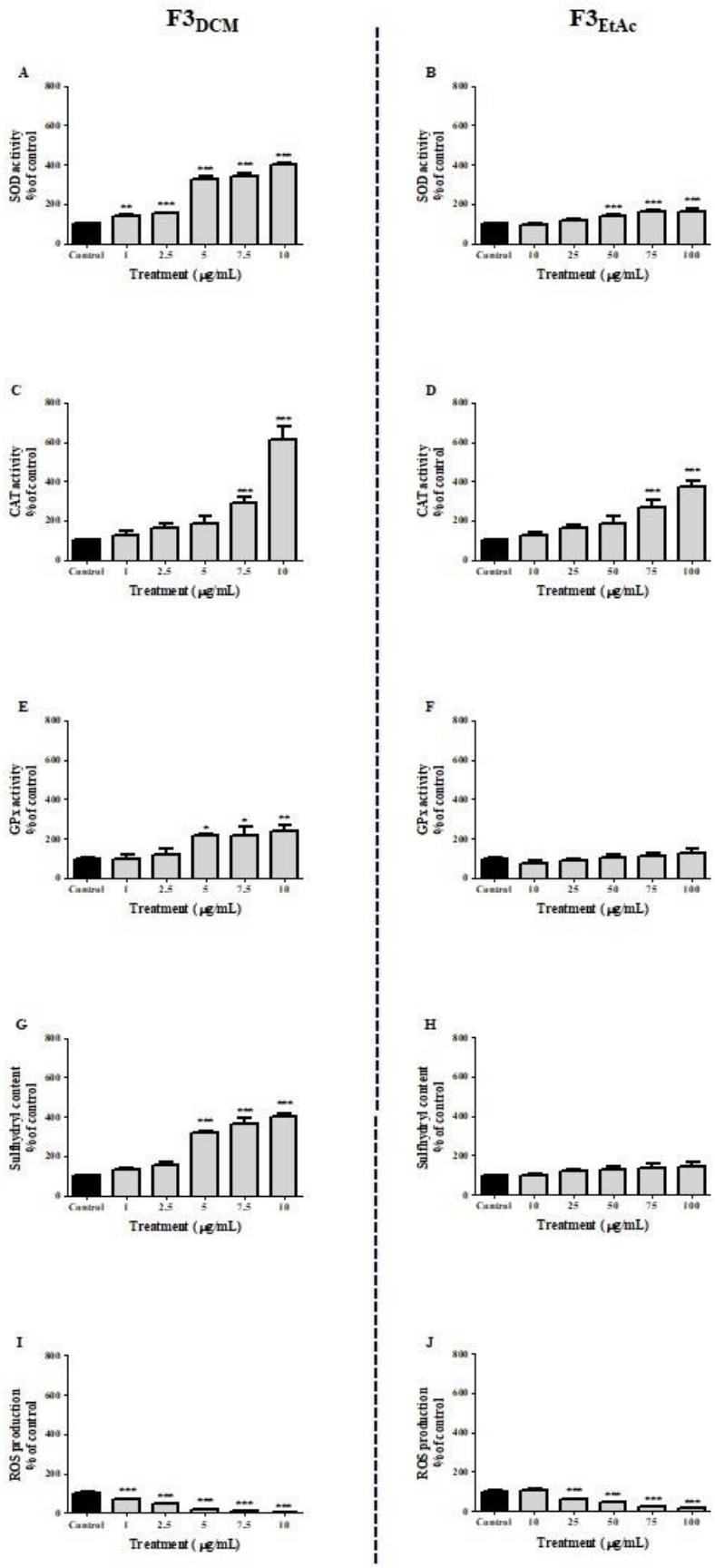

F3DCM
F3EtAc
A
SOD activity %of control
Treatment (µg/mL)
B
SOD activity %of control
Treatment (µg/mL)
C
CAT activity %of control
Treatment (µg/mL)
D
CAT activity %of control
Treatment (µg/mL)
E
GPx activity %of control
Treatment (µg/mL)
F
GPx activity %of control
Treatment (µg/mL)
G
Sulfhydryl content %of control
Treatment (µg/mL)
H
Sulfhydryl content %of control
Treatment (µg/mL)
I
ROS production %of control
Treatment (µg/mL)
J
ROS production %of control
Treatment (µg/mL)

Figure 11

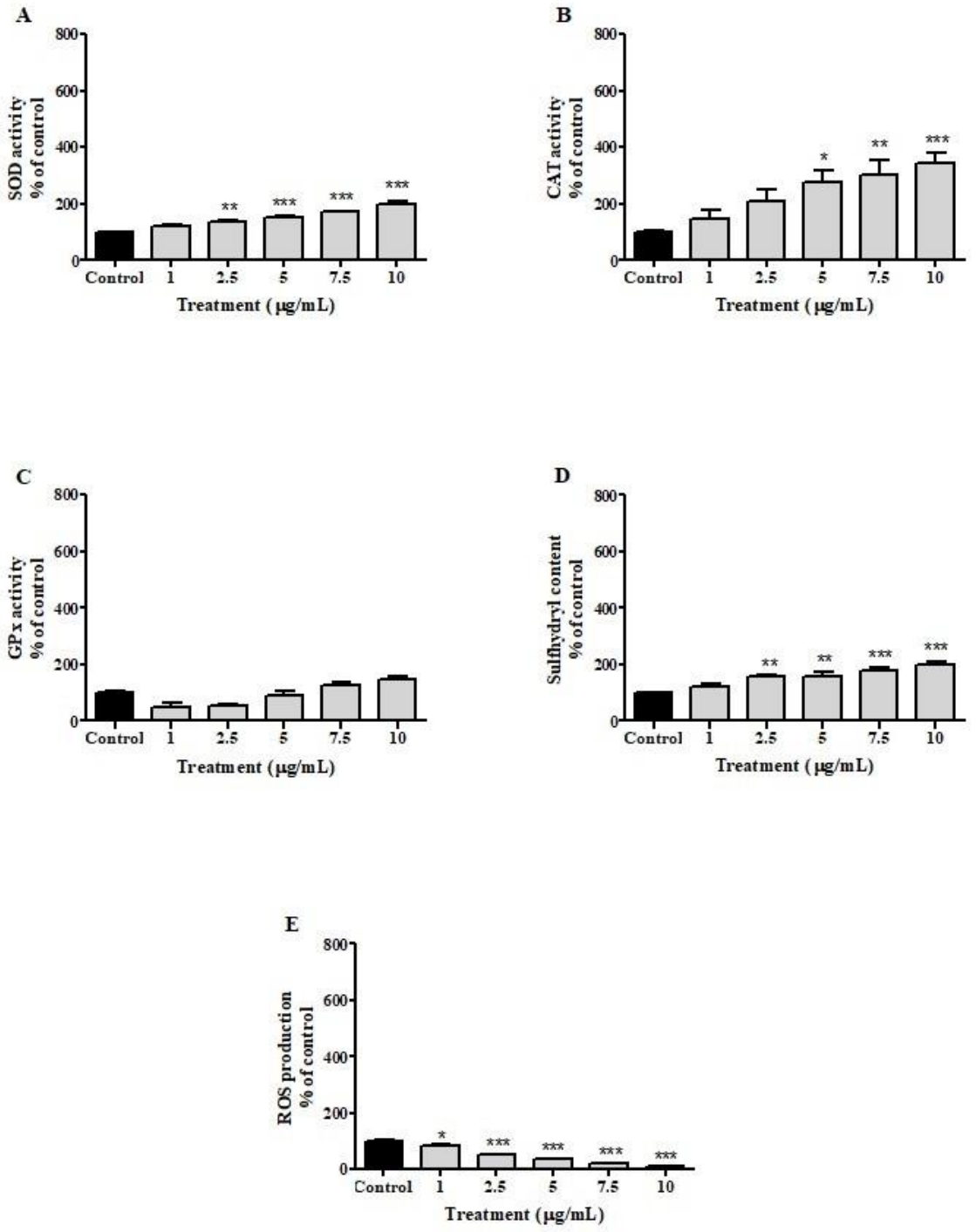

A
SOD activity
% of control
800
600
400
200
0
**
***
***
***
Control
1
2.5
5
7.5
10
Treatment (µg/mL)
B
CAT activity
% of control
800
600
400
200
0
*
**
***
Control
1
2.5
5
7.5
10
Treatment (µg/mL)
C
GPx activity
% of control
800
600
400
200
0
Control
1
2.5
5
7.5
10
Treatment (µg/mL)
D
Sulfhydryl content
% of control
800
600
400
200
0
**
**
***
***
Control
1
2.5
5
7.5
10
Treatment (µg/mL)
E
ROS production
% of control
800
600
400
200
0
*
***
***
***
***
Control
1
2.5
5
7.5
10
Treatment (µg/mL)

Figure 12

5. Conclusão

O presente estudo descreveu o efeito antiglioma de extratos brutos e fracionados, bem como a identificação e atividade da lactona Sch-642305 produzida por fungo endofítico isolado a partir de *A. satureioides*, sendo a primeira evidência a cerca da composição endofítica da Marcela.

- Os extratos brutos de DCM e AcoEt apresentaram citotoxicidade sobre linhagens de glioma C6 e U87, entretanto o eDCM exibiu efeitos anti-proliferativos mais significativos quando comparado ao eAcoEt. Ambos os extratos induziram morte celular por apoptose sobre linhagem de glioma C6.
- As frações $F3_{DCM}$ e $F3_{AcoEt}$ provenientes respectivamente de eDCM e eAcoEt, exibiram propriedades antiglioma sobre linhagem C6 induzindo apoptose e apoptose tardia, respectivamente com acúmulo de células em Sub-G1 e G2/M. Sugere-se ainda, que tais efeitos estão relacionados com o potencial de ambas as frações em modular o sistema redox do tumor. Entretanto, a $F3_{DCM}$ reduziu siginificativamente a viabilidade e proliferação celular, bem como a habilidade formadora de colônias quando comparada à fração $F3_{AcoEt}$.
- Importante ressaltar que os extratos brutos e fracionados não alteraram a viabilidade celular de células não transformadas, exibindo efeito antiglioma seletivo.
- Baseado no superior potencial da fração $F3_{DCM}$, mediante análises químicas, foi possível identificar a presença da lactona Sch-642305, a qual reduziu a proliferação celular de linhagem de glioma C6 induzindo morte celular por apoptose e parada no ciclo em G2/M. Além disso, esta lactona atuou suprimindo a produção de EROs devido ao aumento da atividade do sistema de defesa antioxidante.
- No presente traballho foi possível isolar um composto proveniente da fração $F4_{DCM}$, a qual exibiu efeitos citotóxicos in vitro sobre linhagens de glioma C6 e U87MG. O composto foi identificado como 5-metil-meleína, entretanto estudos são necessários a fim de elucidar seus efeitos antiglioma.

Com base nos dados apresentados neste estudo, pode-se inferir que a atividade antiglioma desencadeada pela fração $F3_{DCM}$, seja devido à presença da lactona Sch-642305. Este estudo corrobora com demais dados da literatura que revelam o elevado potencial dos fungos endofíticos na produção de compostos farmacológicos, destacando o papel de Sch-642305 como uma possível ferramenta para terapia antiglioma, encouranjando a busca de novos compostos bioativos com propriedades terapêuticas.

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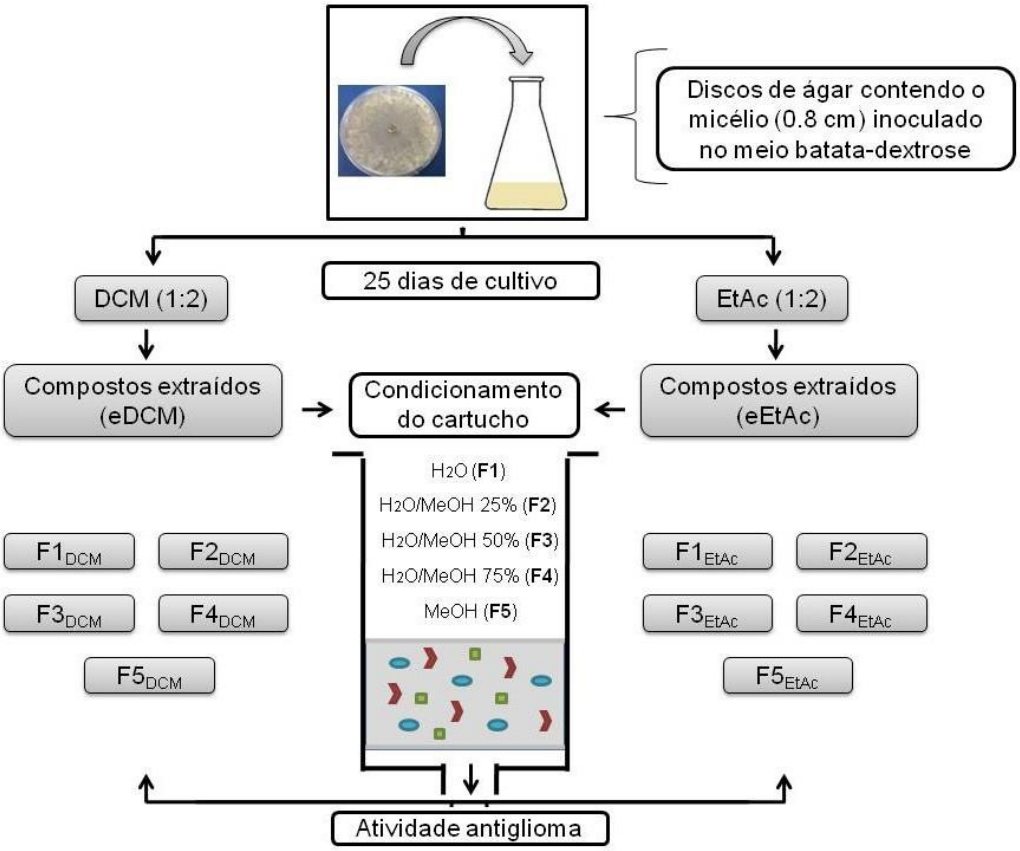
ANEXOS

Anexo A – Esquema representando a produção dos extratos brutos e fracionados



Anexo B – Carta de aprovação no Comitê de Ética em Experimentação Animal (CEEA)

Pelotas, 04 de agosto de 2016

Certificado

Certificamos que a proposta intitulada "**Caracterização e avaliação das atividades antitumoral e antioxidante de extratos de fungo endofítico isolado a partir de *Achyrocline satureoides***", registrada com o nº23110.004755/2016-15, sob a responsabilidade de **Roselia Maria Spanevello**- que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 04/07/2016.

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	15/08/2016 a 15/08/2018
Espécie/linhagem/raça	*Rattus norvegicus*/ Wistar
N° de animais	12
Idade	1-3 dias
Sexo	Machos e Fêmeas
Origem	Biotério Central - UFPel

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao *COBALTO* para posterior registro no *COCEPE* (código para cadastro nº **CEEA 4755-2016**).

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Assinatura do Professor Responsável: Roselia Maria Spanevello Ciente em: 03/10/2016

Anexo C – Elucidação química do composto 1

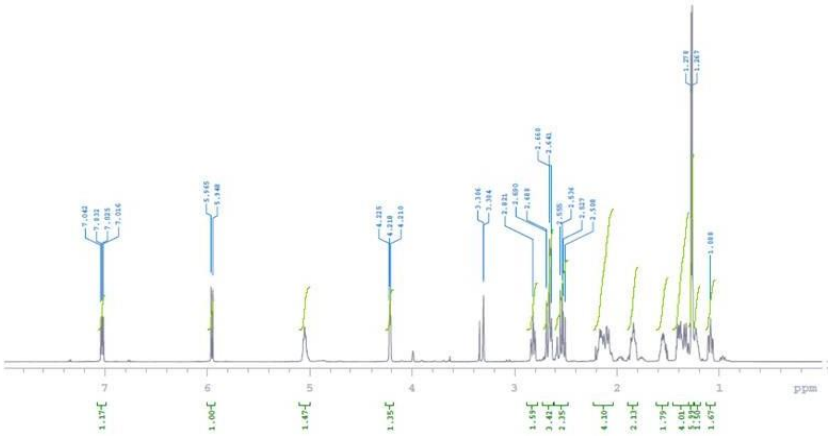


Figura 1. Espectro de RMN de 1H do composto 1 (MeOD, 600 MHz).

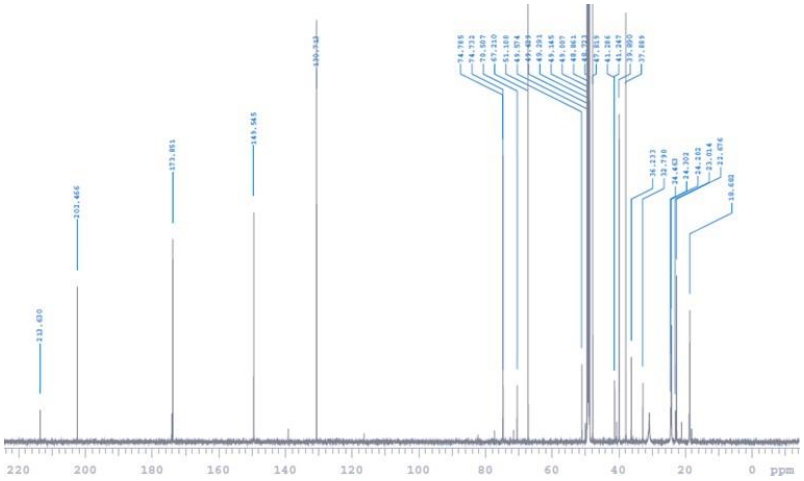


Figura 2. Espectro de RMN de ^{13}C do composto **1** (MeOD, 150 MHz).

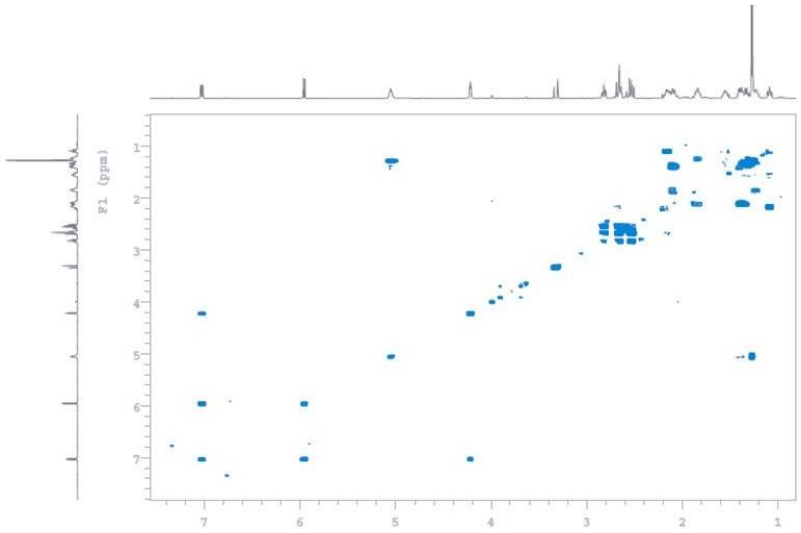


Figura 3. Espectro de RMN- ^{1}H-$^{1}HCOSY$ do composto **1** (MeOD).

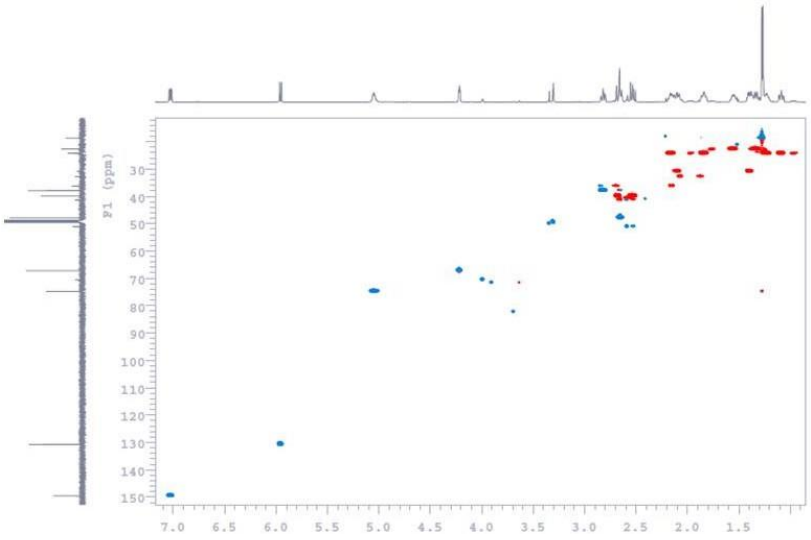


Figura 4. Espectro de RMN- ^{1}H-$^{13}CHSQC$ editado do composto **1** (MeOD).

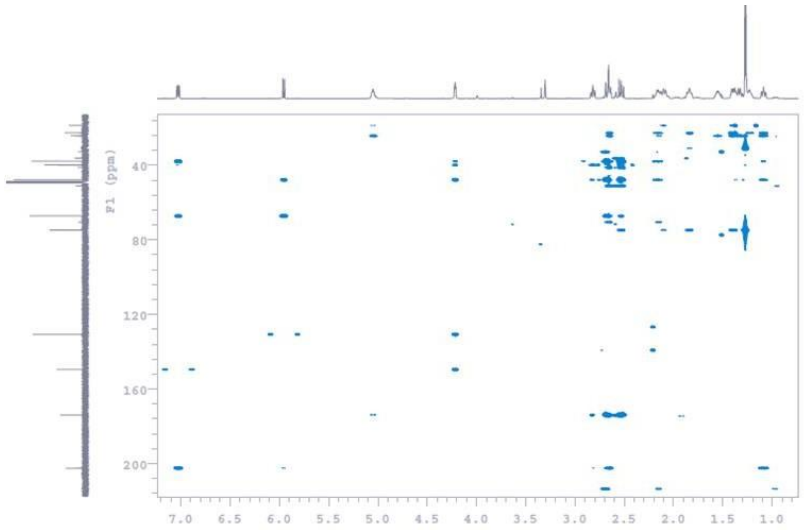


Figura 5. Espectro de RMN- ^{1}H-$^{13}CHMBC$ do composto **1** ($CDCl_3$, 600 MHz).

Anexo D – Elucidação química do composto 2 obtido a partir da fração $F4_{DCM}$

No presente estudo foi possível ainda identificar o composto **2** proveniente de $F4_{DCM}$, fração menos efetiva a qual apresentou valores de IC_{50} variando entre 7.38 µg/mL e 15.20 µg/mL sobre linhagens de glioma C6 e U87, após 48 h de exposição.

O espectro de RMN ^{1}H ($CDCl_3$) do composto **2** (Figura 1) apresentou um singleto largo em δ 10,99 (1H, s, OH-8) característico de hidroxila quelada, dupletosem δ 7.28 (1H, d, H-10, J=8.5 Hz) e 6.81 (1H, d, J=10 Hz, H-7) associados a hidrogênios aromáticos *orto*-posicionados, sinal em δ 4.67 (1H, m, H-3) relacionado a hidrogênio de carbono oxigenado, sinais de hidrogênios metilênicos diasterotópicos em δ 2.94 e 2.71 (2H, dd), além dois de hidrogênios metílicos em δ 2.19 e 1.55 (3H, s, 3-Me e 5 Me). O espectro de RMN ^{13}C (Figura 2) mostrou onze linhas espectrais, das quais uma em δ 170,5 (C-1) foi associada a carbonila de éster/lactona e seis delas na região de δ 160.6-108.3 foram atribuídas a carbonos benzênicos, sendo a ressonância em δ 160.6 (C-8) condizente com carbono sp^2 oxigenado. Além disso, a ressonância em δ 75.3 (C-3) foi referente a carbono sp^3 oxigenado (Tabela 1).

Através do espectro de RMN-^{1}H-^{13}CHSQC editado (Figura 3) do composto **2** foram identificados três carbonos metínicos, um carbono metilênico, dois carbonos metílicos e cinco carbonos não hidrogenados. As informações reunidas até aqui permitiram deduzir a fórmula molecular do composto **2** como sendo $C_{11}H_{11}O_3$, a qual corresponde a um IDH de 6. Então, foi proposta uma estrutura de uma benzolactona.

O espectro de RMN-^{1}H-^{13}CHMBC (Figura 4) permitiu a caracterização do anel fenólico por meio das seguintes correlações à duas e três ligações: sinais dos hidrogênios aromáticos em δ_H 7.28 e 6.81 (H-6 e 7) com os carbonos aromáticos em δ_C 160.6 (C-8), 137.1 (C-10), 124.8 (C-5) e 108.3 (C-9). Os acoplamentos dos hidrogênios em δ_H 10.99 e 2.19 (OH-8 e CH_3-5) com os carbonos em δ_C 160.6 (C-8) e 124.8 (C-5) definiram as posições da hidroxila fenólica e da metila nos carbonos 8 e 5, respectivamente.O anel lactônico foi caracterizado pelos acoplamentos J^4 dos hidrogênios alifáticos em δ_H 2.71 (H-4)

e 1.55 (CH3-3) com a carbonila em δ_C 170.5 (C-1). A junção dos anéis benzênico e lactônico foi confirmada pelo acoplamento dos hidrogênios aromáticos em δ_H 6.81 (H-7) com a carbonila em δ_C 170.5 (C-1) bem como através da correlação dos hidrogênios metilênicos com carbonos aromáticos em δ_C 137.1 (C-10),124.8 (C-13) e 108.3 (C-9) (Figura 5).

Dessa forma, o composto **2** foi determinado como sendo 5-metil-meleina, uma dihidro-isocumarina isolada previamente de *Phomopsis* (CLAYDON et al, 1985; DAI et al., 2005) e *Cephalosporium* (BI et al., 2007). Além disso, os dados de RMN ^{1}H e ^{13}C estão em concordância com os da literatura (BI et al., 2007) (Tabela 2).

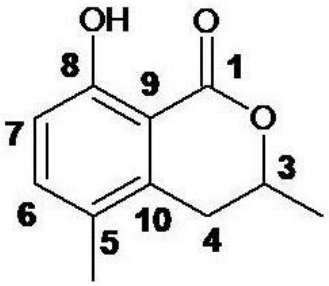


Figura 1. Estrutura química do composto **2**.

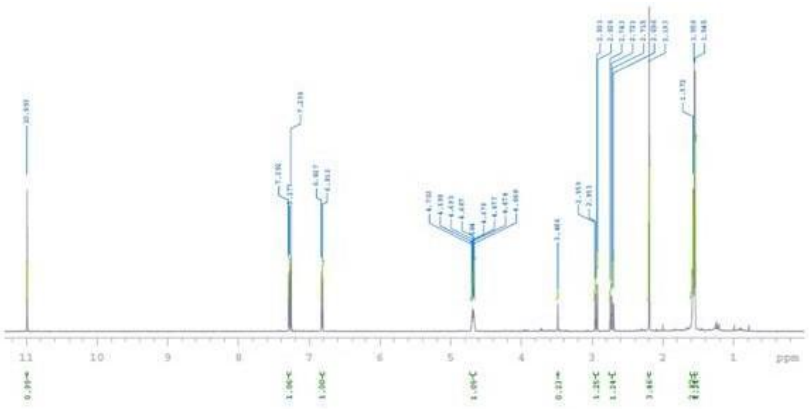


Figura 2. Espectro de RMN de ^{1}H do composto **2** ($CDCl_3$, 600 MHz).

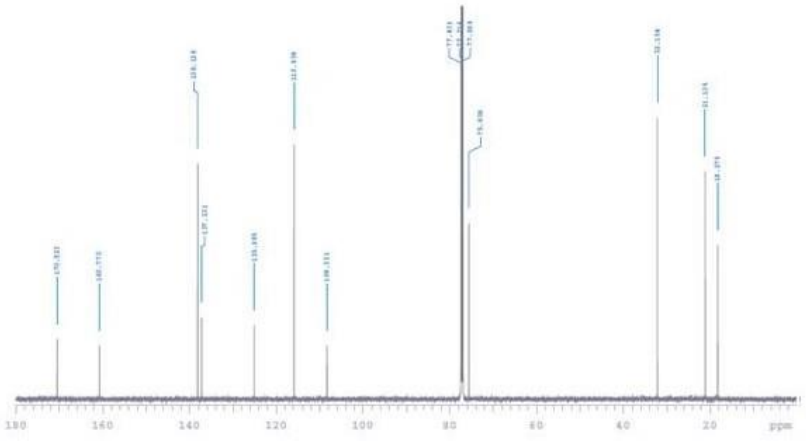


Figura 3. Espectro de RMN de ^{13}C do composto **2** ($CDCl_3$, 150 MHz).

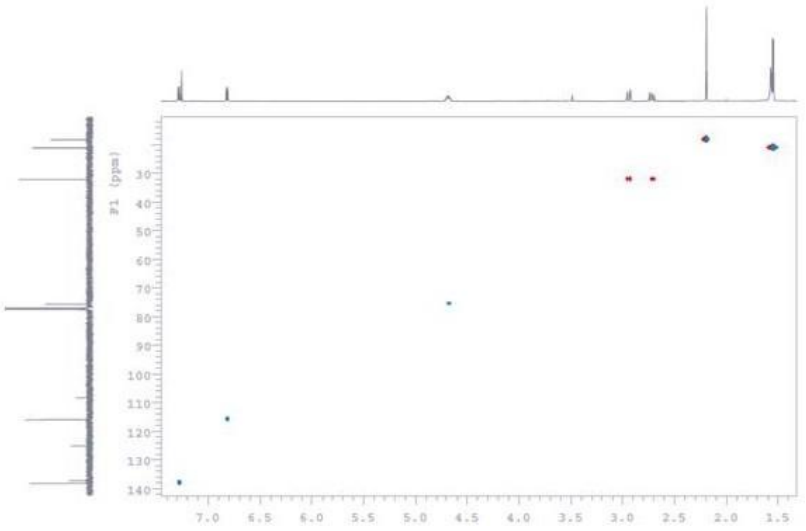


Figura 4. Espectro de RMN- ^{1}H-^{13}CHSQC editado do composto **2** ($CDCl_3$).

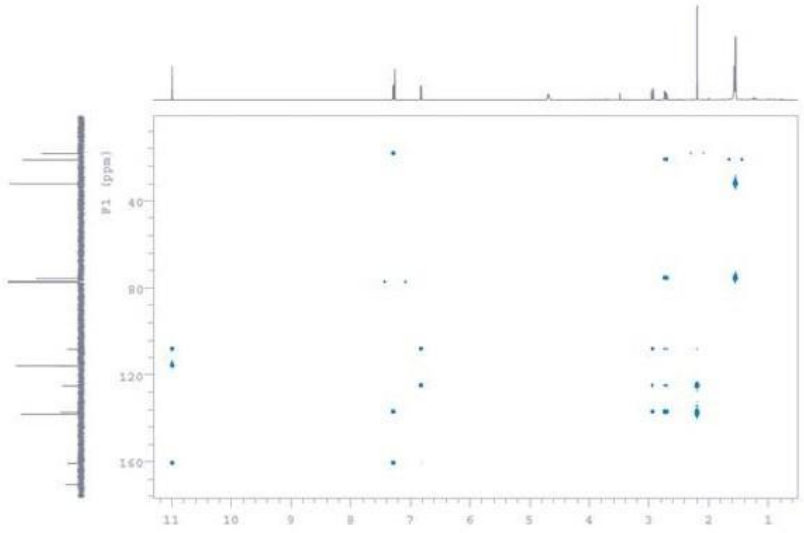


Figura 5. Espectro de RMN- ^{1}H-^{13}CHMBC do composto **2** ($CDCl_3$, 600 MHz).

Tabela 1. Dados de RMN de ^{1}H e ^{13}C (uni e bidimensional) do composto **2** ($CDCl_3$).

#C	**δC composto 2**	**δH composto 2**	**HMBC**	**COSY**
C=O	170,5	-	6,81; 1,55	
=C-O	160,6	-	10,99; 7,28; 6,81; 2,71; 2,19	
=CH	137,9	7,28 (d, 1H, 8,5)	10,99; 2,19	
=C-	137,1	-	**7,28; 2,94; 2,71**; 6,81; 4,67; 1,55	
=C-	124,8		6,81; 2,94; 2,71; 2,19	
=CH	115,7	6,81 (d, 1H, 8,5)	10,99	
=C-	108,3	-	10,99; 6,81; 2,94; 2,71; 2,19	
O-CH	75,3	4,67 (m, 1H)	2,71; 1,55	
CH_2	31,8	2,94; 2,71	1,55	
CH_3	20,8	1,55	2,71	
CH_3	18,0	2,19	7,28	
OH		10,99		

Tabela 2. Comparação dos dados de RMN 1H e 13C do composto **2** com os da 5-metilmeleína (BI et al., 2007).

#C	**δC Composto 2 (150 MHz, $CDCl_3$)**	**δH Composto 2 (600MHz, $CDCl_3$)**	**δC 5-metil-meleina (125 MHz, $CDCl_3$)**	**δH 5-metil-meleina (125 MHz, $CDCl_3$)**
1	170,5	-	169,2	
8	160,6	-	154,4	
6	137,9	7,28 (d, 1H, 8,5)	140,6	7,21 (d, 1H, 8,6)
10	137,1	-	134,6	
5	124,8		126,2	
7	115,7	6,81 (d, 1H, 8,5)	112,8	6,75 (d, 1H, 8,6)
9	108,3	-	106,4	
3	75,3	4,67 (m, 1H)	77,8	4,68 (m, 1H)
4	31,8	2,94; 2,71 (dd)	29,6	2,95; 2,80 (dd)
5-Me	20,8	1,55 (s, 3H)	20,8	1,56 (s, 3H)
3-Me	18,0	2,19 (s, 3H)	19,7	2,18 (s, 3H)
OH		10,99 (s, 1H)		10,97