**UNIVERSIDADE ESTADUAL DE MARINGÁ**

**Departamento de Farmácia**

**Programa de Pós-Graduação em Ciências Farmacêuticas**

**NADLA SOARES CASSEMIRO**

# BIOTRANSFORMAÇÃO DE PRODUTOS NATURAIS E FÁRMACOS NA BUSCA DE NOVOS COMPOSTOS BIOATIVOS

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Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas (Área de concentração: Produtos Naturais e Sintéticos Biologicamente Ativos), da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título em nível de doutorado em Ciências Farmacêuticas.

Orientador: Prof. Dr. João Carlos Palazzo de Mello
Co-orientadora: Profª. Dra. Denise Brentan da Silva

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128ª Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Estadual de Maringá como requisito para obtenção do título de Doutor em Ciências Farmacêuticas.

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Este trabalho foi realizado no Laboratório de Produtos Naturais e Espectrometria de Massas (LaPNEM) da Universidade Federal de Mato Grosso do Sul (UFMS) (Profª Dra Denise Brentan da Silva) e no Laboratório de Biologia Farmacêutica (PALAFITO) da Universidade Estadual de Maringá (UEM) (Prof. Dr. João Carlos Palazzo de Mello), em parceria com o Laboratório de Bioquímica (UFMS) (Prof. Dr Edson dos Santos dos Anjos).

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Nadla Sares Cassemiro, Luana Bonifácio Sanches, Carlos Alexandre Carollo, Edson dos Anjos dos Santos, Denise Brentan da Silva, João Carlos Palazzo de Mello. Biotransformation of natural products and drugs in the search of new bioactive compounds. I International Meeting of Pharmaceutical Sciences, IX Seminário Anual do PCF e I Seminário Anual do PROFAR, 2018.

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A persistência é o menor caminho do êxito”
(Charles Chaplin)

**BIOGRAFIA**

Nadla Soares Cassemiro nasceu no dia 21 de março de 1989 em Campo Grande/MS. Em 2008 iniciou o curso de Farmácia pela Universidade Federal de Mato Grosso do Sul (UFMS) e concluiu em 2012. Durante a graduação participou de projetos de ensino, pesquisa e extensão e no período de 2009-2012 participou do Programa de Iniciação Científica, na área de Química Farmacêutica. Em 2013, iniciou o curso de mestrado no Programa de Pós-Graduação em Ciências Farmacêuticas, pela mesma instituição, sob orientação do Prof. Dr. Adriano Cesar de Morais Baroni, concluindo-o em 2015. O projeto de pesquisa resultou na dissertação intitulada "Síntese de novos 2-Arilindóis com Potencial Atividade Antileishmania". Atualmente é doutoranda do Programa de Pós-Graduação em Ciências Farmacêuticas, da Universidade Estadual de Maringá, sob orientação do Prof. Dr. João Carlos Palazzo de Mello e co-orientação da Profa. Dra. Denise Brentan da Silva. Também é técnica do Laboratório de Produtos Naturais e Espectrometria de Massas (LAPNEM) da UFMS, responsável pelo equipamento Espectrômetro de Massas de Alta Resolução. Participou de disciplinas, cursos, eventos nacionais e internacionais com apresentação de trabalhos, tendo resumos e artigos publicados nas revistas *Journal of the Brazilian Chemical Society*, *Tetrahedron Letters*, *Journal of Ethnopharmacology*, *Journal of Medicinal Food*, *Life*, *Planta Medica International Open* and *Applied Microbiology and Biotechnology*.

CASSEMIRO, N.S. 2021. Biotransformação de produtos naturais e fármacos na busca de novos compostos bioativos. Tese de doutorado, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá. p. 128

**RESUMO**

A síntese química de substâncias bioativas pode ser realizada em larga escala, porém devido a algumas desvantagens desse método outras técnicas já vêm sendo utilizadas. A biotransformação de bioativos usando microrganismos é um método alternativo, simples, de baixo custo, que confere estereoespecificidade reacional, além de ser ambientalmente seguro. Dessa forma, este trabalho teve como objetivo isolar compostos de origem natural e realizar sua biotransformação, bem como de fármacos comerciais, utilizando cepas de fungos como biocatalisadores. O iridoide especiosídeo (das cascas do caule de *Tabebuia aurea*) e um sesquiterpeno oxigenado (do óleo essencial das partes aéreas de *Bacopa gratioloides*) foram isolados e estruturalmente caracterizados por ressonância magnética nuclear (RMN) e Cromatografia líquida de alta eficiência acoplado aos detectores de arranjo de diodo e espectrometria de massas (CLAE-DAD-EM). Além dessas substâncias isoladas, os fármacos fluconazol, meropenem, prednisolona, metilprednisolona e clindamicina foram submetidos a reações de biotransformação utilizando os fungos *Aspergillus terreus, A. niveus, A. niger, A. flavus, A. japonicus, Penicillium crustosum* e *Thermoascus aurantiacus*. A biotransformação de todas as substâncias foi realizada em duplicata, em meio contendo $K_2HPO_4$, $MgSO_4$. $7H_2O$, proteose peptona, glicose e água deionizada, a 30 ºC, sob agitação constante. Alíquotas do meio foram monitoradas por cromatografia em camada delgada (CCD) e CLAE-DAD-EM até cessar o consumo do material de partida. O óleo das partes aéreas da *B. gratioloides* (BGEO), constituído majoritariamente pelo sesquiterpeno oxigenado, foi avaliado quanto sua atividade deterrente alimentar e deterrente contra *Anagasta kuehniella* e *Callosobruchus maculatus*, o qual mostrou-se eficaz. Depois, esse sesquiterpeno foi isolado, identificado e além de se mostrar inédito, apresentou efeito anticâncer contra melanoma murino (B16F10-Nex2), bem como promoveu marcada inibição da migração celular in vitro e morte por apoptose. Posteriormente, esse sesquiterpeno foi biotransformado por *A. niger* e gerou 17 novos metabólitos por reações de hidroxilação, redução e desidrogenação. Dois desses compostos foram isolados e identificados como produtos de hidroxilação régio e estereoseletiva. Já a biotransformação do especiosídeo mediada por fungos resultou em 19 diferentes compostos. O especiosídeo na forma não glicosilada foi o principal metabólito observado. Os outros compostos foram produzidos a partir de reações de hidrólise do éster, hidroxilação, metilação e hidrogenação. Por outro lado, todos os fármacos alvos deste trabalho foram submetidos à biotransformação usando diferentes espécies de fungos e realizadas várias modificações de meio de crescimento, apesar disso, nenhum experimento levou a obtenção de nenhum derivado. Os resultados desse trabalho mostraram o BGEO como promissora fonte para o desenvolvimento de repelentes e deterrentes naturais no controle de pragas. Além disso, o sesquiterpeno isolado deste óleo é inédito e apresentou atividade anticâncer *in vitro*. Sua biotransformação, por sua vez, foi muito eficiente, gerando moléculas inéditas e promissoras anticâncer. Por fim, a biotransformação por fungos mostrou-se uma técnica muito eficiente para os compostos naturais, com alta especificidade, capaz de produzir derivados a partir de variados tipos de reações, e também de compostos que não foram possíveis se obter por síntese química.

**Palavras-chave:** *Bacopa gratioloides*, *Aspergillus* sp, sesquiterpeno, especiosídeo não glicosilado, hidroxilação, anticâncer.

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**ABSTRACT**

The chemical synthesis of bioactive substances can be performed on a large scale, but due to some disadvantages of this method, other techniques are already being used. Bioactive biotransformation using microorganisms is an alternative, simple, low-cost method, which confers reactional stereospecificity, in addition to being environmentally safe. Thus, this work aimed to isolate compounds of natural origin and perform their biotransformation, as well as commercial drugs, using fungal strains as biocatalysts. The speciosid iridoide (from the bark of *Tabebuia aurea*) and an oxygenated sesquiterpene (from the essential oil of the aerial parts of *Bacopa gratioloides*) were isolated and structurally characterized by nuclear magnetic resonance (NMR) and high performance liquid chromatography coupled to array detectors diode and mass spectrometry (HPLC-DAD-MS). In addition to these isolated substances, the drugs fluconazole, meropenem, prednisolone, methylprednisolone and clindamycin were submitted to biotransformation reactions using the fungi *Aspergillus terreus, A. niveus, A. niger, A. flavus, A. japonicus, P. crustosum and Thermoascus aurantiacus.* The biotransformation of all substance was performed in duplicate, in medium containing $K_2HPO_4$, $MgSO_4$. $7H_2O$, proteose peptone, glucose (0.609 g) and deionized water (50 mL), at 30 ºC, under constant agitation (110 rpm). Aliquots of the medium were monitored by thin layer chromatography (TLC) and HPLC-DAD-MS until consumption of starting material ceased. The oil from the aerial parts of *B. gratioloides (*BGEO), mainly constituted by oxygenated sesquiterpene, was evaluated for its food deterrent and deterrent activity against *Anagasta kuehniella* and *Callosobruchus maculatus*, which proved to be effective. Afterwards, this sesquiterpene was isolated, identified and, in addition to being unprecedented, it showed an anticancer effect against murine melanoma (B16F10-Nex2), as well as promoted a marked inhibition of cell migration in vitro and death by apoptosis. Subsequently, this sesquiterpene was biotransformed by *A. niger* and generated 17 new metabolites by hydroxylation, reduction and dehydrogenation reactions. Two of these compounds were isolated and identified as regio- and stereoselective hydroxylation products. The fungal-mediated biotransformation of the specioside resulted in 19 different compounds. Specioside in non-glycosylated form was the main metabolite observed. The other compounds were produced from ester hydrolysis, hydroxylation, methylation and hydrogenation reactions. On the other hand, all target drugs of this work were submitted to biotransformation using different species of fungi and carried out several modifications of the growth medium, despite this, no experiment led to obtaining any derivative. The results of this work showed BGEO as a promising source for the development of repellents and natural detergents for pest control. Furthermore, the sesquiterpene isolated from this oil is new and showed anticancer activity *in vitro*. Its biotransformation, in turn, was very efficient, generating novel and promising anticancer molecules. Finally, biotransformation by fungi proved to be a very efficient technique for natural compounds, with high specificity, capable of producing derivatives from various types of reactions and compounds that were not possible to obtain by chemical synthesis.

**Key words:** *Bacopa gratioloides*, *Aspergillus* sp, non-glycosylated specioside, hydroxylation, anticancer.

## LISTA DE FIGURAS

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| BGEO | *Bacopa gratioloides* essential oil |
| BSA | Bovine Serum Albumin |
| CGEN/MMA | Conselho de Gestão do Patrimônio Genético/ Ministério do Meio Ambiente |
| CLAE-DAD-EM | Cromatografia Líquida de Alta Eficiência acoplada ao Detector de Arranjo de Diodos e Espectrometria de Massas |
| COX-2 | Cicloxigenase-2 |
| EI | Electron Ionization |
| ESI-QTOF | Electrospray - Quadruple Time-Of-Flight |
| FAA | Formaldehyde, acetic acid, and 70% ethanol |
| GC-MS | Gas chromatography-mass spectrometry |
| IL-1b | Interleucina-1b |
| IL-6 | Interleucina-6 |
| LaPNEM | Laboratório de Produtos Naturais e Espectrometria de Massas |
| LAS | Leica Application Suite |
| LC-DAD-MS | Liquid Chromatography- Diode Array Detector- Mass Spectrometry |
| LM | Light Microscopy |
| *m/z* | Razão massa/carga |
| P&D | Pesquisa e Desenvolvimento |
| RI | Retention Indices |
| SEM | Surface Electronic Microscopy |
| STP1 | 7-epi Dihydroartemisinic acid |
| TLC | Thin Layer Chromatographic |
| UFLC | Ultra Fast Liquid Chromatograph |
| UFMS | Universidade Federal de Mato Grosso do Sul |
| UV | Ultravioleta |

**SUMÁRIO**

# 1 INTRODUÇÃO, REVISÃO DE BIBLIOGRÁFICA E JUSTIFICATIVA

A pesquisa e desenvolvimento de fármacos (P&D) está cada vez mais ligada às inovações científicas e tecnológicas, como por exemplo, com o aparecimento da metabolômica, genômica, genômica funcional, proteômica e citômica (Guido et al., 2010). O marco do desenvolvimento da atual geração de fármacos se deu por meio da melhor compreensão de vias bioquímicas e fisiológicas, de alvos moleculares, da técnica de modelagem molecular, de biocatálise, das ciências genômicas e da aplicação da tecnologia do DNA recombinante (2010 Pinto, Diaz, 2011, Guido et al., 2014). Todos esses expressivos avanços da química e biologia, tornaram possível a descoberta de novos fármacos.

Atualmente, a maioria dos fármacos existentes no mercado é de origem natural ou de síntese química inspirada em produtos naturais (Newman e Cragg, 2020). Os metabólitos bioativos estão frequentemente presentes em pequenas quantidades nas espécies vegetais, o que inviabiliza em muitos casos seu isolamento para o desenvolvimento de novos fármacos. Neste contexto, a síntese dessas substâncias pode ser realizada para sua obtenção em larga escala, ou como estratégia de modificação molecular com o intuito de promover melhoras na atividade farmacológica e nas propriedades farmacocinéticas, como também na redução de efeitos tóxicos (Hegazy et al., 2015). Por outro lado, várias desvantagens da síntese química convencional podem ser elencadas como por exemplo, a baixa eficiência catalítica, falta de especificidade enantiomérica para a síntese quiral, necessidade de alta temperatura, alta pressão, várias etapas de reação e purificação, uso de solventes e catalisadores de metais pesados (Perkins et al., 2015), os quais vão contra a estratégia de química verde (Prado, 2003).

Diante preocupação com a conservação do meio ambiente, os processos de reações químicas vêm sendo remodelados para técnicas que empreguem a biocatálise e minimizem a produção de substâncias tóxicas ao meio ambiente com a mesma eficiência e eficácia que as técnicas convencionais, empregando produtos biodegradáveis em menor tempo de reação e em um processo seguro (Mohammed et al., 2020). Esses são alguns dos princípios da química verde que servem como base para a utilização de enzimas produzidas por fungos, o que a possibilitaria a transformação química de compostos em produtos com potencial farmacológico.

A biotransformação é uma ferramenta potencial de tecnologia de desenvolvimento sustentável e inclui-se nos princípios da química verde (Mohammed et al., 2020). Ela pode ser definida como a modificação molecular em um sítio específico de um composto alvo para se obter um produto distinto, com similaridade estrutural ao produto inicial através do uso de catalisadores biológicos incluindo microrganismos como fungos, bactérias e enzimas isoladas. A biotransformação é considerada uma importante ferramenta na obtenção de novos fármacos e vem se tornando cada vez mais usual em aplicações industriais. Como por exemplo, o processo microbiano comercializado na área de esteroides para produção de 11α-hidroxiprogesterona, o qual foi realizado pela primeira vez por Peterson e Murray (1952). 11α-, 11β-, 15α e 16α-hidroxilações são processos atualmente estabelecidos na indústria de esteroides, principalmente para a produção de hormônios do córtex adrenal e seus análogos (Flores et al., 2020).

O uso de biocatalisadores como fungos, bactérias, enzimas, células, por exemplo, podem simplificar, ou mesmo habilitar, o processo de produção de compostos complexos e intermediários. Eles podem adicionar estereoespecificidade ao processo, eliminando a necessidade de etapas complicadas de separação e purificação (Hegazy et al., 2015, Perkins et al., 2015). Desta forma, podemos destacar diversas vantagens do uso das biotransformações microbianas (Perkins et al., 2015; Hegazy et al., 2015), que incluem:

- Ausência da necessidade de proteção dos grupos funcionais do substrato;
- Multiplicidade de tipos de reação como hidroxilação, redução, oxidação, acetilação;
- Redução do número de etapas na obtenção de um composto;
- Condições reacionais brandas (não necessitam de extremos de pH e temperaturas), o que evita a degradação de substâncias instáveis;
- Reações em posições específicas na estrutura, as quais não reagiriam através da aplicação de reações químicas convencionais devido a uma ativação insuficiente ou impedimento estérico, por exemplo.
- Realização de reações com alta especificidade.
- Fungos filamentosos podem agir como agentes de biorremediação tendo um forte impacto ambiental de descontaminação. Uma biorremediação fúngica eficiente gera compostos menos tóxicos, inertes ou totalmente degradados (Lima et al., 2018).

Portanto, uma das vantagens a de ressaltar é a especificidade das biotransformações realizadas por microorganismos, que incluem:

- "Estereospecificidade: um organismo bioconversor normalmente converte apenas um dos enantiômeros quando é exposto a uma mistura racêmica. Também produz um produto opticamente ativo em vez de uma mistura de enantiômeros" (Perkins et al. 2015). As principais bioconversões específicas descritas na literatura são as de esteroides, sendo que os principais tipos de reações são mono-hidroxilação, di-hidroxilação, epoxidação, desidrogenação e hidrogenação (Peterson e Murray, 1952; Hegazy et al., 2015; Mohamed et al. 2017; Putkaradze et al. 2017).
- Especificidade da reação: um microorganismo bioconversor pode realizar apenas um tipo de reação, como no caso da biotransformação da artemisinina por *Streptomices griseus* que gerou somente análogos oxidados (Hegazy et al., 2015).
- Regiospecificidade: um microorganismo bioconversor normalmente ataca apenas uma posição na molécula, como na conversão da progesterona a 11-hidroxiprogesterona usando cepas de *Rhizopus arrhizus* (Peterson e Murray, 1952).

A especificidade reacional mediada por microrganismos na biotransformação é um dos motivos do grande interesse industrial por essa técnica. Os esteroides foram os pioneiros na biotransformação em escala industrial. E hoje, a indústria farmacêutica tem grande interesse na sua biotransformação para a produção desses hormônios esteroides. A estereoquímica complexa dos esteroides requer atenção à regio- e estereosseletividade da reação durante a preparação e, portanto, os métodos biocatalíticos são apropriados para sua produção (Sultana, 2018; Flores et al., 2020).

## 1.1 Biotransformação de compostos de origem natural

Várias classes de metabólitos de produtos naturais bioativos têm sido submetidos ao processo de biotransformação com o intuito de aprimorar suas propriedades farmacológicas, bem como melhorar propriedades farmacocinéticas ou diminuir seus efeitos colaterais. Atualmente, algumas classes desses metabólitos têm sido alvos amplamente estudados em transformações microbianas como: terpenoides, iridoides, esteroides, alcaloides, fenilpropanoides e outros (Birolli et al., 2015; Hegazy et al., 2015; Costa et al., 2016; Sobeh et al., 2016).

Os iridoides, uma das classes estudadas no presente projeto, são metabólitos secundários amplamente encontrados em espécies da família Bignoniaceae. Essa classe de metabólitos é descrita como detentora de diversas atividades biológicas, as quais incluem propriedades neurotróficas, anti-inflamatórias, antivirais, antimicrobianas, antioxidantes e antitumorigênicas (Schröder et al., 2016). Por isso a obtenção de análogos biologicamente ativos através de reações de biotransformação tem sido realizada por vários grupos de pesquisa (Baydoun et al., 2013; Flores et al., 2020).

O iridoide gentiopicrosídeo **(1),** para o qual foram comprovadas atividades antibacteriana, hepatoprotetora e antioxidante, foi submetido a processo de biotransformação usando micélios de *Cordyceps sinensis* e resultou em dois novos metabólitos do tipo pirano[3,4-c]pirano **(2)** e isocromano **(3).** Reações de desglicosilação, isomerização, hidroxilação foram observadas para obtenção dos derivados (Figura 1), (Wang et al., 2007).

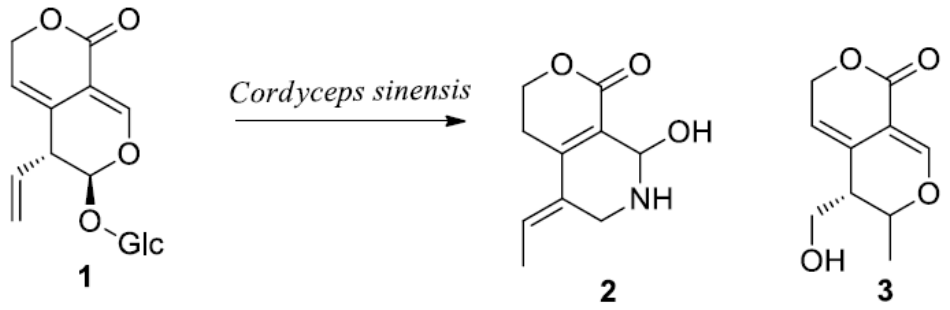



**Figura 1.** Derivados do iridoide genciopicrosídeo obtidos por biotransformação com *Cordyceps sinensis.*

O especiosídeo (Figura 2), por exemplo, é outro iridoide de grande interesse por ser amplamente distribuído no gênero *Tabebuia* (Bignoniaceae), abundante na espécie *T. aurea* (ipê-amarelo) e pelo seu potencial farmacológico, incluindo atividade ati-inflamatória *in vivo* (Santos et al., 2017; Malange et al., 2019, Nocchi et al., 2020). Estudos do nosso grupo de pesquisa (LAPNEM, Laboratório de Produtos Naturais e Espectrometria da Massas, UFMS) têm mostrado promissoras atividades anti-inflamatória e anti-hiperalgésica do extrato hidroetanólico da casca de *Tabebuia aurea* com 80% do iridoide especiosídeo em sua composição (Malange, 2019), além de attividades antihemorrágica e antimiotóxica (Reis et al., 2014). Além disso, mais recentemente, mostramos que o especiosídeo isolado tem potencial anti-inflamatório ao

inibir em 80% o recrutamento leucocitário na cavidade peritoneal após a injeção de carragenina (Nocchi et al., 2020).

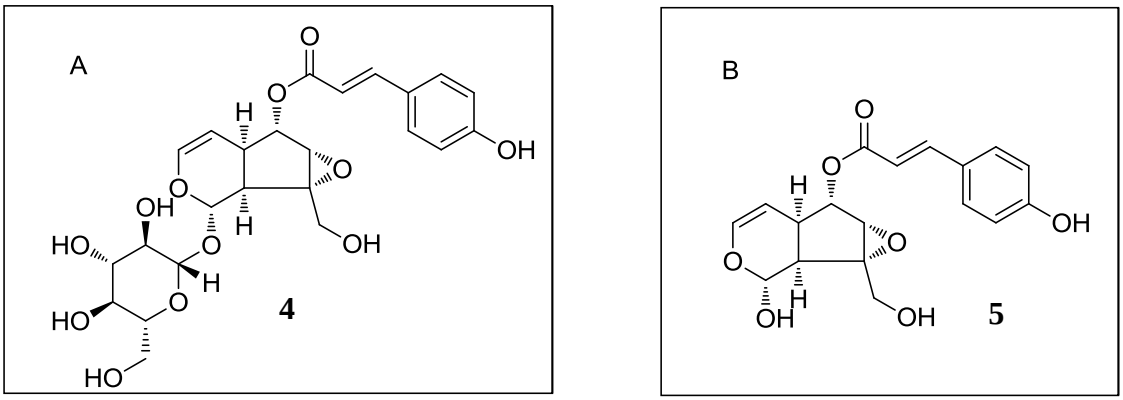



**Figura 2.** Estrutura química do iridoide especisosídeo (A) e especiosídeo não glicosilado (B).

A reação de hidrólise glicosídica por enzimas β-glicosidases obtidas de fungos das espécies *Aspergillus* e *Penicillium* já foi demonstrada para várias classes de compostos, como flavonoides e isoflavonoides glicosídicos, saponinas e iridoides glicosídeos (Parshikov et al. 2015; Yan et al. 2016; Schaub et al. 2020). Estes estudos demonstraram que a aglicona pode ter um efeito biológico maior do que o glicosídeo (Mamma et al. 2003). A forma não glicosilada pode apresentar melhora em suas propriedades farmacocinéticas, pois não envolve a etapa de desglicosilação no metabolismo de primeira passagem (intestino delgado-fígado), muitas vezes necessária para a absorção de glicosídeos (Németh et al. 2003). Izumi et al. (2000) investigaram a diferença na absorção da isoflavona aglicona de soja e dos seus glicosídeos em humanos e mostraram que as isoflavonas agliconas foram absorvidas mais rapidamente e em maiores quantidades do que seus glicosídeos. Assim, esta e outras modificações estruturais com adição de grupos funcionais, podem ser realizadas na biotransformação do especiosídeo (Birolli et al., 2015; Hegazy et al., 2015; Zhan et al., 2015).

Os terpenoides são também compostos de grande interesse em estudos reacionais utilizando microrganismos. Estes compostos têm sido descritos como detentores de diversas atividades biológicas como bactericida, fungicida, antiparasitária, anti-inflamatória, analgésica e outras (Yao et al., 2016). Estruturalmente são componentes com sítios para reações hidroxilação/oxidação por microrganismos, as quais são

dificilmente realizadas por síntese química. Sua modificação química por biocatálise, por sua vez, poderia gerar derivados com atividade biológica potencializada, os quais não seriam obtidos por outra técnica (Hegazy et al., 2015; Birolli et al., 2015).

Recentes estudos provaram que o sesquiterpeno quelerina **(6),** o principal componente de *Ferula sinkiangensis* K.M. Shen (Apiaceae), revelou ser um promissor agente terapêutico para o tratamento da doença de Alzheimer atuando na inibição de citocinas inflamatórias de óxido nítrico (NO), fator de necrose tumoral a (IL-6), (TNF-a), ciclooxigenase-2 (COX-2), interleucina-6 (IL-6) e interleucina-1b (IL-1b) em células microgliais BV2 de ratos (Xing et al., 2016). Porém, sua produção em maior escala por síntese química não é possível devido aos múltiplos centros quirais presentes em sua estrutura e a sua instabilidade química. Este sesquiterpeno foi submetido ao processo de biotransformação utilizando calos de *Angelica sinensis* (Oliv.) Diels para a obtenção e avaliação de seus análogos. Esta biocatálise resultou em 14 derivados, dentre os quais, 4 inéditos: **(7)** 14′-hydroxy-(3′S, 4′R, 5′S, 8′R, 9′S, 10′R)-kellerin**, (8)** 6′-ene-14′-hydroxy-(3′S, 4′R, 8′R, 9′S, 10′R)-kellerin**, (9)** 5′, 6′-ene-(3′R, 8′R, 9′S, 10′R)-ferukrin, **(10)** 14′-hydroxy-(3′S, 4′R, 5′S, 8′R, 9′S, 10′R)-deacetylkellerin**)** (Figura 3). Seis deles exibiram melhor atividade anti-inflamatória *in vitro* que o controle positivo, minociclina (Zhou et al. 2016).

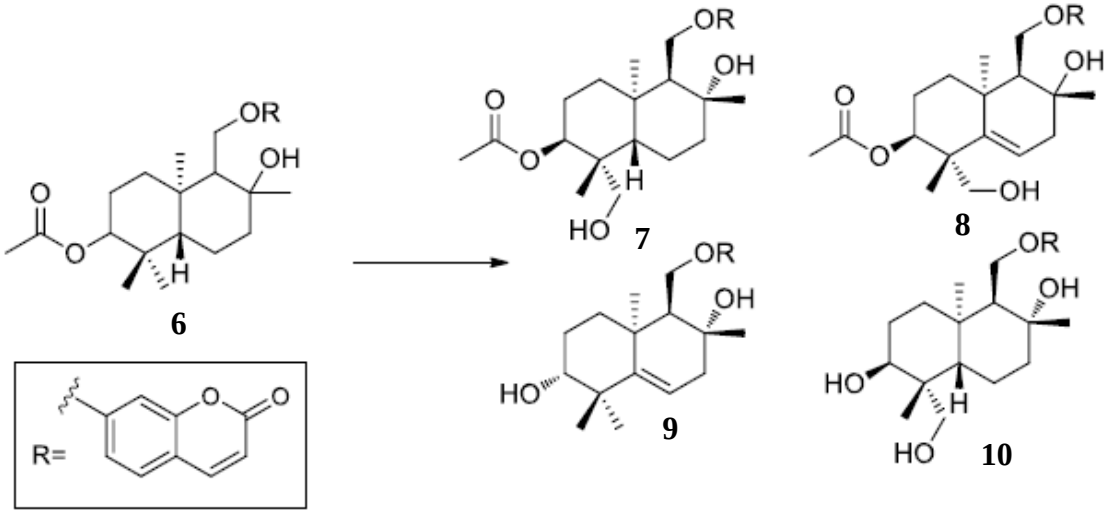



**Figura 3.** Compostos inéditos **(7-10)** obtidos por biotransformação de quelerina **(6)** usando *Angelica sinensis* (Oliv.) Diels.

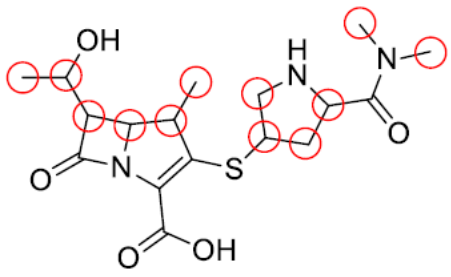
## 1.2 Biotransformação de fármacos

A busca por medicamentos mais potentes e eficazes tem sido alvo de estudos por pesquisadores e indústrias farmacêuticas há décadas. A bioconversão microbiana pode ser utilizada para induzir modificações em estruturas de fármacos já existentes, levando à produção de análogos aos compostos originais. Biotransformações de fármacos anti-inflamatórios, anticânceres e antibimicrobianos tem sido reportado por vários grupos de pesquisa, como meloxicam, exemestano, clindamicina (Ozaki et al., 1972; Shyam et al., 2009; Baydoun et al., 2013; Siddiqui et al., 2020;).

Na área de antimicrobianos, é importante ressaltar que a resistência a esses medicamentos é um dos principais problemas de saúde pública no mundo, o que impulsiona a busca por novos medicamentos. O aparecimento da cepa da bactéria *Staphylococcus aureus* resistente à penicilina, levou a uma imensurável epidemia hospitalar com esses agentes. O antibiótico vancomicina é um dos únicos fármacos que ainda pode ser aplicado para tratar infecções por *S. aureus* resistente à penicilina (Cong, 2020). Diante desse quadro, a biotransformação de antimicrobianos já existentes é uma importante alternativa para criação de novos antibióticos mais potentes e assim novos análogos podem impactar e promover a manutenção da saúde da população.

Espécies de fungos do gênero *Aspergillus*, conhecidas por promover reações de oxidação, hidroxilação, redução, epoxidação (Birolli et al., 2015; Hegazy et al., 2015; Sura et al., 2015; Zhan et al., 2015), podem ser aplicadas para a obtenção desses análogos (Zhan et al., 2015).

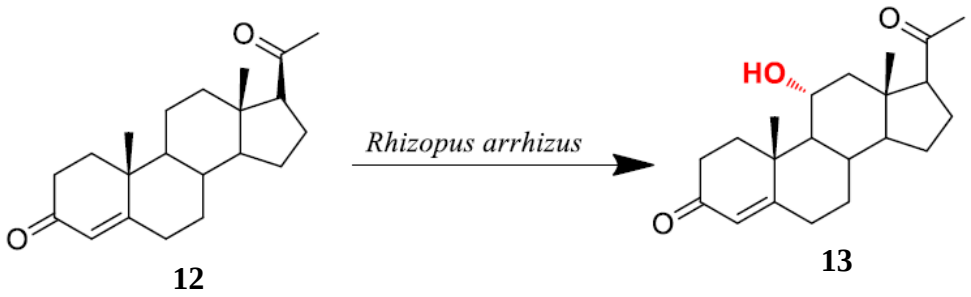
O meropenem (Figura 4), um carbapenêmico que atua sobre bactérias gram-negativas e anaeróbios, também foi um alvo deste trabalho, já que este pode sofrer modificações por microrganismos nos carbonos hibridizados $sp^3$ cíclicos ou acíclicos. Estas reações já foram observadas em estudos de biotransformação de alguns fármacos, como meloxicam e danazol (Shyam et al., 2009).




**11**

**Figura 4.** Estrutura química do fármaco Meropenem, destacando em vermelho carbonos com configuração $sp^3$, prováveis sítios de reação por microrganismos.

Os compostos esteroidais são alvos importantes deste trabalho, já que estes foram um dos pioneiros nos estudos de transformação microbiana e com aplicação bem-sucedida em processos industriais de larga escala (Fernandes et al., 2003). O interesse pelos métodos de biotransformação por via microbiológica se deu em meados de 1952 pelos pesquisadores Peterson e Murray com a utilização de fungos da espécie *Rhizopus* no processo de hidroxilação do substrato progesterona em 11α-hidroxiprogesterona (**12**) (Figura 5) (Asha e Vidyavathi, 2009). Essas reações de 11α-hidroxilação de esteroides também já foram descritas através da aplicação de cepas de *Aspergillus ochraceus*, *A. awamori* e *A. fumigatus* (Fernandes et al., 2003).




**12** **13**

**Figura 5.** 11α-hidroxilação da progesterona (**12**) catalisada por cepas de fungos *Rhizopus arrhizus.*

A estrutura química dos esteroides é geralmente composta por quatro anéis cicloalcanos condensados (Figuras 5 e 6), o que torna difícil a introdução de grupos funcionais em posições específicas por reações químicas convencionais, mas isso foi possível por transformações microbianas (Hegazy et al., 2015; Mohamed et al., 2017; Putkaradze et al., 2017). Os fármacos esteroidais disponíveis no mercado têm uma vasta

gama de propósitos terapêuticos, tais como anti-inflamatórios, imunossupressores, progestacionais, agentes diuréticos, anabolizantes e contraceptivos. Porém, uma grande variedade de efeitos colaterais é observada durante o tratamento, principalmente com o uso prolongado, como ganho de peso, hiperglicemia, alterações oftalmológicas, hipertensão, insuficiência cardíaca, osteoporose e vários outros (Ericson e Kaye, 2014; Pereira et al. 2010).

O fármaco esteroidal prednisolona, o qual já foi produzido por biotransformação do cortisol e é um dos alvos deste trabalho. Ele é importante no tratamento de diversos processos inflamatórios e em doenças autoimunes, porém também apresenta os efeitos indesejáveis dos corticoides (Fernandes et al., 2003). Por isso alguns estudos de biotransformação já têm sido realizados no intuito de obter análogos com redução de efeitos colaterais e maior potência. O trabalho de Mohamed e colaboradores (2017) mostrou a conversão de prednisolona (**14**) por cepas de *Penicillium aurantiacum* em quatro diferentes derivados (**15, 16, 17** e **18**), sendo que 98% da prednisolona foi convertida, o que demostra sua potencial aplicação em escala industrial. Esses derivados foram obtidos principalmente por reações de hidroxilação, metoxilação e metilação (Figura 6).

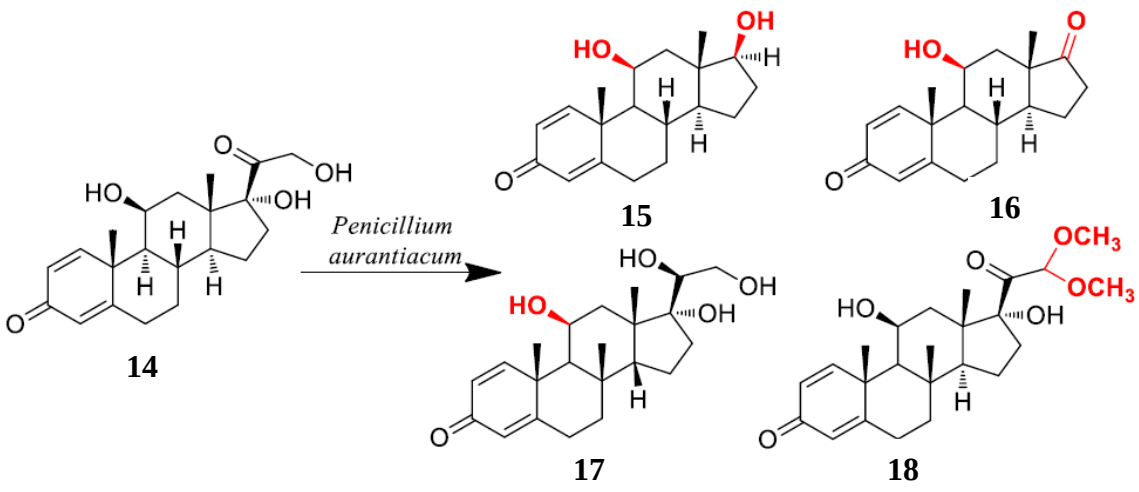



**Figura 6**. Derivados da prednisolona (**14**) obtidos por cepas de *Penicillium aurantiacum.*

Embora a química tenha experimentado um desenvolvimento notável no Brasil, a aplicação de microrganismos e de enzimas para a realização de transformações de compostos orgânicos pode ainda ser mais explorada. Esses apontamentos justificam nosso grande interesse na obtenção de análogos com potencial atividade biológica

produzidos por biotransformação utilizando como alvos importantes fármacos e compostos de origem natural.

# 2 OBJETIVOS

## 2.1 Objetivos gerais

Explorar modificações estruturais de compostos com interesse farmacológico, tanto de origem natural quanto sintética, através da técnica de biotransformação usando diferentes cepas de fungos.

## 2.2 Objetivos específicos

- Extração, isolamento, purificação e caracterização estrutural de metabólitos bioativos (alvos) de plantas, incluindo um iridoide (especiosídeo, casca do caule de *Tabebuia aurea* (Silva Manso) Benth. & Hook. F. ex S. Moore) e um sesquiterpeno oxigenado (óleo das partes aéreas da *Bacopa gratioloides* (Cham.) Edwall).
- Biotransformação dos compostos supracitados e dos seguintes fármacos: meropenem, clindamicina, fluconazol, prednisolona e metilprednisolona utilizando diversas cepas de fungos.
- Isolamento dos compostos de biotransformação de interesse e caracterização estrutural por técnicas de ressonância magnética nuclear e espectrometria de massas.
- Avaliar as atividades biológicas de interesse para análogos obtidos e isolados como antibacteriana, antifúngica, anti-inflamatória e anticâncer de acordo com cada precursor.

# Capítulo 1

# 3 NEW DERIVATIVES OF THE IRIDOID SPECIOSIDE FROM FUNGAL BIOTRANSFORMATION

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**Abstract**

Iridoids are widely found from species of *Bignoniaceae* family and exhibit several biological activities, such as anti-inflammatory, antimicrobial, antioxidant and antitumor. Specioside is an iridoid found from *Tabebuia* species, mainly in *Tabebuia aurea*. Thus, here fungus-mediated biotransformation of the iridoid specioside was investigated by seven fungus. The fungus-mediated biotransformation reactions resulted in a total of nineteen different analogs by fungus *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus japonicus*, *Aspergillus terreus*, *Aspergillus niveus*, *Penicillium crustosum* and *Thermoascus aurantiacus*. Non-glycosylated specioside was the main metabolite observed. The other analogs were yielded from ester hydrolysis, hydroxylation, methylation and hydrogenation reactions. The non-glycosylated specioside and coumaric acid were yielded by all fungi-mediated biotransformation. Thus, fungus applied in this study showed the ability to perform hydroxylation and glycosidic, as well as ester hydrolysis reactions from glycosylated iridoid.

**Keywords:** non-glycosylated specioside, *Aspergillus niger*, ester hydrolysis, methylation, hydroxylation.

**Key points**

- The biotransformation of specioside by seven fungi yielded nineteen analogs
- The non-glycosylated specioside was the main analog obtained
- Ester hydrolysis, hydroxylation, methylation, hydrogenation reactions were observed

## Introduction

Iridoids are widely distributed in plants from different families, such as *Bignoniaceae, Scrophulariaceae, Rubiaceae, Verbenaceae, Oleaceae, Acanthaceae, Pedaliaceae, Labiatae, Loganiaceae, Ericaceae, Liliaceae, Apocynaceae* and *Euphorbiaceae* (Cao et al. 2019). Several biological activities have been reported for iridoids, such as anti-inflammatory, antiviral, antimicrobial, neurotrophic, antioxidant and antitumor. For this reason, they are studied for many research groups for the determination of new biological active compounds (Schröder et al. 2016).

Specioside is an iridoid found in *Tabebuia* species (*Bignoniaceae*), and it is abundant in *Tabebuia aurea* (Silva Manso) Benth. & Hook. F. ex S. Moore (Cao et al. 2019; Malange et al. 2019). Its pharmacological potential includes antioxidant (Elusyian et al. 2011) and anti-amoebic (Bharti et al. 2006) activities, as well as it also demonstrated to alleviate stress and to prolong lifespan of the free-living nematode *Caenorhabditis elegans* (Ashtana et al. 2015). In addition, high anti-inflammatory, anti-hemorrhagic and anti-myotoxic properties have also been reported for specioside and extracts from *Tabebuia aurea* stem bark with high amount (80%) of specioside (Reis et al. 2014). Recently, an inhibition of 80% of the recruitment of leukocytes into the peritoneal cavity in mice injected with carrageenan was reported for specioside (Nocchi et al. 2020).

The production of analogs from bioactive compounds is an interest technique to obtain new derivatives. Chemical synthesis, a conventional approach, can be used to induce chemical modifications, but they can yield artefacts in reactions from labile sites of molecules and produce toxic chemical waste (Mohammed et al. 2020), beyond the aimed analogs cannot be produced. Specioside, for example, can be cleaved in labile bonds, such as coumaric acid ester, epoxide and glycosidic bonds by conventional chemical reactions and an alternative for this purpose is biotransformation reactions.

The biotransformation by microorganisms (fungi and bacteria), enzymes and cells is another approach to induce chemical modifications and to yield analogs from pharmaceutical and bioactive compounds. It is considered an important tool to produce new drug analogs and it has become increasing uses in industrial applications (Flores et al. 2020; Chandra et al. 2020). Differently of chemical methods, the biotransformation reactions are commonly stereo- and regio-selective, besides they are lower costs, environmental friendly, the reactions are milder conditions and with simple methods (Yousuf et al. 2019; Hegazy et al. 2015; Perkins et al. 2015).

Fungi have been applied as biocatalysts and simplify, or even enable, for the production of complex and intermediate analogs, introducing substituents and modifications in nonactivated positions with stereospecificity. Besides they can also eliminate various stages of chemical synthesis by a single step of biotransformation (Hegazy et al. 2015; Perkins et al. 2015). Numerous studies have shown the ability of fungi (such as *Thermoascus aurantiacus* and species of *Aspergillus* and *Penicillium*) to produce several enzymes such as β-glucosidases, which are able to hydrolyze (1,3)-, (1,4)- and (1,6)-β-glycosidic bonds, as well as aryl β-glucosides (Kudou et al. 1991; Yan et al. 2016; Schuerg et al. 2017). These enzymes promote efficiently β-glucosides hydrolysis from primary metabolites, such as disaccharides and oligosaccharides, and from secondary metabolites, such as flavonoids, steroids and saponins. In addition, they can also degrade several biopolymers, such as starch, hemicellulose, pectin and proteins (Kudou et al. 1991; Yan et al. 2016, Gao et al. 2014; Mamma et al. 2004).

Other types of reactions have also been reported for the production of analogs by fungi-mediated reactions, including hydroxylation, structural rearrangements, oxidation, ester hydrolysis, methylation, and hydrogenation (Kostrzewa-Susłow and Janeczko 2014; Zhong et al. 2008; Parshikov and Sutherland 2015; Zohri and Mostafa 2000; Schuerg et al. 2017; Zhan et al. 2015; Peart et al. 2013). Therefore, the use of fungi would enable the formation of different products, since the target compound of the present work, the iridoid specioside, has several sites available for molecular modifications.

Thus, the objective this work was to perform molecular modifications on iridoid specioside, by fungi-mediated biotransformation reactions applying the new strains *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus japonicus*, *Aspergillus terreus*, *Aspergillus niveus*, *Penicillium crustosum* and *Thermoascus aurantiacus*, as well as its reaction by microwave, acidic and basic hydrolysis.

## Materials and Methods

### *Plant Material*

The stem bark of *Tabebuia aurea* were collected in Campo Grande, MS, (20°26′37″ S, 54°38′52″ W). It was identified by Flavio Macedo Alves and a voucher was deposited in the CGMS herbarium of the Federal University of Mato Grosso do Sul, under

number 74328. The access of plant material was registered at Sistema Nacional de Gestão do Patrimônio Genético (SISGEN) under number A15EB96.

*Extraction and purification of specisoside*

2.9 kg of *T. aurea* stem barks were dried and powdered, subsequently this material was exhaustively extracted by percolation using ethanol and water 7:3 (v/v) (flow of 30 drops/ min and 2,6 L of extractor solvent) for 48 h. The extract was concentrated by rotary evaporator and lyophilized to yield a crude extract (606.49 g). This crude extract (189.11 g) was solubilized at methanol, maintained with the resin Amberlite XAD2 for 2 h under agitation. Then, this Amberlite XAD2 resin was washed with approximately 5 L of deionized water and followed by washing with methanol (5 L) to obtain a fraction without sugar.. The methanol fraction was submitted to precipitation appling cold deionized water and the precipitate was recristalized to obtain the isolated iridoid specioside (18.65 g, 94% chromatographic purity), which was analyzed by LC-DAD-MS and NMR to characterize its structure, as described by Nocchi et al. (2020).

*Identification of microorganisms*

Seven fungal strains were used in the biotransformation reactions, which were identified by morphology and molecular biology, and they provided from the library of Federal University of Mato Grosso do Sul (UFMS). The strains *Aspergillus terreus*, *Aspergillus niveus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus japonicus*, *Penicillium crustosum*, and *Thermoascus aurantiacus* were morphologically identified by MSc Clarice Rossato Marchetti (according to Bergey's Manual of Determinative Bacteriology). After morphological identification procedures, the fungi were cultivated in inclined tubes containing PDA (potato, 100 g; dextrose, 7.5 g; agar, 9.1 g; water, 500 mL) and replanted periodically for the maintenance of the cultures, which were incubated for a period of 4 to 5 days at 30 ˚C.

The molecular identification of fungi followed the experimental procedures of the methodological pipeline for molecular analysis previously described in the literature, where the 16S ribosomal DNA genes were amplified by PCR from the genomic DNA after phenol-chloroform method (Garces et al. 2016). Each genomic DNA extracted was quantified and performed as template for PCR using specific oligonucleotides ITS1 (5′-

TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The PCR amplification steps were performed by the initial cycle of denaturation at 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec, 15 sec at 55˚C for ITS1 and ITS4 primer annealing, extension at 72˚C (2 min) and a final cycle at 72 ºC for 7 min for the extension. The amplified products were purified and sequenced by Sanger technology in a 3500 Genetic Analyzer sequencer (Applied Biosystems) by Senai Co. Ltd. (Rio de Janeiro, Brazil).

The ITS DNA sequences obtained in this study and other sequences, obtained from literature, were aligned using the CLUSTAL W program (Larkin et al. 2007). The Geneious software (Kearse et al. 2012) version 6. (Biomatters) was used to analyze the ITS DNA sequences of all strains obtained and they were compared using the BLASTN tool (http://blast.ncbi.nlm.nih.gov) with other specie-types fungi previously deposited in the NCBI (National Center for Biotechnology Information) GenBank (Agarwala et al. 2018). In this case, each species was BLASTed and identified from the best-scoring reference sequence data of the blast output with an identity >98.5% compared for query sequenced.

The phylogenetic analyses were performed using the MEGA v 6.0 software (Tamura et al. 2013) using the *neighbor-joining* algorithm and the Kimura 2-P nucleotide replacement model (Kimura 1980). The stability of the relationships in the phylogenetic tree was obtained using resampling (Bootstrap) with 1.000 repetitions and the region sequences was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model (Harris and Stocker, 1998). After completed analysis, the sequence data from new strains were compared with sequences of specie-types of fungi previously deposited in NCBI (*A. terreus* ATCC 1012, *A. niveus* NRRL 5505, *A. niger* ATCC 16888, *A. flavus* ATCC 16883, *A. japonicus* CBS 114.51, *P. crustosum* FRR 1669, *T; aurantiacus* ATCC 204492) (Agarwala et al. 2018) and then the ITS of new strains were deposited at NCBI GenBank under the following accession numbers: *A. terreus* (MW592440), *A. niveus* (MW592376), *A. niger* (MW603826), *A. flavus* (MW590627), *A. japonicus* (MW587714), *P. crustosum* (MW600274), and *T. aurantiacus* (MW603821).

The fungal strains were deposited in the libraries URM (University Recife Mycology) of Federal University of Pernambuco (UFPE) and MMBF (Micoteca Mario Barreto Figueiredo), which are inserted in the World Data Centre for Microorganisms

under number WDCM 604 and 942, respectively. *T. aurantiacus* (CBMAI 756) was previously deposited at Brazilian Collection of Microorganisms from the Environment and Industry CBMAI – WDCM 823. *A. niveus* and *A. terreus* were deposited in URM at numbers URM 7545 and URM 7461, and *A. niger*, *A. japonicus* and *P. crustosum* in MMBF at numbers MMBF 02/2021, 01/2021, and 03/2021.

*Biotransformation assay*

Liquid culture medium was prepared with the following components: $K_2HPO_4$ (0.075 g), $MgSO_4.7H_2O$ (0.075 g), proteose peptone (0.500 g), glucose (0.609 g) and deionized water (50 mL). After fungi inoculation, the medium was incubated at 30 °C under constant stirring (110 rpm) for 48 h and specioside (50 mg prepared in 200 µL of methanol) was added to the culture medium. After 15h of reaction, aliquots of 400 µL of the medium were extracted with ethyl acetate (200 µL, three times) and analyzed by liquid chromatography coupled to diode array detector and mass spectrometer (LC-DAD-MS) and thin layer chromatography (TLC) (Silica gel, $UV_{254}$, Sigma-Aldrich). For TLC, the eluent solvent was composed by hexane, ethyl acetate, and methanol (50:30:20 v/v) added and formic acid (1%). Then, the reactions were monitored every 2h until total consumption of the starting material. The biotransformation reactions were performed in duplicate. The reactions without specioside (contained only the culture medium and the strains) were used as blank and the controls were composed by the culture medium and specioside (without fungi). The blank and control samples were analyzed by LC-DAD-MS to confirm the products determined here were yielded from biotransformation reactions of specioside.

After total consumption of the starting material (determined by TLC analyses), the mycelium was filtered, and the culture medium was extracted with ethyl acetate. The organic phase was dried over $Na_2SO_4$ and the solvent removed under reduced pressure.

*Analyses by LC-DAD-MS*

The samples (biotransformation reactions, blanks and controls) were solubilized in methanol (1 mg/mL) and water deionized (7:3, v/v), filtered on syringe filters (PTFE, Millex 0.22 µm, Millipore®) and 1 µL was injected into the chromatographic system. For these analyses, a Shimadzu LC-20AD UFLC chromatograph coupled to a diode array

detector and a mass spectrometer ESI-QTOF (MicroTOF III, Bruker Daltonics, Billerica, MA, USA) were used. The analyses were monitored between the wavelenghts 240-800 nm and acquired initially in negative and positive ion modes (*m/z* 120-1200), and after only negative ion mode was applied, because more peaks were detected in this ion mode. MS/MS spectra were acquired by automatic method using a collision energy 45 to 65 eV and nitrogen was applied as collision gas. Kinetex C-18 chromatography column (Phenomenex, 2.6 μm, 150 x 2.1 mm) was used and the mobile phase was composed by ultrapure water (solvent A) and acetonitrile (solvent B), both added 0.1% formic acid. The gradient elution profile was the following: 0-2 min - 3% of B; 2-25 min - 3-25% of B; 25-40 min - 25-80% of B; 40-43 min - 80% of B. The flow rate was 0.3 mL/min and the oven temperature was 50 ˚C.

Data were processed by Data Analysis software version 4.2 (Bruker) and compounds were identified based on spectral data (UV, MS and fragmentation profile). The molecular formulas were determined based on the accurate mass considering errors up ±5 ppm and mSigma below 30.

*Acidic and alkaline hydrolysis*

In a two-necked round-bottom flask equipped with a magnetic stirring bar were added 0.201 mmol (102.28 mg) of specioside and 10 mL of HCl (1M). The mixture was stirred at room temperature for 1 h. The whole mixture was extracted with ethyl acetate (10 mL × 3). The combined organic phases were washed with distilled water, dried over $Na_2SO_4$, and concentrated *in vacuo*. A crude product was analyzed by LC-DAD-MS.

The same procedure was used for basic hydrolysis, however, using 10 mL of NaOH (1 M).

*Microwave hydrolysis*

To oven-dried microwave vial (10 mL, CEM Focused Microwave Synthesis System, model Discover) a solution of 0.1 mmol (51.14 mg) of specioside in 5 mL of ethanol was added. The reaction tube was sealed and irradiated at a ceiling temperature of 70 °C using 100 W microwave power for 30 min. The reaction mixture as cooled with

an air flow. The mixture was extracted with ethyl acetate (10 mL x 3). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure.

## Results

### *Identification of microrganisms*

The fungal strains were morphological analyzed and subsequently they were also molecularly identified comparing the sequences described in NCBI/GeneBank for ITS sequences with higher identity than 97%. Thus, the sequences of these comparisons with NCBI were the following of reference sequence or accession numbers: *Aspergillus terreus* ATCC 1012 - NG_055723, *Aspergillus niveus* NRRL 5505 – NR_137476.1, *Aspergillus niger* ATCC 16888 – NR_111348.1, *Aspergillus flavus* ATCC 16883 – NR_111041.1, *Aspergillus japonicus* CBS 114.51 – NR_131268.1, *Penicillium crustosum* FRR 1669 – NR_077153.1, *Thermoascus aurantiacus* ATCC 204492 – KC585413.1*.

### *Fungi-mediated biotransformation reactions*

The biotransformation of the specioside was carried out by the fungi *A. terreus, A. niveus, A. niger, A. flavus, A. japonicus, P. crustosum* and *T. aurantiacus* and they resulted in a total of nineteen different analogs annotated (Fig. 1, Table 1). Each fungus produced a different number of metabolites, but common compounds were also observed among them. The blanks and controls do not exibhited theses peaks, confirming that they were obtained by fungi-mediated biotransformation and thus they are not degradation products from speciocise, as observed in the analyses of control samples (Fig. S1 – Supplementary). The main products were yielded from glycosidic and ester hydrolysis, hydroxylation, methylation and hydrogenation reactions.

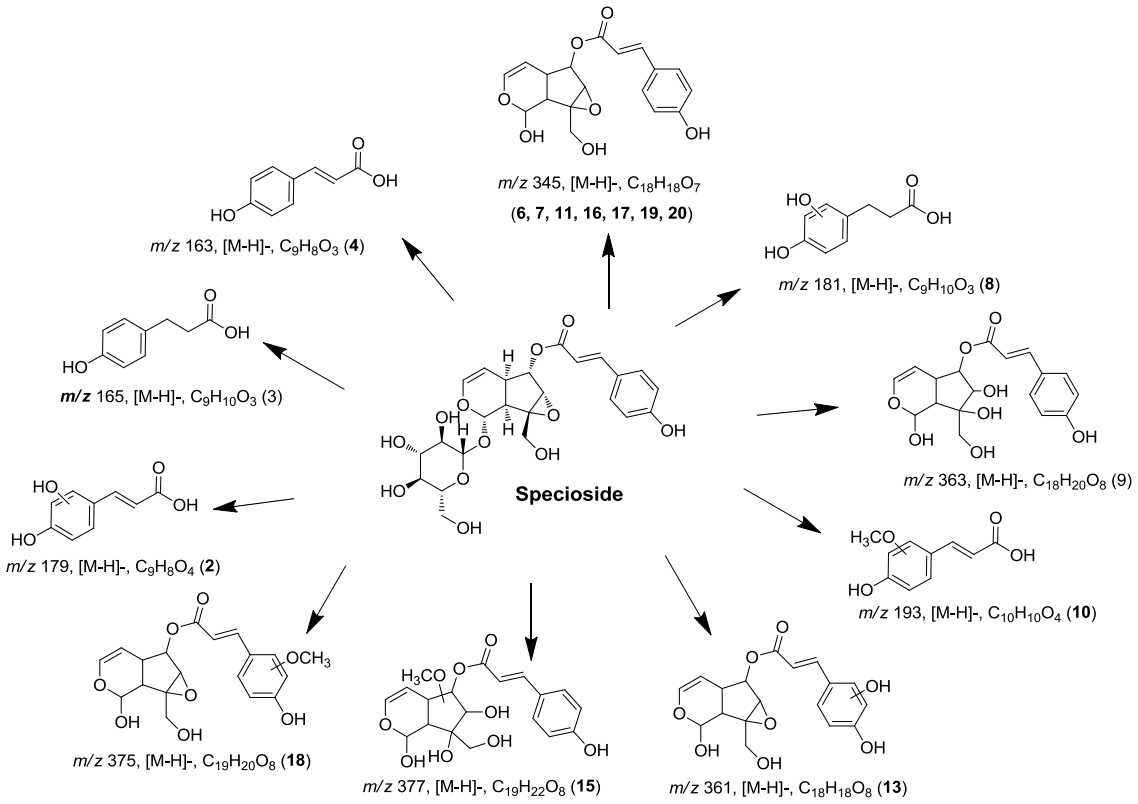


**Fig. 1** Chemical structures of the iridoid specioside derivatives obtained by fungal biotransformation reactions.

The biotransformation reactions were analyzed by LC-DAD-MS. The data from products were summarized on **Table 1** and all the spectra are illustrated in Supplementary Material (**Fig. S2-S25**)

The specioside (Table 1, peak **14**) displayed an ion at *m/z* 507.1498 $[M-H]^-$ with the molecular formula $C_{24}H_{28}O_{12}$. Its main fragment ions were at *m/z* 345.0974 obtained through the cleavage of the glycosidic bond $[M-H-162]^-$, *m/z* 231.0658 yielded by the ring-open reaction on the basis of the isomerization of the hemiacetal group, and *m/z* 163.0398 and 145.0293 indicated the existence of the coumaroyl group (Li et al. 2017).

The chromatogram of all fungus-mediated biotransformation exhibited an intense peak relative to non-glycosylated specioside (Fig. 2, peak **16**). This compound demonstrated an intense ion at *m/z* 345.0968 $[M-H]^-$ consistent with the molecular formula $C_{18}H_{18}O_7$ and exhibited the fragment ions *m/z* 163.0242 and 145.0284 relative to coumaric acid. In addition, the UV spectrum of **16** revealed absorption wavelength bands at 297 and 312 nm that indicated the existence of the coumaroyl group in its structure.

The compounds **6**, **7**, **11**, **17**, **19** and **20** exhibited the same molecular formula, fragmentation pathway and UV spectra observed for **16**; $C_{18}H_{18}O_7$ (*m/z* 345 $[M-H]^-$), suggesting isomers of **16**. Thus, deglycosylation and isomerization reactions could be observed in these fungus-mediated biotransformations. These compounds were not produced by all fungi tested. Peak **6**, for example, was obtained by *A. flavus* and *A. niveus*, the compound **7** was obtained by *A. niger*, *A. flavus*, *A. niveus* and *A. japonicus*, whereas the **11** was obtained by *A. niger*, *A. flavus*, *A. japonicus* and *A. niveus*. Finally, the compounds **17**, **19** and **20** were only produced by *A. niger* and *A. japonicus*.

The analog **9** (*m/z* 363.1132, $[M-H]^-$, $C_{18}H_{19}O_8$) was observed with 18 *u* added to **16**. After of the glycosidic hydrolysis, the epoxide ring opened by hydration, explaining the presence of an additional hydroxyl group. The fragment ions *m/z* 163.0398 and 145.0293, as well as UV spectrum ($\lambda_{max} \approx 290$ and 312 nm) indicated the existence of the coumaroyl group without modifications from **9**. This compound was obtained by *A. niger* and *A. flavus*-mediated biotransformation. The peak **15** presented an intense ion at *m/z* 377.1243 $[M-H]^-$ consistent with the molecular formula $C_{19}H_{22}O_8$ and exhibited an additional methyl group in relation to the **9**. Its fragment ion at *m/z* 345 $[M-H-32]^-$ is yielded by methanol molecule loss, which indicated a non-aromatic methoxyl group. In addition, the product ions at *m/z* 163.0398 and 145.0293, relative to coumaric acid, also indicated no modification on coumaroyl group, corroborating with a methoxylation on iridoid structure part. Thus, analog **9** was putatively identified as coumaroyl-methoxy-iridoid, and it was observed from *A. niger*-, *A. flavus*- and *A. niveus*-mediated biotransformations.

The compound **13**, produced by *A. niger*, *A. flavus* and *A. niveus*, exhibited an ion at *m/z* 361.0915 $[M-H]^-$, confirming the molecular formula $C_{18}H_{18}O_8$ and showed absorption bands at the wavelengths 291 and 327 nm from UV spectrum. Beyond the sugar loss by hydrolysis, the fragment ions at *m/z* 179 and *m/z* 161 suggested the hydroxylation on coumaric acid region, since they are compatible with hydroxy-coumaric acid. The peak **18**, *m/z* 375.1114 $[M-H]^-$ ($C_{19}H_{20}O_8$), was observed from biotransformation reactions by *A. niger* and *A. japonicus*, and it showed an additional methyl group compared to analog **13**. The methylation ocurred in an hydroxyl group of hydroxy-coumaric acid unit, since its fragmentation yielded the product ion *m/z* 175 ($C_{10}H_7O_3^-$) relative to methoxy-coumaric acid and subsequently it lost a radical methyl ($^{\bullet}CH_3$, 15 *u*) yielding the ion *m/z* 160.

The peak **4** showed an UV spectrum similar with coumaric acid chromophore ($\lambda_{max}$ =299 and 307 nm) (Caderby et al. 2013) and presented the molecular formula $C_9H_8O_3$ compatible to coumaric acid. All fungi tested produced this compound, except *T. aurantiacus*. The compound **3** (*m/z* 165.0550, $[M-H]^-$, $C_9H_{10}O_3$) was obtained from *A. japonicus*-, *A. terreus*- and *T. aurantiacus*-mediated biotransformations. It showed the presence of two additional hydrogens and different UV spectra compared to analog **4**. So, analog **3** was identified as dihydrocumaric acid (Owen et al. 2003), which was yielded from reduction of double bond in coumaric acid by hydrogenation reaction.

The compound **2**, produced by *A. japonicaus, A. niger and T. aurantiacus*-mediated biotransformations, exhibited the deprotonated ion at *m/z* 179.0347 confirming the molecular formula $C_9H_8O_4$. This compound showed an additional hydroxy group compared to analog **4** and it was identified as caffeic acid by comparison with authentic standard (Guo et al. 2008). The peak **8** (*m/z* 181.0494 $[M-H]^-$) was observed from reactions with *A. terreus* and *A. niveus*, revealing the additional two hydrogens compared to compound **2.** Thus, the analog **8** was putatively annotated as hydroxy-dihydro-coumaric acid, as already described by Guy et al. (2009), yielded from the reduction of double bond linked to carbonyl group. Whereas the compound **10** (*m/z* 193.0533, $[M-H]^-$, $C_{10}H_{10}O_4$) produced by *A. terreus, A. japonicas, A. niger* and *A. niveus*, presented a methyl group additional in relation to **2** and showed UV spectrum similar to ferulic/caffeic acid chromophore ($\lambda_{max}$ = 292 and 330 nm) (Arni et al. 2010). The analog **10** was annotated as methoxy-coumaric acid.

**Table 1** Compounds identified from biotransformation of specioside by *A. niger*, *A. terreus*, *A. niveus*, *A. japonicus*, *A. flavus*, *P. crustosum*, and *T. aurantiacus*.

| Peak | RT (min) | UV ($\lambda_{max}$) | MF | Negative ion mode (*m/z*) | | Compound | Type reactions | Fungi |
|---|---|---|---|---|---|---|---|---|
| | | | | MS [M-H]$^-$ | MS/MS | | | |
| 1 | 9.4 | 280 | $C_8H_{20}O_9$ | 259.1058 | - | Unknow | - | *A. niger* |
| 2 | 10 | 291, 323 | $C_9H_8O_4$ | 179.0347 | - | Hydroxycoumaric acid | Hydrolysis, hydroxylation | *A. japonicus; T. aurantiacus* |
| 3 | 12.6 | 288 | $C_9H_{10}O_3$ | 165.0550 | - | Dihydrocoumaric acid | Hydrolysis, hydrogenation | *A. japonicus; A. terreus; T. aurantiacus* |
| 4 | 13.5 | 299, 309 | $C_9H_8O_3$ | 163.0384 | | Coumaric acid | Hydrolysis | *A. niger; A. terreus, A. niveus; A. japonicus; A. flavus; P. solitum* |
| 5 | 15.7 | 285 | $C_{21}H_{16}NO_2$ | 314.1175 | - | Unknow | - | *A. niger* |
| 6 | 16.1 | 299, 310 | $C_{18}H_{18}O_7$ | 345.1003 | 145. | Coumaroyl iridoid | Hydrolysis | *A. flavus; A. niveus* |
| 7 | 17.3 | 299, 310 | $C_{18}H_{18}O_7$ | 345.0957 | 145; 163. | Coumaroyl iridoid | Hydrolysis | *A. niger; A. flavus; A. japonicus; A. niveus* |
| 8 | 17.9 | 287 | $C_9H_{10}O_4$ | 181.0494 | - | Hydroxy-dihydro-coumaric acid | Hydrolysis, hydroxylation, hydrogenation | *A. terreus; A. niveus* |
| 9 | 18.0 | 290, 313 | $C_{18}H_{20}O_8$ | 363.1132 | 145; 159; 163; 181; 187; 345 | Coumaroyl- hydroxy-iridoid | Hydrolysis, hydroxylation | *A. niger; A. flavus* |
| 10 | 20.3 | 292, 330 | $C_{10}H_{10}O_4$ | 193.0533 | 175 | Methoxy-coumaric acid | Hydrolysis, hydroxylation, methylation | *A. terreus; A. japonicus; A. niveus.* |
| 11 | 21.0 | 293, 311 | $C_{18}H_{18}O_7$ | 345.0984 | 145; 163. | Coumaroyl iridoid | Hydrolysis | *A. niger; A. flavus; A. japonicus; A. niveus* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 12 | 21.2 | 280, 320 | $C_{22}H_{28}NO_3$ | 354.2059 | 169; 180; 197; 199; 223 | Unknow | - | *A. niger* |
| 13 | 21.4 | 291, 327 | $C_{18}H_{18}O_8$ | 361.0915 | 161; 175; 179; 185; 187; 203. | Hydroxy-Coumaroyl iridoid | Hydrolysis, hydroxylation | *A. niger; A. flavus; A. niveus* |
| 14 | 22.0 | 299, 312 | $C_{24}H_{28}O_{12}$ | 507.1493 | 345; 327; 231; 203; 187; 163; 145 | Specioside | - | *A. niger; A. niveus; A. flavus; A terreus; A. japonicus; P. solitum; T. aurantiacus* |
| 15 | 23.0 | 292, 310 | $C_{19}H_{22}O_8$ | 377.1243 | 145; 163; 187; 203, 345 | Coumaroyl-methoxy-iridoid | Hydrolysis, hydroxylation, methylation | *A. niger; A. niveus; A. flavus; A terreus; A. japonicus* |
| 16 | 24.9 | 297, 312 | $C_{18}H_{18}O_7$ | 345.0968 | 145; 158; 163; 172; 187; 203; 231 | Coumaroyl iridoid | Hydrolysis | *A. niger; A. terreus, A. niveus; A. japonicus; A. flavus; P. solitum; T. aurantiacus* |
| 17 | 25.7 | 292, 315 | $C_{18}H_{18}O_7$ | 345.1015 | 145.0276 | Coumaroyl iridoid | Hydrolysis | *A. niger; A. japonicus* |
| 18 | 26.4 | 290, 330 | $C_{19}H_{20}O_8$ | 375.1114 | 160.0157; 175.0393; 202.0668 | Methoxy-coumaroyl iridoid | Hydrolysis, hydroxylation, methylation | *A. niger; A. japonicus* |
| 19 | 27 | 293, 310 | $C_{18}H_{18}O_7$ | 345.0984 | - | Coumaroyl iridoid | Hydrolysis | *A. niger; A. japonicus* |
| 20 | 28.3 | 290 316 | $C_{18}H_{18}O_7$ | 345.0997 | - | Coumaroyl iridoid | Hydrolysis | *A. niger; A. japonicus* |

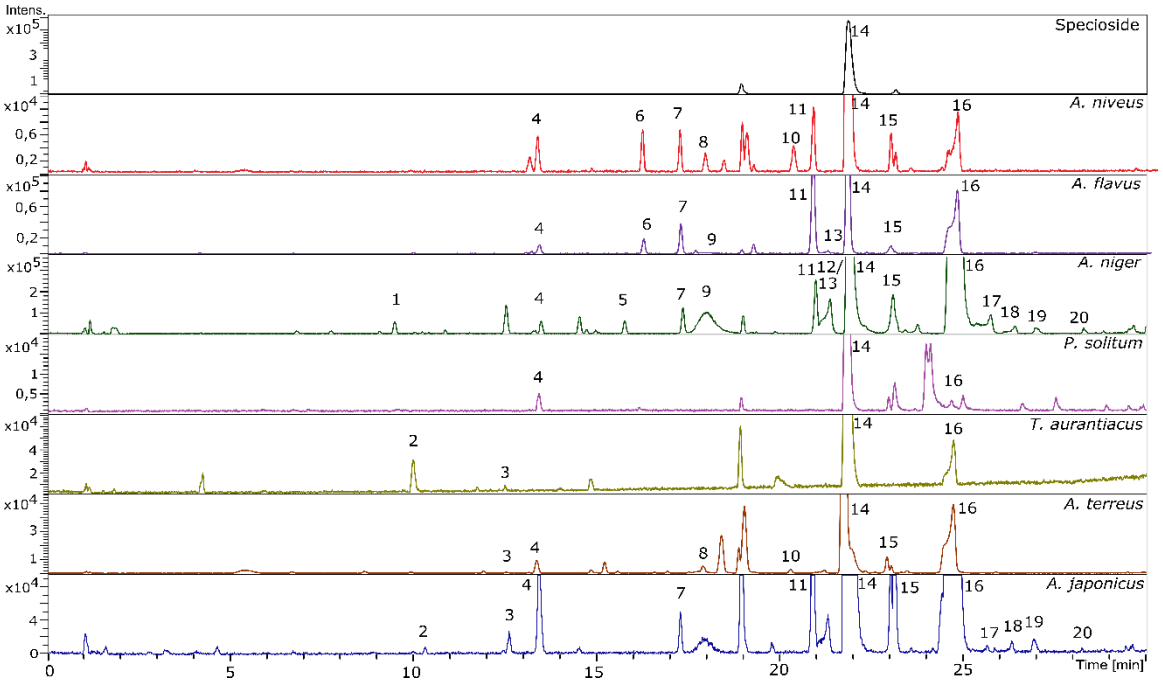


**Fig. 2** Base peak chromatogram obtained by LC-DAD-MS analyses from fungi-mediated biotransformations of specioside. The first chromatogram is related to iridoid specioside isolated from the stem bark of the *Tabebuia aurea*. The spectral data and annotation of derivatives are described in Table 1.

*Reaction Kinetic by A. niger-mediated biotransformation*

The compound **16** (Fig. 1), specioside in the non-glycosylated form, was the main metabolite observed in all fungi-mediated biotransformation reactions. Thus, we monitored the decay from specioside and production of its main analogs. The first monitoring was carried out with 15 h of reaction and then every 2 h.

The biotransformation reaction was monitored by LC-DAD-MS and the non-glycosylated specioside (**16**) was verified after 15 h reaction, which reached its greatest intensity after 46 h. At this time, small ion intensities of other analogs were also observed, as well as a significant decay of specioside. After 48 h of incubation, the metabolite **16** started to be catalyzed and at the same time the intensity of peak **4** (coumaric acid) increased (Fig. 3).

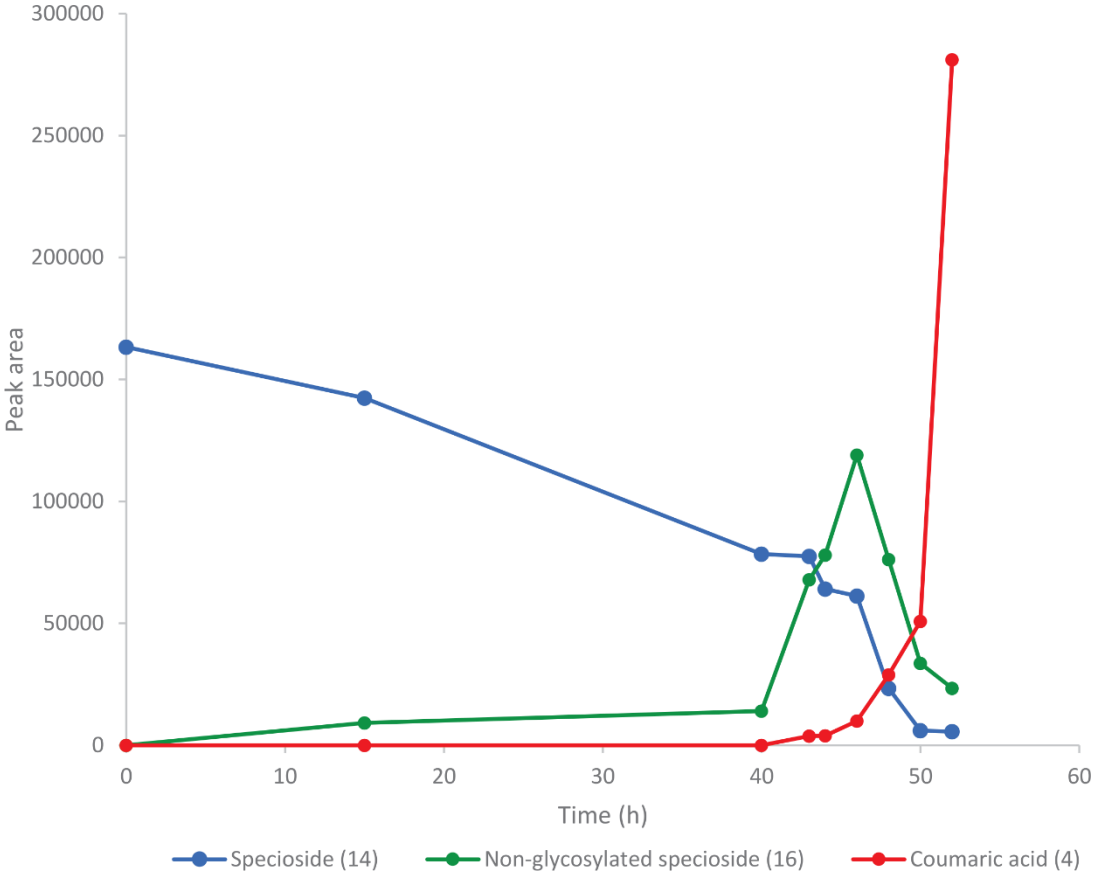


**Fig. 3** Specioside (**14**) decay curve and production of non-glycosylated specioside (**16**) and coumaric acid (**4**) by biotransformation by *Aspergillus niger*.

*Hydrolysis reactions by acid, alkaline and microwave*

The iridoid specioside was also reacted using acid, base and hydrolysis by microwave to verify if the hydrolysis products observed from fungi-mediated biotransformation reactions could be the same from these reactions. The products of each reaction were analyzed by LC-DAD-MS, but the iridoid specioside was completely degraded in all reactions and did not yield the desired non-glycosylated product. In the basic hydrolysis reaction, the presence of coumaric acid was verified, but no other analog was observed, suggesting the degradation from specioside in the reaction medium.

## Discussion

In this study, we initially evaluated fungi-mediated biotransformation reactions of specioside applying the fungi *A. terreus*, *A. niveus*, *A. niger*, *A. flavus*, *A. japonicus*, *P.*

*crustosum*, and *T. aurantiacus.* Fungi have a highly specific enzymatic apparatus, which could carry out reactions such as glycosidic hydrolysis, preserving other reactive sites.

Several studies have shown the ability of fungi, such as *T. aurantiacus* and species of *Aspergillus*, and *Penicillium*, to produce β-glucosidase enzymes that able to hydrolyze β-glycosidic bonds (Kudou et al. 1991; Yan et al. 2016; Schuerg et al. 2017). Yan et al. (2016) identified, purified, and characterized an extracellular β-glucosidase produced by *A. terreus* and they were applied for the hydrolysis of soybean glycosylated isoflavones. The enzyme efficiently converted isoflavone glycosides to aglycones, with a hydrolysis rate of 95.8% for daidzin, 86.7% for genistin, and 72.1% for glycitin. In present work, the presence of β-glucosidases could also be suggested since all fungi-mediated biotransformation reactions afforded the non-glycosylated specioside (**16**) (*m/z* 345.0968 $[M-H]^-$, $C_{18}H_{18}O_7$), the main compound observed in the reactions, preserving other reactive sites in the molecule.

The obtention of deglycosylated metabolites by enzymes β-glycosidases from fungi of the species *Aspergillus* and *Penicillium* has already been demonstrated for several classes of compounds, such as flavonoids and isoflavonoid glycosides, saponins and iridoid glycosides (Yan et al. 2016; Parshikov et al. 2015; Schaub et al. 2020). Studies have shown that aglycone may have a greater biological effect than glycoside (Mamma et al. 2004). The non-glycosylated form may show improvement of pharmacokinetic properties, since it does not involve the deglycosylation step in the first-pass metabolism (small intestine-liver) necessary for the absorption of glycosides (Németh et al. 2003). Izumi et al. (2000) investigated the difference in the absorption of soy isoflavone aglycone and glycosides in humans and showed that isoflavone aglycones were absorbed faster and in greater amounts than their glycosides. These appointments suggest the importance of the non-glycosylated specioside performed efficiently here by fungus-mediated biotransformation.

Other derivatives were obtained with lower ion intensities than **16**, such as ester hydrolysis, hydroxylation, methylation and hydrogenation. We could observe by time course of biotransformation that glycosidic hydrolysis analogs were yielded and followed by other reactions. The compounds **2, 8, 9, 10, 13, 15** and **18**, for example, were obtained secondarily by hydroxylation. Besides hydroxylation, the compounds **10, 15** and **18** were also methylated, getting the methoxy group. While the hydrolysis of the ester of specioside molecule was confirmed by the formation of **2, 3, 4, 8** and **10**. Finally, we could observe the hydrogenation of the coumaric acid double bond to afford compounds

**3** and **8**. All these derivatives were observed only in the chromatograms of fungus-mediated biotransformation from the specioside. Thus, both the blanks (culture medium with fungus without the specioside) and the controls (culture medium with the specioside) did not show the chromatographic peaks related to these substances. Therefore, these data confirmed that the biotransformation analogs described here were in fact yielded by fungal biotransformation reactions.

Numerous studies in literature have shown the ability of the fungi evaluated here to perform the reactions described in our study (glycosidic and ester hydrolysis, hydroxylation, methylation and hydrogenation) (Liu et al. 2010; Kostrzewa-Susłow and Janeczko 2014; Zhong et al. 2008; Parshikov and Sutherland 2015; Zohri and Mostafa 2000; Schuerg et al. 2017). Liu et al. (2010) showed that ginsenoside Rf, a ginseng saponin, was deglycosylated by a crude extract using a strain of *A. niger* to produce its aglycone (*20S*)-Protopanaxatriol. 5-hydroxyflavone was hydroxylated yielding 5,4′-dihydroxyflavone by *A. niger*- and *Penicillium chermesinum*-mediated biotransformation reactions (Kostrzewa-Susłow and Janeczko 2014). These results of deglycosylation and hydroxylation reactions, suggested the specificity of the reaction carried out by the machinery enzymatic of fungi and demonstrates once again the relevance of biotechnological processes in obtaining bioactive compounds.

Considering the efficacy of the fungi-mediated biotransformation determined for glycosidic hydrolysis, we carefully evaluated the biotransformation reactional kinetic by *A. niger*, since it yielded the highest ion intensities of non-glycosylated specioside **16**. At 46 h of reaction time, we observe the highest intensity of **16**, at the same time, a decay of 62.5% of specioside (Fig. 3). Our results also demonstrated that the long reaction time led to the consumption of the analog **16** and an increase in the formation of coumaric acid. This was probably due to the lack of nutrients in the medium as an energy source for the fungus, which started to use the products themselves for this purpose.*Aspergillus* and *Penicillium* are filamentous and endophytic fungus that has been successfully applied to biotransformation reaction from natural products (Parshikov and Sutherland 2015). Here, we demonstrate that these fungi have a broad ability to transform specioside in unique structures. This ability may be related to the endophytic nature of this fungus, which make them promise new sources of biocatalysts for the biotransformation of natural products. Endophytes can produce diverse secondary metabolites indicating that the biosynthetic pathways contain enzymes with versatile functions. In addition, the environmental stress

may lead them to produce specific enzymes for detoxification, which may also contribute to their capability in transforming natural products (Ying et al. 2014).

In this work, we also investigated the glycosidic hydrolysis from specioside by common chemical methods using acid, base and microwave. However, the results showed that the iridoid was completely degraded and the analogs, yielded from fungi-mediated biotransformation reactions, were not observed, including the non-glycosylated specioside. Beyond glycosidic bond, specioside has other more suitable and reactive sites, such as the ester bond with phenylpropanoid and the epoxy group, which are sites easily hydrolyzed and attached by chemical methods. These results revealed the requirement of specific previous multi-step reactions, such as reactions with protection of reactive functional groups, which would be necessary to obtain the non-glycosylated specioside by chemical synthesis. However, these processes are not aimed in several studies since they are costly, time-consuming and with low yields.

Another highlight of this work is that no reports involving fungi biotransformation from specioside was found in the literature, in addition, several metabolites obtained are unprecedented. Our research group has showed the great biological value of specioside. More recently, we showed its anti-inflammatory potential by inhibiting the recruitment of leukocytes into the peritoneal cavity in mice (Nocchi et al. 2020). Thus, the fungal biotransformation technique proved to be very efficient and important to produce analogs from a glycosylated iridoid. These biotransformation reactions are simple, low-cost and sustainable, performing various types of reactions in a single step. In addition, the deglycosylated product was successfully produced, which was not possible by conventional chemical methods.

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**Declarations**

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**Conflict of Interest**

All authors declare that they have no conflict of interest.

**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

**Data and material availability statement**

The strains are deposited in the library URM (University Recife Mycology) of Federal University of Pernambuco (UFPE) and MMBF (Micoteca Mario Barreto Figueiredo). The data can be found at http://www3.ufpe.br/micoteca/nova/fazerBusca.php and http://www.biologico.sp.gov.br/page/colecoes/fungos-fitopatogenicos.All MS/MS data obtained in this study was summarized in table 1 and the spectra are illustrated in supplementary information files.

**Author's Contributions**

NSC, JCPM, and DBS conceived and designed research. ESA supplied the strains of the fungi. NNK and CAC collected the plant and isolated the specioside iridoid. NSC, NNK and LBS conducted experiments. RR analyzed the ITS sequence and identified the microorganisms. NSC, CAC, ESA and DBS analyzed the chemical data. NSC and DBS wrote the manuscript. All authors read and approved the manuscript.

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# Capítulo 2

# 4 A NEW SESQUETERPENE FROM *Bacopa gratioloides* DISPLAYS CYTOTOXICITY AGAINST MURINE MELANOMA CELLS

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**ABSTRACT**

Natural products are still a valuable source to find bioactive compounds, and organisms that produce high levels of the target compounds are a good strategy to simplify and facilitate the optimization of analytical process to get them. *Bacopa gratioloides* (Cham.) Edwall. (Plantaginaceae) is an aquatic species underexplored and it occur in the inhospitable biome Chaco, a steppe savanna. In the present work, we isolate high levels of a new sesquiterpene, which was structurally characterized by NMR, MS, and X-ray diffraction and its potential against B16F10-Nex2 melanoma cancer cells were evaluated. The new sesquiterpene was characterized as 7-epi Dihydroartemisinic acid and presented promising activity against B16F10-Nex2, also promoting a marked inhibition of cell migration *in vitro* and death by apoptosis. No toxic effects were observed for the healthy cell line, MRC5. Therefore, a promising inhibition rate achieved by 7-epi Dihydroartemisinic acid against B16F10-Nex2, which was obtained high amounts from *B. gratioloides*.

**Keywords:** Cell migration, *Bacopa gratioloides*, *sesquiterpene*.

## INTRODUCTION

Nature is a rich source to search new active compounds and candidates for drugs. In the last 39 years, 36% of all new small molecules discovered correspond to natural substances and inspired by natural products. In the antitumor area, the number of small-molecule anticancer drugs in this same period were 41% [1]. This shows that natural products still hold out the best options for finding novel agents/active templates.

The unmodified marine natural product Aplidine was the latest molecule approved for the cancer treatment. It is a depsipeptide isolated from the Mediterranean tunicate *Aplidium albicans* and it is used to multiple myeloma treatment [1]. Another molecule approved in recent years is the homoharringtonine, a small molecule alkaloid derived from *Cephalotaxus fortunei* Hook. used in the treatment of chronic myeloid leukemia. Homoharringtonine probably holds record for the longest time of development of an anticancer agent until FDA approval, more than 40 years [1, 2]. This confirms the urgent need to research new anticancer molecules, given all the difficulties on the way to their approval.

Alkaloids, terpenes, flavonoids, lignans, saponins, vitamins, minerals, glycosides, and terpenes play significant roles in impairing cancer cell physiology, enzymes and signaling pathways. These effects contribute to imbalance of mitochondrial membrane potential and release of pro-apoptotic factors, culminating in caspase activation and mediated-apoptosis cell death [3]. One of the most important examples of antineoplastic compounds developed from plants are the alkaloids vinblastine and vincristine that are obtained from *Catharanthus roseus* (L.) G. Don. These alkaloids have been used in the treatment of Hodgkin's lymphoma, Kaposi's sarcoma, cancer of ovary and testicular and infantile acute lymphoblastic leukemia [4].

Terpenes are also highlighted secondary metabolites for cancer treatment, such as taxol. This diterpene is a natural product firstly obtained from barks and leaves of *Taxus baccata* L., *Taxus canadensis* Marshall, and *Corylus avellana* L. Taxol has been used in the treatment of different cancer types, including ovarian, breast, and lung cancer. The first-generation of taxanes drugs is marketed under the name Paclitaxel®. Betulinic acid, an acid triterpenoid isolated from *Ziziphus mauritiana* Lam., *Ziziphus rugosa* Lam., *Ziziphus oenoplia* (L.) Mill., and *Betula* sp. (Betulaceae), is a cytotoxic agent for a wide range of cancer, including melanoma [3]. Furthermore, the sesquiterpene β-elemene is a

promising anticancer agent, showing antitumor effects against lung, brain, breast, ovarian, and prostate cancer, beyond the capability to reverse the resistance of drug-resistant tumors [5].

*Bacopa* genus is distributed in America, and several species have shown effective properties in the treatment of a wide range of diseases. *Bacopa monnieri* (L.) Pennell, a species the most studied in the *Bacopa* genus, is commonly used for medicinal purposes such as the treatment of cognitive disorders, anxiety, depression, and concentration deficit [6,7]. Recent studies demonstrated the effectiveness of both standardized extract and of their isolated phytochemicals against different cancer types through *in vitro* and *in vivo* assays. The secondary metabolites from *B. monnieri* exert anticancer properties against colon, breast, liver, prostate, and neurological cancers. The anticancer properties are attributed to the mixture of triterpene saponins Bacoside A and B, the triterpene Cucurbitacin, and the triterpene saponins Bacopaside I, II and VII [6, 7].

Despite the medicinal use and the relative knowledge about the chemical constituents from *Bacopa* species, studies on the bioactive compounds from *Bacopa gratioloides* (Cham.) Edwall are lacking. This species possesses records in Paraguay, Bolivia, Venezuela, and Guyana. In Brazil, the most records are described in the Northeast and Midwest, where its occurrence is observed in the Chaco biome [8]. Thus, the study of the chemical profile of this species, as well as its pharmacological properties, can lead to the discovery of substances not yet studied, with potential application to treatment of diseases that represent a threat to public health, including cancer. Therefore, we aimed to extract of essential oil from the aerial parts of *B. gratioloides*, to optimize the isolation procedures of its main constituent, and to evaluate its anticancer properties against melanoma, the type of skin cancer with the highest mortality death rate.

## MATERIAL AND METHODS

### *Plant material and collection area*

The aerial parts of *B. gratioloides* were collected from plants growing in temporary ponds, in Porto Murtinho municipality, Brazilian Humid Chaco (21°41’56”W 57°52’57”S). Prof. Rosani do Carmo de Oliveira Arruda identified the vegetal material,

and a voucher was deposited on CGMS Herbarium of UFMS under accession number 66620 and SisGen number A1FB919.

***Extraction and purification of essential oil***

Fresh aerial parts of *B. gratioloides* (786 g) were extracted by hydrodistillation using a Clevenger-type apparatus for 4 h/day for 5 days. The essential oil of *B. gratioloides* (BGEO) was obtained with a yield of 0.15% (1.16 g). After reaching room temperature, the oil solidified into crystals. It was then purified by cold recrystallization using methanol and deionized water (7:3), and yielded 73% of the compound 1.

***Gas chromatography-mass spectrometry (GC-MS)***

The compound 1 was analyzed by gas chromatography coupled to mass spectrometer (GC-MS). This equipment was a chromatograph GC-MS Shimadzu QP2010equipped with autoinjector COA-20i and a RTx-5MS capillary column (30 m x 0.25 mm x 0.25 μm) was applied. The carrier gas was helium, and the pressure was 79.7 kPa. The injection temperature was 250 °C, and the temperature program was the following: 60-240 °C increasing 3 °C.min$^{-1}$, 240-310 °C increasing 15 ˚C.min$^{-1}$, and 310°C for 10 min in an isothermal step. The mass spectra were obtained by electron ionization (EI), applying the energy 70 eV. The *B. gratioloides* essential oil (BGEO) and compound 1 were solubilized in dichloromethane (1 mg.mL$^{-1}$) and 1 μL was injected on the chromatographic system with a split ratio of 1:10. Retention indices (RI) were calculated using C8-C28 alkane standards (Sigma Aldrich).

***LC-DAD-MS***

The compound 1 was solubilized in methanol (1 mg.mL$^{-1}$) and water deionized (7:3, v/v) and 1 μL was injected into the chromatograph system LC-20AD UFLC (Shimadzu Corp.) coupled to a diode array detector and a mass spectrometer with electrospray ionization and analyzers quadrupole and time-of-flight (MicrOTOF III, Bruker Daltonics, Billerica, MA, USA). The analyses were monitored at 240-800 nm and acquired in negative and positive ion mode (*m/z* 120-1200). The MS/MS spectra were acquired by automatic method using a collision energy 45 to 65 V. A chromatograph column Kinetex C-18 (Phenomenex, 2.6 μm, 150 x 2.1 mm) was used for the analyses and the mobile phase was composed by ultrapure water (solvent A) and acetonitrile

(solvent B), both containing 0.1% formic acid. The gradient elution profile was the following: 0-2 min - 3% of B; 2-25 min - 3-25% of B; 25-40 min - 25-80% of B; 40-43 min - 80% of B. The flow rate was 0.3 $mL.min^{-1}$ and the oven temperature adjusted to 50 ˚C.

The raw data were processed by Data Analysis software version 4.2 (Bruker) . The molecular formula was determined based on the accurate mass considering errors up ±5 ppm and mSigma below 30.

***NMR***

The NMR experiments were performed on a DPX-500 Bruker instrument ($^{1}H$: 500 MHz; $^{13}C$: 125 MHz), using methanol-D4 as solvent. Chemical shifts ($\delta$) are given in parts per million (ppm) downfield from tetramethyl silane or deuterated solvent as the internal standard. The coupling constants (*J*) were expressed in Hertz (*Hz*).

***Melting point***

The melting temperature of compound 1 was determined using a Fisaton-430 D equipment. The analysis was carried out in thin-walled capillary melting point tubes sealed at one end in triplicate (2 mg).

***Evaluation of compound 1 on cellular viability***

The effects of compound 1 on human lung fibroblasts (MRC-5) and murine melanome (B16F10-Nex2) were evaluated according to Mosmann [9] based on the enzymatic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were cultured in 96-well microplates at their respective media and density: MRC-5, high-glucose DMEM, $6 \times 10^3$ $cells.well^{-1}$; B16F10-Nex2, RPMI, $5 \times 10^3$ $cells.well^{-1}$. The media were supplemented with 10% fetal bovine serum (FBS), 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ of streptomycin (Gibco, Brazil) at 37 ˚C in an atmosphere containing 5.0 % $CO_2$. Cells were washed with PBS and incubated with a serial dilution of compound 1, prepared in the respective media, from 100 to 3.12 µg/mL.

After 24 h, 100 µL of MTT solution (0.5 mg.mL$^{-1}$ diluted in culture medium) were added. Following 4 h of incubation, the formazan crystals were resuspended with 100 µL of dimethylsulfoxide (DMSO) and read at 630 nm. At least three independent experiments were performed in triplicate.

### *Evaluation of compound 1 on migration cells*

The cells were cultured in 24-well microplates at their respective media and density: MRC-5, high-glucose DMEM, 1 × 10$^5$ cells.well$^{-1}$; B16F10-Nex2, RPMI, 1 × 10$^5$ cells.well$^{-1}$. The analysis of cell migration was carried out using the methodology of wound healing assay, as described by Grada and collaborators [10]. The microplates were kept in the incubator until the formation of a confluent monolayer. A sterile P200 micropipette tip was used to create a cell-free zone across the cell monolayer in each well. After creating the scratch, the monolayer cells were washed with the respective media to remove cell debris and new media were replaced containing either 0 or 6.2 µg.mL$^{-1}$ of the sesquiterpene. In each well, three points were marked with a fine permanent marker tip to compare the same the reference points in time 0 and 24 h. The migration was documented by digital photographs in x20 magnification, obtaining three images per well per timepoint. The areas were calculated using the ImageJ software.

### *Statistical analysis*

The data were expressed as means ± SEM, where appropriate. The one-way analysis of variance (ANOVA), with post-test Tukey $P < 0.05$ was considered significant.

## RESULTS

### *Analytical obtaining the compound 1*

The essential oil from the aerial parts of *B. gratioloides* (BGEO) was extracted by the convention hydrodistillation method using a Clevenger apparatus and it was analyzed by GC-MS. The results of the GC analysis showed the presence of a major compound in BGEO (data not shown), which was submitted to cold recrystallization using methanol

and deionized water (7:3), yielding the compound 1 (Figure 1) as white crystals. The chemical analysis by CG-MS do not hit with available libraries indicating to be a rare or unprecedented compound.

***Structural determination of compound 1***

The melting point of compound 1 was determined reaching a range of 108-109 °C. The MS spectra showed a deprotonated ion peak at *m/z* 235.1697 compatible with molecular formula $C_{15}H_{24}O_2$ (err ppm 2.8, mSigma1.9). This compound was also submitted to NMR analysis and the experiments $^{1}$H NMR (δ in ppm; *J* in Hz), $^{13}$C NMR, COSY, J-*Res*, HSQC, HMBC and NOESY (Table 1).

The $^{1}$H NMR spectrum showed three signals relative to three methyl groups at δ 0.91, 1.06, and 1.65. The signals at δ 0.91 and 1.06 are two doublets with coupling constant of 6.8 and 7.2 and they coupled with the hydrogens δ 2.84 (H-10) and 1.63 (H-11) and the correlations observed in the HMBC spectra confirmed that they are from H-13 and H-14 positions, such as the correlation with C-1/C-9/C-10, and C-7/C-11/C-12, respectively. In addition, the olefinic methyl at δ 1.65 showed a broad singlet and correlations with C-3, C-4, and C-5.

A double quartet at δ 5.45 (relative to 1 H) indicated the presence of a double bond. The $^{13}$C NMR spectrum showed two signals with characteristic shift of $Csp^2$ at 123.6 and 135.6 ppm corresponding to C-5 and C-4, respectively.

The COSY experiment showed correlations between H-11 ($\delta_H$ 2.84) with H-7 ($\delta_H$ 1.94) and at δ 1.06 (H-13), besides H-6 ($\delta_H$ 1.83) showed correlations with H-1 ($\delta_H$ 1.67) and the olefinic hydrogen at δ 5.45 (H-5), and the methyl group in C-15 ($\delta_H$ 1.65) coupled to H-5 ($\delta_H$ 5.45). The HMBC experiment (Figure 1, Table 1) confirmed correlations of the three-methyl groups with the carbons proposed that their bond (Table 1). The H-14 ($\delta_H$ 0.91) showed correlations with $\delta_C$ 39.7 (C-1), $\delta_C$ 29.3 (C-9), and C-10 ($\delta_C$ 35.4); the H-13 with C-7 ($\delta_C$ 41.1), C-11 ($\delta_C$ 39.5) and C-12 ($\delta_C$ 182.4); and the olefinic methyl group H-15 ($\delta_C$ 23.8), showed correlation with C-3 ($\delta_C$ 31.2); C-4 ($\delta_C$ 135.6), C-5 ($\delta_C$ 123.6) and C-7 ($\delta_C$ 41.1). A typical carbon for carboxylic acid was observed at 182.4 ppm, which correlate to the H-11 ($\delta_H$ 2.84) and methyl group 13 ($\delta_H$ 1.06), this confirms

the position of the carboxyl in the isopropyl group. In NOESY spectra, correlations between H-1 ($\delta_H$ 1.67) and methyl group H-14 ($\delta_H$ 0.91); H-5 ($\delta_H$ 5.45) and methyl group H-13 ($\delta_H$ 1.06) contributed to the determination of the relative configuration of this compound (Figure 1, Table 1). Thus, all these spectral data allowed the identification of compound 1 as an unprecedented acid sesquiterpene with a muurolene skeleton, the 7-epi Dihydroartemisinic acid.

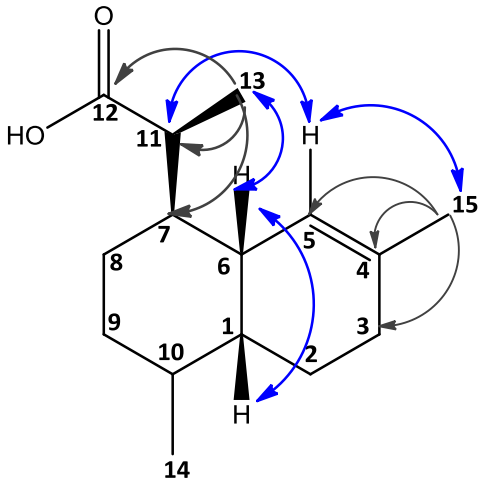


**Figure 1.** HMBC (grey arrow), and NOESY (blue arrow) correlations keys of compound 1.

**Table 1.** $^{1}H$ (300 MHz) and $^{13}C$ (75 MHz) NMR spectral data of compound 1

| H/C | δ $^{1}H$ (int., mult., *J*)[a] | δ $^{13}C$ | COSY | HMBC | NOESY |
|---|---|---|---|---|---|
| **1** | 1.67 (1 H, *dt*, 12.9, 3.6) | 39.7 | H-2, H-6 | C-6 | H-2 |
| **2** | 1.55 (1 H, *m*) 1.50 (1 H, *m*) | 17.1 | H-1, H-2, H-3 | C-1, C-3, C-4, C-6, C-15 | H-3, H-14 |
| **3** | 1.97 (2 H, *m*) | 31.2 | H-2, H-5 | C-1, C-2, C-4, C-5, C-15 | H-2 |
| **4** | - | 135.6 | - | - | - |
| **5** | 5.45 (1 H, *dq*, 9.7, 2.1) | 123.6 | H-3, H-6, H-15 | C-1, C-3, C-15 | H-6, H-11, H-13, H-15 |
| **6** | 1.83 (1 H, *m*) | 39.2 | H-1, H-5, H-7 | C-1, C-2, C-4, C-5, C-7 | H-1, H-5, H-13 |

| | | | | | |
|---|---|---|---|---|---|
| **7** | 1.94 (1 H, *m*) | 41.1 | H-6, H-8, H-11 | C-2, C-6, C-4, C-5 | H-2, H-8/9, H-11 |
| **8** | 1.42 (1H, *m*)<br>1.16 (1H, *m*) | 26.90 | H-7, H-9 | C-1, C-6, C-7, C-9, C-13 | H-9, H-14 |
| **9** | 1.42 (1H, *m*)<br>1.16 (1H, *m*) | 29.3 | H-8, H-10 | C-7, C-8, C-10, C-14 | H-8, H-14 |
| **10** | 1.63 (1 H, *m*) | 35.4 | H-9, H-14 | C-6, C-7, C-14 | H-2, H-7, H-6, H-8/9 |
| **11** | 2.84 (1H, *qd*, 3.5, 7.2) | 39.5 | H-7, H-13 | C-7, C-8, C-12, C-13 | H-5, H-7, H-13 |
| **12** | - | 182.4 | - | - | - |
| **13** | 1.06 (3 H, *d*, 7.2) | 9.1 | H-11 | C-7, C-11, C-12 | H-14 |
| **14** | 0.91 (3 H, *d*, 6.8) | 19.6 | H-10 | C-1, C-9, C-10 | H-2, H8/9, H-13 |
| **15** | 1.65 (3 H, *br s*) | 23.8 | H-5 | C-3, C-4, C-5 | H-5 |

[a]500 MHz, $CDCl_3$; 125 MHz, $CDCl_3$; br: broad.

**Effects of compound 1 from *B. gratioloides* on cellular viability**

To evaluate the effects of the compound 1 on cellular viability, the assays were carried out on melanoma (B16F10-Nex2) and healthy cell lines (MRC5). The increase of concentration of sesquiterpene showed a dose-dependent reduction on viability of B16F10-Nex2 cells (Figure 2A), revealing an. $IC_{50}$ of 43.93 µg.mL$^{-1}$, corresponding a concentration of 186 µM. At the highest concentration assayed (100 µg.mL$^{-1}$), the B16F10-Nex2 viability was reduced by 92.1%. The assays also carried out on healthy human fibroblast cell line (MRC5), and the dose-dependent effect of cell viability was not noticed (Figure 2B). At low concentration, the viability of MRC5 cells was unaltered following the incubation with the sesquiterpene 1. At the highest concentration evaluated, the viability of MRC5 cells around 77%, implying that, at the concentration assayed, no cytotoxicity toward healthy cells was observed. This result suggests that the sesquiterpene 1 has a higher selectivity against cancer cells.

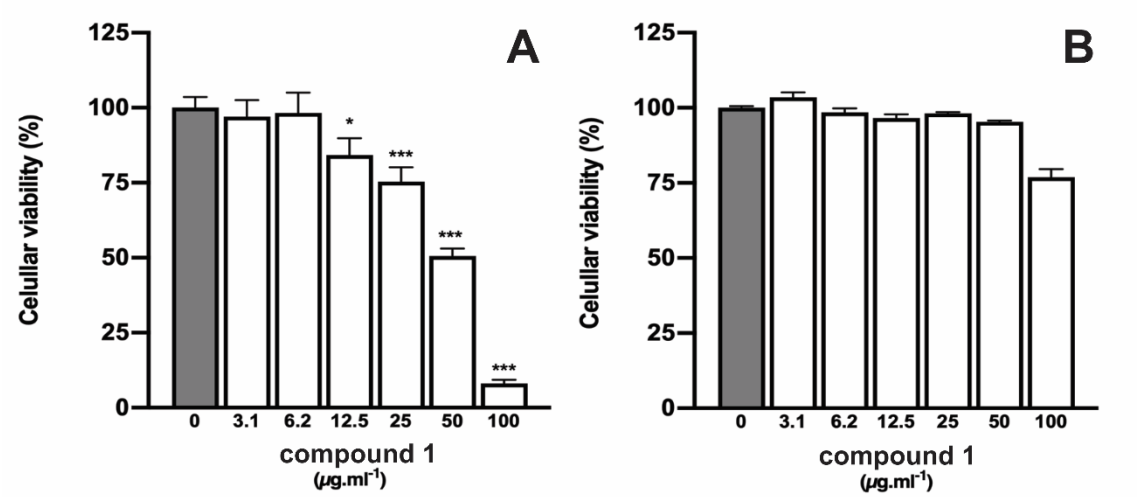


**Figure 2.** Effects of compound 1 on cellular viability. This sesquiterpene was incubated with (**A**) melanoma (B16F10-Nex2) or (**B**) healthy human fibroblasts cells (MRC5) during 24 h. The cellular viability was determined by assay MTT and the results represent the average of three independent assays, carried out in triplicates. Analysis of variance (ANOVA), with post-test Tukey P< 0.05 (*), 0.01 (**), and 0.001 (***).

We also investigated the effect of compound 1 on migration of cells using *in vitro* wound healing assay, and we measured the cell migration rate for a denuded created during a period of 24 h. Both melanoma and fibroblast cells were incubated with 6.2 $\mu g.mL^{-1}$ of compound 1, since this concentration did not affect the cell viability of both cells in earlier assay. The migration of melanoma cells was remarkedly impaired in presence of sesquiterpene, reaching an inhibition of migration rate of 71.6% (Figure 3A). The representative image at time 0 and 24 h is showed in Figure 5. Nonetheless, the sesquiterpene did not affect the migration of MRC5 cells (Figures 3B and 5), when compared with the cells harvested in absence of the compound, reinforcing the absence of negative effects on physiology of healthy cells.

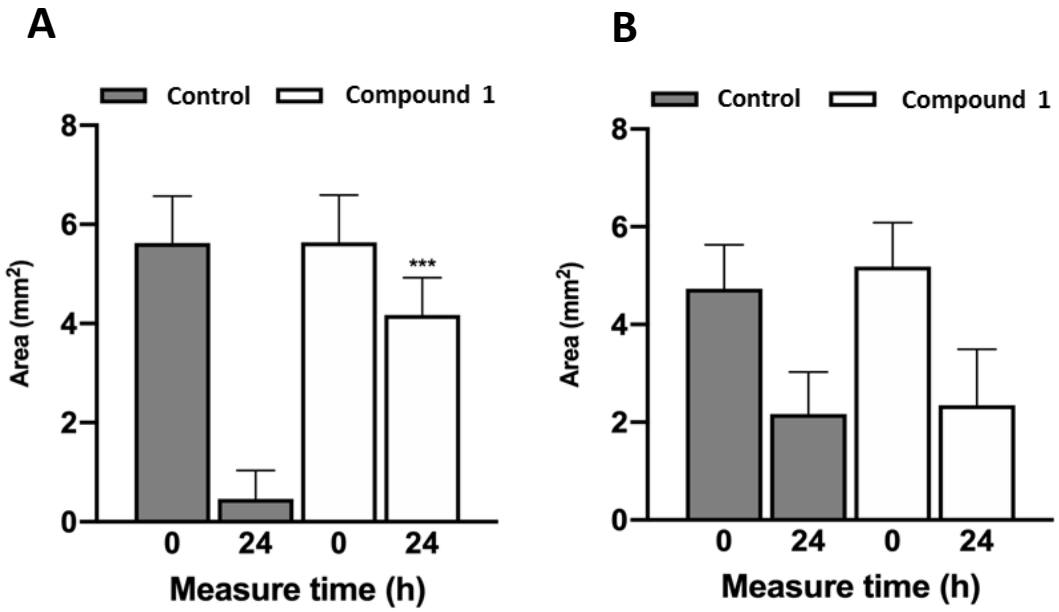


**Figure 3.** Effects of compound 1 on cell migration. The compound 1 was incubated with (**A**) melanoma (B16F10-Nex2) or (**B**) healthy human fibroblasts cells (MRC5) during 24 h. The cell migration was measured on the basis of the bordering cells refilled the denuded area after 24 h. The calculation of area was carried with aid of ImageJ software. Analysis of variance (ANOVA), with post-test Tukey P< 0.05 (*), 0.01 (**), and 0.001 (***).

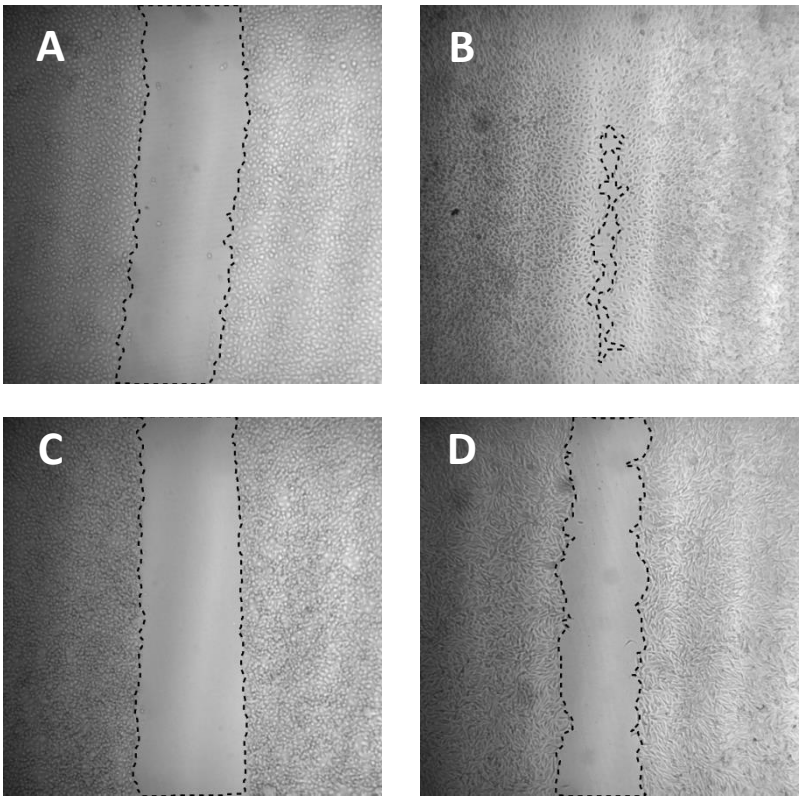

**Figure 4.** Representative images of effect of compound 1 on migration of B16F10-Nex2 cells. Cells incubated with RPMI media at 0 h (A) and 24 h (B), and cells incubated with RPMI containing 6.2 μg.mL$^{-1}$ of sesquiterpene at 0 h (C) and 24 h (D).

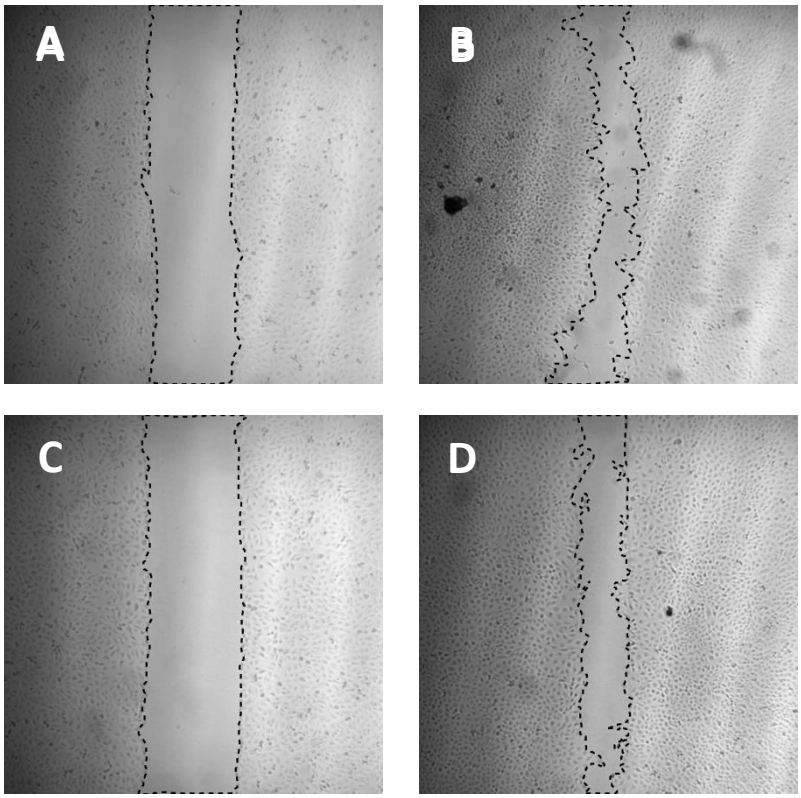


**Figure 5.** Representative images of effect of compound 1 on migration of MRC5 cells. Cells incubated with DMEM media at 0 h (A) and 24 h (B), and cells incubated with DMEM containing 6.2 μg.mL$^{-1}$ of sesquiterpene at 0 h (C) and 24 h (D).

The effects of compound 1 on morphology of B16F10-Nex2 were observed through fluorescence microscopy. The images were obtained after 24 h of incubation with the compound 1 at $IC_{50}$ (43.93 μg.mL$^{-1}$), contributing with investigation regarding the type of cell death triggered by this sesquiterpene. The nucleus cells were stained with NucBlue™ Live ReadyProbe and the mitochondria were stained with MitoTracker™ Deep Red FM. The control cells showed a typical B16F10-Nex2 cellular morphology, with homogeneous distribution of fluorescence of nucleus surrounded by mitochondria (Figure 6A). Compared with control cells, B16F10-Nex2 cells incubated with the

compound 1 showed marked morphological differences (Figure 6B). The presence of genetic material diffused outside of cells, and a minor fluorescence of chromatin are signals of DNA damage. Some cells apparently lost the blue staining, suggesting DNA fragmentation. Another effect observed was the diffused distribution of mitochondria, observed by lower red fluorescence signals, suggesting altered mitochondrial functions. Together, these finding supporting the hypothesis that the sesquiterpene 1 is triggering apoptosis-mediated cell death.

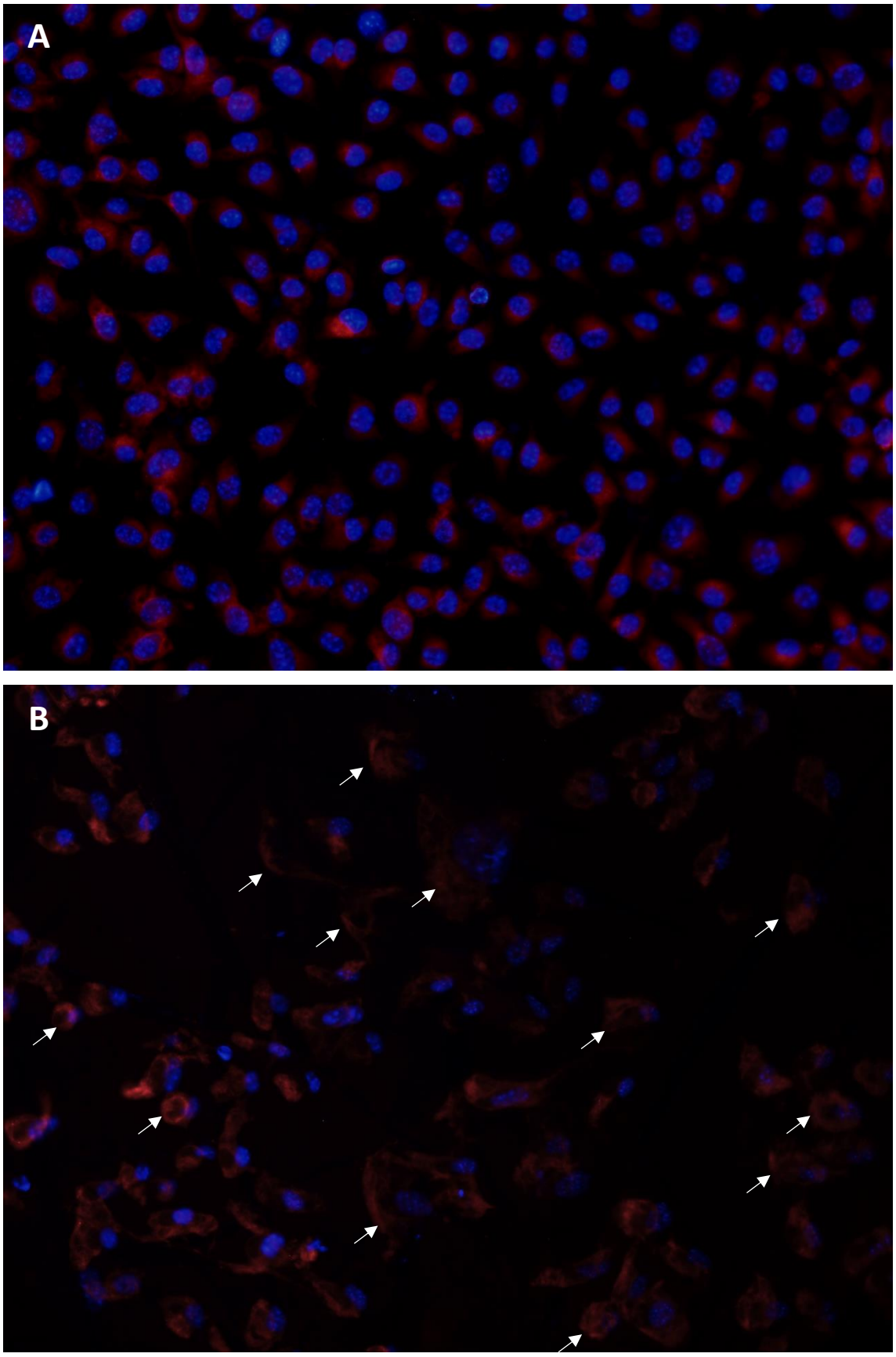


**Figure 6.** Fluorescence microscopy of B16F10-Nex2 cells incubated with compound 1 at IC50 (43.93 µg.mL$^{-1}$). The nucleus cells were stained with NucBlue™ Live ReadyProbe

and the mitochondria were stained with MitoTracker™ Deep Red FM. (A) B16F10-Nex2 cells harvested in RPMI media and (B) B16F10-Nex2 cells harvested in RPMI media containing compound 1at IC50. The cells with apparent DNA fragmentation lost the blue staining and are marked with arrows.

**Discussion**

No study on chemical profile and biological properties of *B. gratioloides* oil has been reported in the literature to date. In this work, we isolated, identified and evaluated the activity of compound 1, a new compound, against melanoma and evaluated its anti-migration melanoma cell propreties.

Terpenoids constitute the largest class of natural products, comprised by a diversity of classes, structures and functions. Sesquiterpenoids represent the most prevailing class of terpenoids and present a wide range of biological activities, including antimicrobial [11], anti-inflammatory [12], immunomodulatory [13], and chemotherapeutic [14,15]. Regarding anticancer activity, several sesquiterpenoids trigger cancer cells inducing apoptosis, mediated by arrest in cellular cycle, damages to intracellular structures, alteration of mitochondrial function, DNA damage, and caspase activation [16-18]. For this reason, the interest by new terpenoids and their biological properties are still highlighted.

Several studies from *B. monnieri* have showed bioactive secondary metabolites, such as components Bacoside A and B, Cucurbitacin and Bacopaside I, II and VII with anticancer effect on different types of cancer like colon, breast, liver, prostate and neurological cancers. The main class of metabolites attributed to its anticancer properties is terpenoid [5,6,19]. Cucurbitacin, a tetracyclic triterpenoid of *B. monnieri*, showed cytotoxic effect when its combination with betulinic acid in DCM fraction were evaluated against two breast cancer cell lines MCF-7 and MDA-MB231 [20]. Bacopaside I and II, triterpenoid saponins, are capable of rendering positive effect on reducing proliferation and hindering migration and invasion of breast cancer cell line when applied synergistically [21]. In addition, the triterpenoid saponins Bacoside E and Bacopaside VII have shown cytotoxic effects against human tumor cell lines MDA-MB-231, SHG-44, HCT-8, A549 and PC-3M assay in vitro, and showed 90.52 % and 84.13 % inhibition in

mouse implanted with sarcoma S180 *in vivo* at the concentration of 50 μmol/ kg, respectively [22].

In this work, a new sesquiterpene 7-epi Dihydroartemisinic acid (1) were isolated and structurally characterized, and its cytotoxic effects against melanoma cancer cells were determined. Furthermore, no cytotoxicity forwards the healthy cell line, MRC5, was observed, suggesting selectivity of compound 1 for cancer cells, which showed an $IC_{50}$ of 43.93 $\mu g.mL^{-1}$ or 180 μM. The sesquiterpene β-ionone displayed cytotoxic activity against DU145 and LNCaP prostate cancer cell lines exhibiting $IC_{50}$ of 210 and 130 μM, respectively [23]. Concomitantly, β-ionone-induced apoptosis and cell cycle arrest at the G1 phase in DU145 and PC-3 cells. The sesquiterpene ferutinin revealed cytotoxic against HT29 and CT26 colon cancer cell lines displaying $IC_{50}$ of 29 (HT29) and 26 $\mu g.mL^{-1}$ (CT26). Ferutinin prompted apoptosis in treat cells, reducing the size of tumor in mice model in comparison to the control groups.

Different researchers have shown that terpenoids trigger apoptosis in cancer cells. These conclusions have been obtained through different approaches, such as the use of pharmacological inhibitors, fluorescent probes, flow cytometry, western blot, and fluorescence microscopy analysis. Yang and collaborators [24], showed that the incubation of caspase inhibitor, Z-VAD-FMK, in human MDA-MB-468 breast cancer cells reverted the apoptosis prompted by sesquiterpene lactone from *Eupatorium lindleyanum* DC. A similar result was observed for a glioma cell line, GBM 8401, treated with zerumbone, a sesquiterpene and cyclic ketone [25], suggesting that even against different cancer cell lines, the anticancer effects are mediated by apoptosis. The results from fluorescence microscopy of compound 1 in B16F10-Nex2 showed signals of apoptosis-mediated cell death, such as shrinking of cells, fragmentation of nuclear chromatin, altered mitochondrial function with damage of intracellular structures. Similar results were described for guai-2-en-10α-ol, a guaiane sesquiterpene derivative, purified from *Ulva fasciata* Delile [16], shrinking of cells and fragmentation of chromatin.

The high lethality of melanoma is mainly due to its great metastatic potential. Among other characteristics, compounds capable of modulating cell migration have the potential to control progression and metastasis, being alternatives for the development of future drugs for aggressive forms of cancer. The 7-epi Dihydroartemisinic acid from *B. gratioloides* showed a marked inhibition of migration for melanoma cells. Our results are

encouraging, however, the mechanisms involved with the inhibition of migration and invasion into cancer cells by terpenoids vary. Among the possibilities already described, there is the inhibition of VEGF, the vascular endothelial growth factor [26], inhibition of transforming growth factor beta (TGF-β1) induced epithelial mesenchymal transition (EMT) [27], the negative regulation of metallopeptidases and other pathways dependent upon regulation of p38 MAPK, NF-κB, and Nrf2 [28]. Anyhow, the inhibition rate achieved by 7-epi Dihydroartemisinic acid against B16F10-Nex2 was promising, supporting future studies to determine the mechanism by which sesquiterpene inhibits cell migration, allowing preclinical assays to be performed, since the B16F10 strain has been widely used and recognized as a model for studies of melanoma in mice.

This work demonstrated by first time the chemical composition and anticancer potencial of the *B. gratioloides* oil and its main component, a new sesquiterpene, 7-epi Dihydroartemisinic acid. This molecule can be a promisor candidate to anticancer drug, since showed a good effect against B16F10-Nex2 and do not have toxic for the healthy cell line, MRC5. Another highlight is the obtaining of the oil from the aerial parts of *B. gratioloides* with high yields and a simple purification process by recrystallization afforded the sesquiterpene with 73%, showing to be a promising source for obtaining of this molecule on a large scale. It is important to emphasize the relevance of these findings since this is the first phytochemical and biological studies from *B. gratioloides*. Further studies will be carried out to investigate the role of chemical modifications in the structure of the new sesquiterpene, the effects on the selectivity as well anticancer properties.

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**Declarations**

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**Conflict of Interest**

All authors declare that they have no conflict of interest.

**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

**Author's Contributions**

NSC, JCPM, and DBS conceived and designed research. RCOA collected the plant. NSC, CFRO and LBS conducted experiments. NSC and DBS analyzed the chemical data. CFRO, ELS and KPS analyzed the biological data. NSC and DBS wrote the manuscript. All authors read and approved the manuscript.

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# Capítulo 3

# 5 MOLECULAR NETWORKING OF ANTIFEEDANT AND DETERRENT COMPOUNDS FROM *Bacopa gratioloides*

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**Abstract**

The genus *Bacopa* (Plantaginaceae) presents several biological activities. However, the chemical composition and biological properties of essential oil from *Bacopa gratioloides* is unknow. Thus, our goal was to perform histochemical and anatomical analyses of *B. gratioloides* leaves, to analyze the *B. gratioloides* essential oil (BGEO) and to investigate its antifeedant and deterrent activities. The anatomical study showed that *B. gratioloides* leaf epidermis is composed of common epidermal cells, stomata, and trichomes and is covered by a thin and striated cuticle. Analysis of BGEO by GC-MS revealed the presence monoterpenes, hydrocarbons and sesquiterpenes. The incorporation of BGEO into an artificial diet (at 1.4%) revealed antifeedant activity against the Mediterranean flour moth, *Anagasta kuehniella*, prompting a reduction on both larval weight and survival rate by 76% and 26%, respectively. Coating cowpea with BGEO at 0.005 % was able to prompt a significant reduction in oviposition by *Callosobruchus maculatus*, demonstrating that BGEO possesses deterrent activities. In addition, BGEO was promisor as a source for the development of natural repellent and deterrent agents effective in pest control, alternative synthetic products, reducing the environmental impact.

**Keywords:** Plantaginaceae, molecular networking, sesquiterpene, *Callosobruchus maculatus*, *Anagasta kuehniella*.

## INTRODUCTION

The *Bacopa* genus (Plantaginaceae) is constituted by roughly a hundred species, around 60 of them are spread in Brazil. These plants are found in wet, damp, and marshy regions in tropical and subtropical areas (Tungphatthong et al., 2018; Tropicos, 2020). Numerous studies have demonstrated the biological properties of compounds isolated from *Bacopa* species, such as antibacterial, antioxidant, analgesic, anti-inflammatory, sedative, and anti-epileptic (Dulger and Hacioglu, 2009; Tungphatthong et al; Saesong et al, 2019, Stough et al., 2001). *Bacopa monnieri* (L.) Wettst is the most used species for medicinal purposes, for treatment of memory disorders, epilepsy, insomnia, improvement of cognitive abilities, and treatment of anxiety (Govindarajan et al., 2005; Tungphatthong et al., 2018; Gubbannavar et al., 2013). The beneficial effects of *B. monnieri* are attributed to the triterpene saponins bacoside A and B. Furthermore, metabolites such as alkaloids (hydrocotyline, brahmine and herpestine), flavonoids (apigenin and luteonin), tannins, fatty acids, glycosides (asiaticoside and thanakunicide), amino acids, and other volatile components, including sesquiterpenes, have been reported in *B. monnieri* (Mathew et al., 2010).

Despite the medicinal use and the relative knowledge about the chemical profile of some *Bacopa* species worldwide, there are no studies on the characterization of chemical constituents of *Bacopa* species with high incidence in Brazil. The lack of studies with Brazilian species associated with high rates of deforestation in most Brazilian biomes puts in risk the description of our biodiversity. Consequently, the discovery of new molecules with biotechnological applications is seriously threatened. *Bacopa gratioloides* (Cham.) Edwall has most of its records in the Northeast and Midwest of Brazil, where its occurrence is observed in the Chaco biome (SpeciesLink, 2021).

Recently, essential oils from aromatic plants with low toxicity to mammalians

have been introduced in the food industries such as supplements, natural repellents, and insecticides for market packing (Falleh et al., 2020; Lee et al., 2020; Arjmand and Dastan, 2020). Also, the main compounds described from essential oils (monoterpenes, sesquiterpenes, and phenolic derivatives) are commonly related to pollinators attraction (Giuliani et al, 2018), plant defenses against insect (Norris, 1986), and antifeedant (Harmatha and Nawrot, 1984; Asakawa et al., 1988; Ley and Toogood, 1990; Paruch, 2001; Nawrot, 2009). The research of new natural insecticides and repellents is essential since the toxicity of traditional chemical insecticides are frequently high. Thus, the research with natural products represents a new biotechnological alternative to deal with insect pests, especially those who attack stored products, where the use of chemical insecticides is worrying.

*Anagasta kuehniella* (Lepidoptera: Pyralidae), known as the Mediterranean flour moth, is an insect pest of primary economic importance, prompts severe losses in flour mills (Oliveira et al., 2014), this pest also feeds stored grains and fruits. Another pest with great economic importance is *Callosobruchus maculatus* (Coleoptera: Bruchidae). Known as the cowpea weevil, *C. maculatus* larvae consume the whole endosperm of grains during their development, making the seed inviable for planting and consumption (Sanon et al., 2010).

Therefore, the present study aimed to increase the knowledge about Brazilian *Bacopa* species, carrying out the anatomical, histochemical, and volatile chemical composition of *B. gratioloides* leaves. Investigate the essential oil deterrence and oviposition reduction effects against *A. kuehniella* and *C. maculatus*, respectively.

## MATERIAL AND METHODS

### *Plant material and collection area*

The aerial parts of *B. gratioloides* (Cham.) Edwall were collected from plants growing in temporary ponds, in Porto Murtinho municipality, Brazilian Humid Chaco (21°41'56"W 57°52'57"S). Professor Rosani do Carmo de Oliveira Arruda identified the vegetal material, and a voucher was deposited on CGMS Herbarium of UFMS under accession number 66620 and SisGen number A1FB919.

### *Anatomical procedures*

For anatomical, histochemical and micromorphological analyses, about 20 leaves were processed from five individuals. Leaves were fixed in FAA (3.7% formaldehyde, acetic acid, and 70% ethanol (1:1:18), as described by Johansen 1940), during 72 h, washed in tap water and conserved in ethanol 70%. Leaf segments were embedded in plastic resin (Leica®); sections 0.5-0.8 µm thick were stained with Toluidine Blue 1% (O'Brien et al., 1965). Epidermal peels were prepared using leaf segments dissociated in hydrogen peroxide and glacial acetic acid solution (1:1) and heated in an oven at 60 ºC for 12 h (Franklin 1945). These samples were stained with 0.25% ethanolic solution of basic fuchsin (20 s), washed in distilled water, and mounted in 50% glycerin. Analyses with scanning electron microscopy (SEM) were performed on dry leaves about 0.4 $cm^2$ coated with a thin layer of gold (Denton Vacuum Desk IV Standard Sputter Coater). The specimens were examined in a JEOL JSM-6380LV scanning electron microscope (JEOL, Japan).

For histochemical analysis, free-hand and plastic resin-embedded sections (Leica, Heidelberg, Germany) obtained with a rotary microtome (0.5-0.8µm thickness) were prepared and exposed to the following reagents: Sudan IV for total lipids (Pearse, 1972);

Nile blue for acidic lipids (Cain, 1947) and Nadi for essential oils and terpenoids (David & Carde, 1964). All samples were rinsed and mounted in distilled water on slides with coverslips. Control sections were performed simultaneously. Photomicrographs (with scale bars) were obtained with the Leica DM5500 B microscope and Leica Application Suite (LAS) V3 Program.

*Extraction of essential oil*

The fresh aerial parts of *B. gratioloides* (786 g) were extracted by hydrodistillation using a Clevenger-type apparatus for 4 h. The *B. gratioloides* essential oil (BGEO) was obtained with yielded 0.15% (1.16g), and it was maintained at -12 ºC until GC-MS analyses and biological assays.

*Gas chromatography-mass spectrometry (GC-MS) analysis*

The essential oil BGEO was analyzed by gas chromatography (Shimadzu QP2010) coupled to a mass spectrometer (CG-MS) equipped with autoinjector COA-20i and using a RTx-5MS capillary column (30 m x 0.25 mm x 0.25 μm). The carrier gas used was helium, and the pressure was 79.7 kPa. The injection temperature was 250 ºC, and the temperature program was the following: 60-240 ºC increasing 3 ºC.min$^{-1}$, 240-310 ºC increasing 15 ˚C.min$^{-1}$, and 310 ºC for 10 min (isothermic). The mass spectra were obtained by electron ionization (EI), applying the energy 70 eV. BGEO was solubilized in dichloromethane (1 mg.mL$^{-1}$) and 1 μL was injected on the chromatographic system with a split ratio of 1:10. Retention indices (RI) were calculated using C8-C28 alkane standards (Sigma Aldrich). The identification of constituents was performed comparing the mass spectra registered with NIST, WILEY, FFNSC, and GNPS libraries, and the retention indices with the literature (Adams & Spackman, 2007).

***Molecular networking***

The GC-MS data were transformed in software GCMS solution (Shimadzu), and the molecular network was obtained by Library Search/Molecular Networking GC workflow in GNPS (https://gnps.ucsd.edu) (Wang et al., 2016, Aksenov et al., 2020). For the construction of molecular networking, edges were filtered with a cosine score above 0.7 and considered more than 6 matched. The precursor ion mass tolerance was set to 2 Da and the MS/MS fragment ion tolerance to 0.5 Da. The maximum size of the molecular family was considered 100, and the library spectra were similarly filtered. The matches (network spectra and library spectra) were required to score above 0.5, and at least 6 matches peaks. The molecular networking was processed and visualized in software Cytoscape 3.8. (Shannon et al., 2003).

*Antifeedant activity against the larvae of Anagasta kuehniella*

The colony was reared in laboratory standard conditions (25 ± 1 °C, 40 ± 10% relative humidity with a photoperiod of 16 h). The eggs were collected, and the neonate first-instar larvae were used in assays. BGEO was incorporated into an artificial diet composed of wheat germ and whole flour in a ratio of 3:2 (w/w). The concentrations of BGEO evaluated were 0.8, 1.2, and 1.4% (w/w). The control treatment was fed with a diet without BGEO. Each treatment was set up in 250 mg plastic containers containing 4 larvae (n= 40), as previously described (Oliveira et al., 2017). The assays were finished when control larvae reached the $4^{th}$ instar under standard conditions. Then, the weights and numbers of larvae were determined.

*Deterrent activity on oviposition of adults of Callosobruchus maculatus*

A binary oviposition chamber was developed, according to Laudani & Swang (1954) and Sadegi (2006). The oviposition chamber was composed of a plastic box (15 cm long x 15 cm wide x 7 cm high) with 5 plastic containers with 5 cm diameter. Containers were disposed in a specific arrangement, one in the center of the chamber and the four others equidistantly from this central position. The chamber was kept in laboratory standard conditions during the assays. Cowpea (*Vigna unguiculata*) was obtained in a local market, seeds of the same size were selected, dipped into BGEO solution for 1 min, and air-dried for 30 min. The BGEO were dissolved with ethanol 70% at final concentrations of 0.05, 0.005 and 0.0005% (w/v). Control beans were dipped in a solution of bovine serum albumin (BSA) at the same concentrations used for BGEO. After evaporation of the solvent, the chambers were prepared.

In the binary choice oviposition chamber, two samples of 10 seeds treated with BGEO were placed in two containers positioned opposite each other. Samples of 10 control seeds were deposited in the two remaining containers. At the beginning of the experiment, 20 or 30 female adults of *C. maculatus* were transferred to the center of the oviposition chamber. After 24 h, the adult beetles were removed from the chamber and the numbers of eggs attached to each bean counted under a stereoscopic microscope (TL3000 Ergo, Leica Microsystems).

## RESULTS

### *Anatomical and histochemical analyses*

*B. gratioloides* is a small herbaceous plant, about 50-60 cm, with opposite leaves, sessile, aromatic, with crossed opposite phyllotaxis (Fig. 1A). The flowers are zygomorphic, bilabiate, hermaphrodites, lilac, with purple lines on the anterior petal, and yellow spot on the posterior petal, hairy on the outer portion of the floral tube; the stem

is greenish, and the roots have a spongy consistency with ample development of aerenchyma.

The anatomical study showed that the leaf epidermis is composed of typical epidermal cells, stomata, and trichomes and is covered by a thin and striated cuticle, except for over the trichomes (Fig. 1B-C). The leaf is amphistomatic, and the stomata are anomocytic type, organized in rows, more numerous on the underside (Fig. 1D). Trichomes are formed by a basal cell, and a group of five cells, organized radially and that constitute the secretory region (Fig. 1D). A single, thin cuticle covers the whole set of secretory cells.

In cross-section, the epidermis is uniseriate, formed by voluminous cells; stomatal cells are located above the rest of the tissue (Fig. 1G-H). The glandular trichomes are implanted below the level of the epidermal cells and are inserted in the epidermis through a basal cell. Secretory cells are characterized by containing lipophilic substances, as evidenced by Sudan IV (Fig. 1F). The Nadi test confirmed the occurrence of terpenoids in the glandular trichomes, accumulated under the cuticle (Fig. 1E).

The leaf presents a heterogeneous mesophyll, with loose and short palisade parenchyma cells on both sides, and spongy parenchyma in the central portion (Fig. 1G). The vascular system is formed by collateral bundles, surrounded by an evident parenchyma sheath, including in the leaf margin region; a thicker cuticular layer covers it (Fig. 1H).

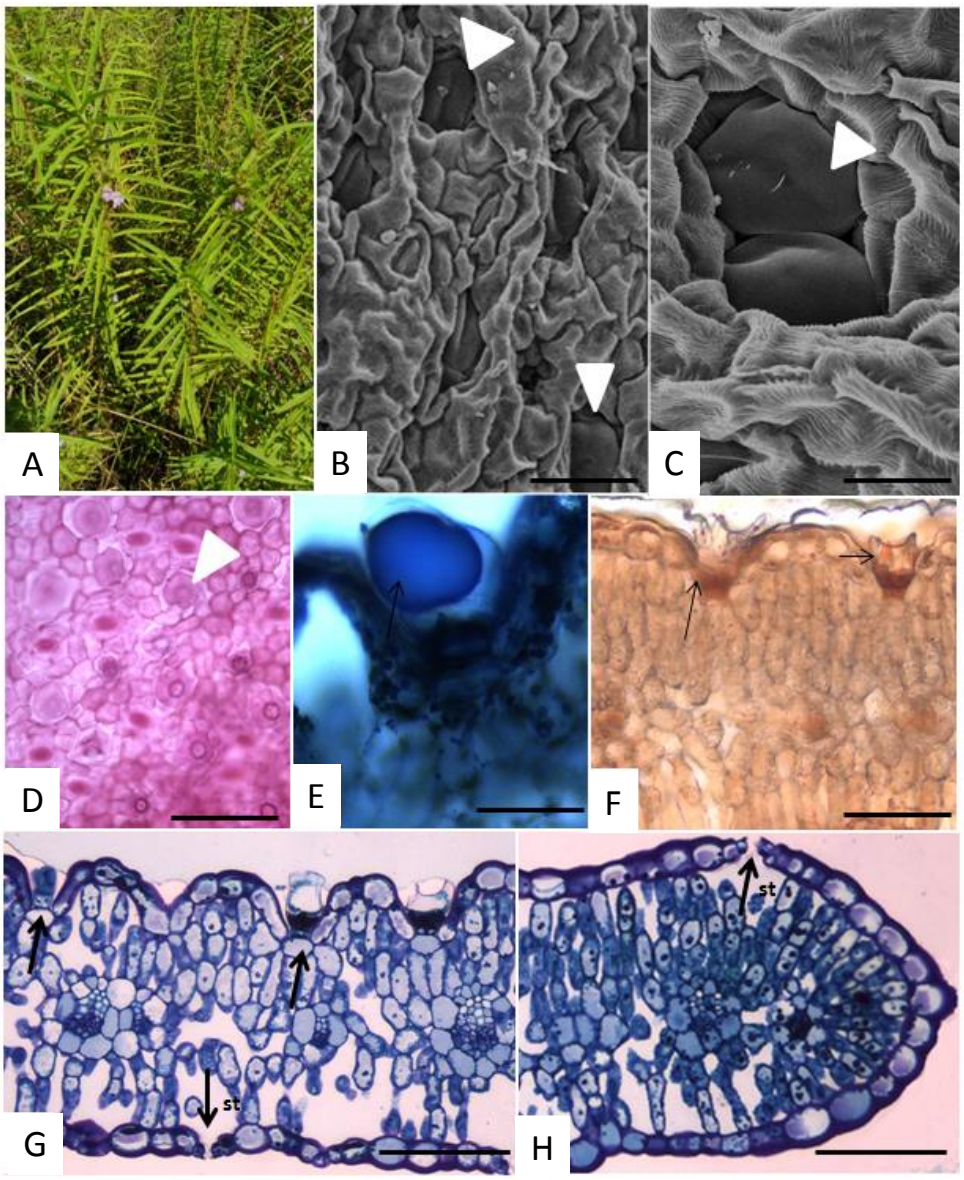



**Fig. 1.** *Bacopa gratioloides* (Plantaginaceae). Plant habit and leaf anatomical structure. (A) Plant habit in a temporary pond. (B) Leaf epidermis under surface electronic microscopy (SEM): glandular trichomes (arrowhead), stomata, and common epidermal cells. (C) Leaf epidermis under SEM: striated leaf cuticle and glandular trichomes (arrowhead). (D) Abaxial surface under light microscopy (LM): stomata and glandular trichome. (E) Histochemical test with Nadi showing terpenes in glandular trichomes apical cells (LM). (F) Histochemical test with Sudan IV: lipophilic drops are evidenced in glandular trichome cells (arrows, LM). (G) Leaf in cross section

(LM): uniseriate epidermis, with glandular trichomes, stomata (st), palisade, and spongy parenchyma, vascular bundles are surrounded by parenchyma sheath. (H) Margin leaf cross section showing thick cuticle, stomata, and palisade parenchyma under the epidermis, including the edge (LM).

*Chemical analyses by GC-MS and molecular networking of BGEO*

The chemical analysis of BGEO revealed several volatile constituents. Thirteen compounds could be annotated using the library of equipment (NIST, FFNSC, and Wiley) and the libraries on GNPS platform. Several compounds do not hit with available libraries and could be new or rare compounds.

The compounds annotated from BCEO were the oxygenated monoterpenes linalool, α-terpineol and geraniol, the sesquiterpenes α-copaene, α-guaiene, β-acoradiene, α-muurolene, δ-cadinene, and (*E*)-nerolidol, the oxygenated hydrocarbon octen-3-ol, and the hydrocarbonates tricosane, pentacosane, and heptacosan (Table 1). The metabolite in 29 min. showed the highest relative percentage (58.2 %) which was isolated and identified in a previous work by our research group as 7-epi Dihydroartemisinic acid.

The molecular networking was built from MS data and the edges/connections between nodes were represented by lines that their thickness is relative to cosine score, revealing similar MS data (Fig. 2). A node cluster family was observed between the peaks 1 (1-octen-3-ol) and 35-38, but the edge was thinner with peak 1, revealing lower similarity of this compound with others in this node. The peaks 35, 36, and 37 were annotated as tricosane, pentacosane, and heptacosane; thus five other components (RT 61.06, 62.41, 64.18, 66.25, and 67.06) were in this hydrocarbon cluster. Other node cluster families showed peaks 19, 22-23, 25-26, and 29-30, which are non-annotated

compounds, but they revealed similarities to the sesquiterpene 29 that is the main compound of BGEO.

The peaks relative to oxygenated monoterpenes 2 (linalool), 3 (α-terpineol), and 4 (geraniol) were present in a node cluster, which revealed other compounds with spectral similarities (Fig. 2). Besides, the non-oxygenated sesquiterpenes 8 (α-copaene), 10 (α-guaiene), 11 (β-acoradiene), 12 (α-muurolene), and 13 (δ-cadinene) were grouped in the same cluster, revealing chemical similarities between these compounds and others that could not be annotated.

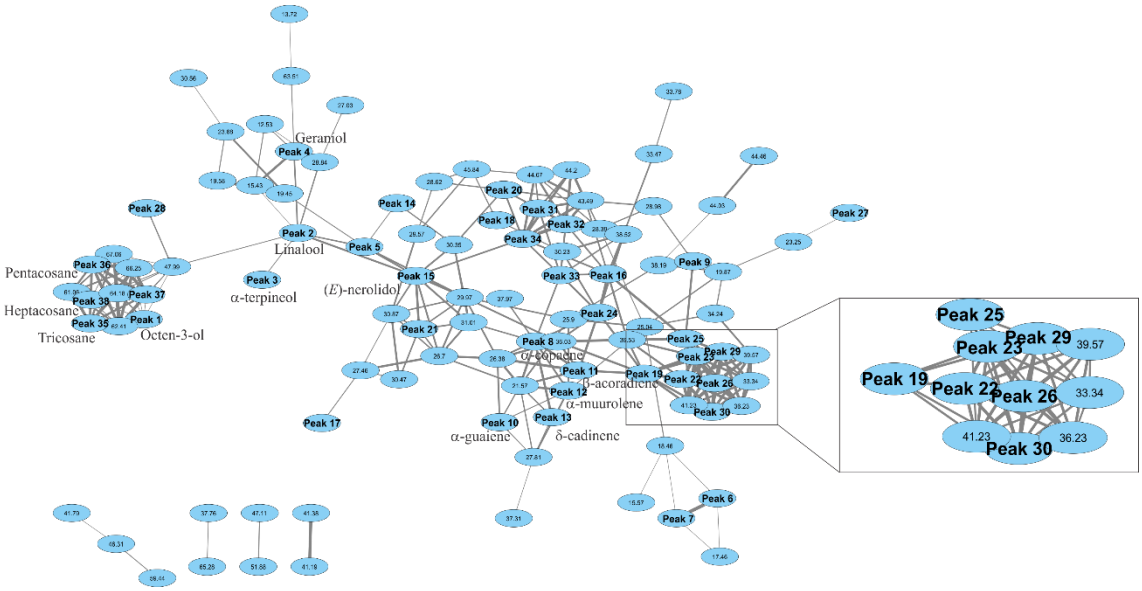



**Fig. 2.** Molecular network of BGEO displaying the compounds described in Table 1, highlighting the node cluster family with the oxygenated sesquiterpene 29.

**Table 1.** Constituents identified of the aerial parts essential oil from *Bacopa gratioloides* (BGEO) by GC-MS

| Peak | RT (min) | Compound | Area % | RI |
|---|---|---|---|---|
| 1 | 6.24 | Octen-3-ol | 0.25 | 974 |
| 2 | 10.29 | Linalool | 0.10 | 1097 |
| 3 | 14.07 | α-terpineol | 0.05 | 1191 |
| 4 | 16.53 | Geraniol | 0.06 | 1249 |
| 5 | 16.87 | NI | 0.13 | 1256 |
| 6 | 18.85 | NI | 0.63 | 1303 |

| | | | | |
|---|---|---|---|---|
| 7 | 19.01 | NI | 0.43 | 1307 |
| 8 | 21.87 | α-copaene | 0.27 | 1374 |
| 9 | 23.47 | NI | 0.44 | 1413 |
| 10 | 24.34 | α-guaiene | 0.01 | 1434 |
| 11 | 25.27 | β-acoradiene | 0.70 | 1457 |
| 12 | 26.87 | α-muurolene | 0.02 | 1496 |
| 13 | 27.68 | δ-cadinene | 0.25 | 1517 |
| 14 | 28.01 | NI | 1.28 | 1526 |
| 15 | 29.24 | (*E*)-Nerolidol | 0.40 | 1558 |
| 16 | 29.73 | NI | 0.74 | 1570 |
| 17 | 31.29 | NI | 0.14 | 1620 |
| 18 | 31.70 | NI | 0.42 | 1622 |
| 19 | 31.93 | NI | 0.49 | 1628 |
| 20 | 32.33 | NI | 0.14 | 1639 |
| 21 | 32.42 | NI | 0.21 | 1642 |
| 22 | 34.12 | NI | 3.28 | 1688 |
| 23 | 34.49 | NI | 5.40 | 1698 |
| 24 | 35.19 | NI | 1.10 | 1717 |
| 25 | 36.86 | NI | 1.41 | 1765 |
| 26 | 37.08 | NI | 0.37 | 1771 |
| 27 | 39.11 | NI | 0.59 | 1830 |
| 28 | 39.41 | NI | 0.21 | 1839 |
| 29 | 41.01 | 7-epi Dihydroartemisinic acid | 58.29 | 1887 |
| 30 | 41.42 | NI | 1.53 | 1899 |
| 31 | 42.47 | NI | 0.76 | 1931 |
| 32 | 43.32 | NI | 18.34 | 1958 |
| 33 | 43.64 | NI | 0.79 | 1968 |
| 34 | 43.79 | NI | 0.72 | 1972 |
| 35 | 53.50 | Tricosane | 0.01 | 2301 |
| 36 | 58.79 | Pentacosane | 0.02 | 2500 |
| 37 | 64.85 | Heptacosane | 0.01 | 2708 |
| 38 | 65.52 | NI | 0.14 | 2725 |

NI: not identified; RT: retention time; RI: retention indices on DB-5 capillary column.

***Antifeedant and deterrent activity***

The BGEO was added into artificial and offered to *A. kuehniella* larvae in different concentrations, ranging from 0.8 to 1.4 % (w/w). At the end of 4$^{th}$ instar (25 days), the larval weight and survival were recorded. Figure 3 shows the effect of BGEO on larval development. At 0.8 %, BGEO reduced the larval weight by 38 %. Increasing the

concentration to 1.2 and 1.4 % did not differ significantly the average weight between them, showing a reduction of 78 and 76 % in larval weight, respectively (Fig. 3A). Even showing a dose-dependent effect on larval weight, the survival rate was not affected in the same proportion. At 0.8 and 1.2 %, larval survival was reduced by 14 %, on average, while at the highest concentration of BGEO (1.4 %), the reduction in survival was about 26 %. Only the highest concentration caused a significant reduction in larval survival ($P > 0.05$). Through the representative image of larvae from different treatment and analysis of the head capsule, we note the difference among the larval instars. While the control larvae reached the $4^{th}$ instar, oil-fed larvae showed retard on development, keeping in $3^{rd}$ in 0.8 % and 1.4 % and $2^{nd}$ instar when fed at 1.2% (Fig. 3C). Our results show that BGEO in an artificial diet inhibited the larval feeding, demonstrating that the oil exhibits antifeedant properties.

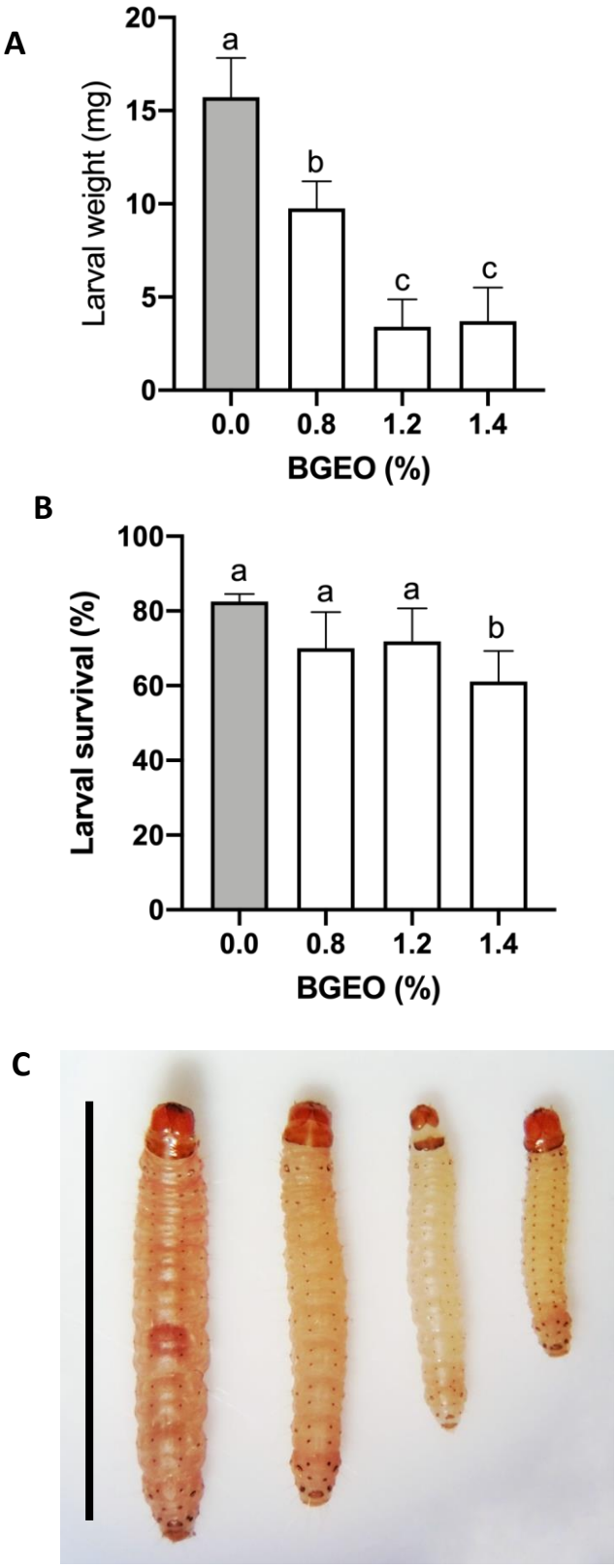
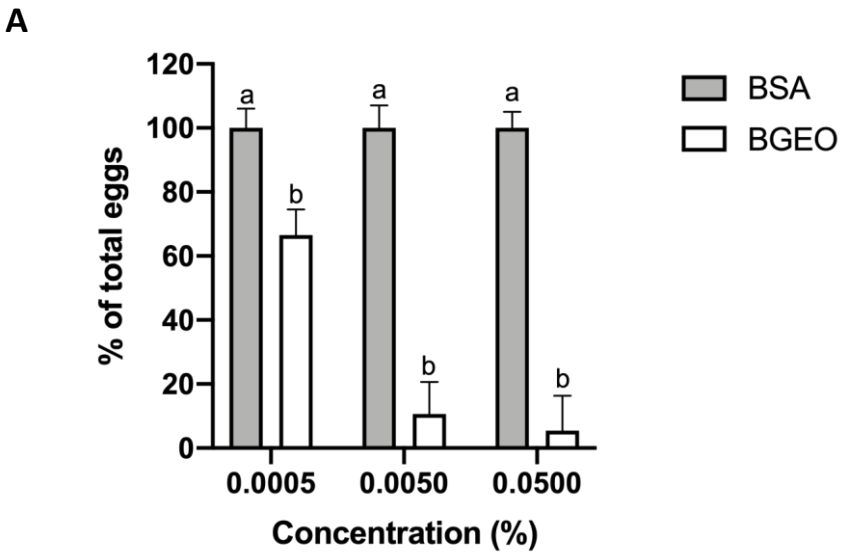



**Fig. 3.** Effects of *B. gratioloides* essential oil (BGEO) feeding on *A. kuehniella* development. The effects of BGEO was evaluated on larval weight (A) and survival (B). Data are expressed as means ± SD, based on two independent experiments, consisting of n= 40 for each treatment. (C) Representative image of larvae of each treatment, in sequential order from the left: control-fed larvae, larvae fed with 0.8%, 1.2%, and 1.4% of BGEO. Bar= 1 cm.

Further experiments were carried out to investigate whether the adverse effects observed by BGEO go beyond the antifeedant properties. We developed a binary choice experiment evaluating the ability of insects to avoid BGEO-coated cowpea for oviposition. Seeds of cowpea were coated with BGEO were prepared in different solutions, 0.05; 0.005, and 0.0005% (w/v) and exposed to 20 females *C. maculatus* adults. Beans also were coated with BSA at the same concentrations in the control treatment. The results indicated that all concentrations of BGEO significantly reduced the *C. maculatus* laying (Fig. 4A). BSA coated bean did not inhibit the laying egg, suggesting that activity is specific and observed for specific molecules.

Finally, we carried out an experiment increasing the density of females, from 20 to 30 adults, to investigate the effect of density in the choice of laying sites. The exposition of beans to 30 females completely abrogated the deterrent activity observed previously (Fig.4-B). None concentration reduced the *C. maculatus* laying egg, with no significant differences between BGEO and BSA oviposition.



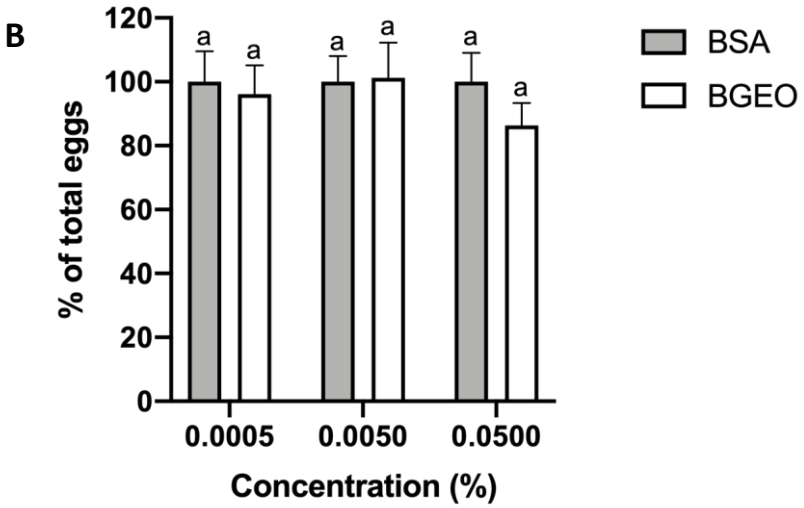



**Fig. 4.** Distribution of eggs deposited in the oviposition chamber by (A) 20 or (B) 30 *C. maculatus* female adults. The cowpeas were coated with BSA (control) or BGEO in different concentrations. The total number of eggs laid in each treatment was set at 100%. Data are expressed as means ± SD, based on two independent experiments, consisting of 3 oviposition chambers.

## DISCUSSION

The aromatic plant *B. gratioloides* is still little studied and has records of collections in some regions of Brazil. In this work, this plant was collected in the Chaco region located in the western region of Brazil, where Chaquean vegetation occupies an area of approximately 70,000 km² (Arruda et al., 2019). The absence of studies on the phytochemistry and pharmacological properties of *B. gratioloides* has aroused our interest in the composition and properties of the essential oil of this plant.

The anatomical characterization was performed for *B. gratioloides,* and the results corroborate with some information presented by Metcalfe & Chalk (1950) for species of Plantaginaceae. *Bacopa* is a genus of plants recognized for presenting species with medicinal value, including *Bacopa floribunda* (R.Br.) Wettst and *B. monnieri* used in Ayurveda for Brahmi (Gubbannavar et al., 2013). For *B. monnieri*, a plant widely used as a medicine in India, some similar descriptions of our observations are mentioned such as

the presence of glandular, multicellular trichomes, anomocytic stomata, uniseriate epidermis (Anju et al. 2017, Gubbannavar et al., 2013). Some elements mentioned, however, were not detected in *B. cochlearia* (Huber) L.B.Sm, such as calcium oxalate crystals, mesophyll containing subepidermal layers, diacitic stomata, indicating that the attributes, described here for the first time, have diagnostic value.

In the present work, BGEO showed in its composition monoterpenes sesquiterpenes, and hydrocarbons. The sesquiterpene 7-epi Dihydroartemisinic acid displayed a highly relative percentage (58.29%) and other similar sesquiterpenes present in the same node cluster family cannot be annotated. Plant essential oils are commonly complex mixtures of monoterpenes, sesquiterpenes, and aromatic compounds, and they have been widely used as bactericidal, virucidal, fungicidal, antiparasitic, insecticidal, medicinal, and for cosmetic applications. Several monoterpenoids exhibit toxicities against stored product and urban pests, which are good spatial repellents, insecticidal, and larvicidal and could be used in pest control (Gonzalez-Coloma et al., 2013). The monoterpene linalool, one of the compounds of BGEO, has shown repellent activities against female *Aedes aegypti* in laboratory and field studies (Kwon et al., 2011), and larvicidal activities against *Anopheles* and *A. aegypti* (Nour Et al., 2009; Fujiwara et al., 2017)**.**

Sesquiterpenes also influence insect physiology. In contrast to the monoterpenes, mostly toxic (fumigants), the sesquiterpenes have more antifeedants and deterrents activities (Gonzalez-Coloma et al., 2013; Schultz et al., 2006). Nerolidol, also present in BGEO, showed adulticide and ovicidal activities against *Pediculus capitis* (head lice) and *Pediculus humanus,* and antifeedant activities against gypsy moth larvae from *Melaleuca leucadendron* (Priestley et al., 2006; Di Campli et al., 2012).

Thus, sesquiterpenes, the class of the main compound identified in BGEO, can also be related to its antifeedant and deterrent activities. These results corroborate with data from the literature that show that essential oil that has both monoterpenes (acting as an excellent spatial repellent) and sesquiterpenes (good contact repellent) are incredibly effective via both modes of action and show potential for repellent, insecticidal and antifeedant action from a natural product (Schultz et al., 2006). Moreover, several studies have shown the synergetic activity of terpenoids against insects, which may be related to the different mechanisms of action of each compound (Mossa, 2016). Monoterpenes act on many insect targets, with different mechanisms (as acetylcholinesterase inhibition and neurotoxicity) (Isman, 2000). Gallardo *et al.* reported that a mixture of citronellol, geraniol, citronellyl formate, and linalool from *Geranium maculatum* L. essential oil has a synergistic effect against *Pediculus humanus capitis*, the removal of any of the four constituents produced a decrease in the efficacy. This suggests that the monoterpenes and sesquiterpenes identified in BGEO in this work, may also be acting synergistically, which may lead to a broad spectrum and less toxic insecticide.

The antifeedant and deterrent activities of the BGEO against insect pests from storage food, the Mediterranean flour moth, and the cowpea weevil, was highlighted. Our first experiment, using *A. kuehniella* larvae, brought information regards non-choice assay, since the only difference between treatments was the concentration of BGEO used in an artificial diet. So, we decided further to investigate, through a binary choice assay, a possible repellent or deterrent activity of BGEO using insects that do not feed during the adult stage, such as *C. maculatus*. The results from non-binary assay demonstrated that the incorporation of oil in low concentration exerts antifeedant activity. Moreover, the binary choice assay demonstrated that the application of BGEO on cowpea reduces the oviposition by *C. maculatus.*

Despite the lack of mechanism of action for terpenes on feeding deterrence, studies suggest that action on taste receptors might mediate the observed effects. For lepidopteran larvae, the binding caused by drimane sesquiterpenes blocks the stimulatory effects of glucose, sucrose, and inositol on chemosensory receptor cells from mouthparts (Gershenzon & Dudareva, 2007). Regards the effects on egg-laying, it was apparent the ability of the females to distinguish the oil-coated cowpea from seeds coated with BSA. It is well known that sensory mechanisms are an essential modulator of insect behavior for survival and reproduction, including the selection of mates, location of food resources, and selection of oviposition sites (Hekmat-Scafe et al., 2002; Ban et al., 2003; SADEGHI, 2006). This finding corroborates with Messina et al. (1987 a,b) that described the location of organs related to hosting discrimination and egg-spacing behavior in maxillary and labial palpi of *C. maculatus.*

We observed that the insect density modulated the deterrent activity. A possible argument that explains this finding was raised by Sadeghi et al. (2006) that observed a similar result. According to the authors, the increase in density of adults increases the movement on the seeds during the exploration searching egg-laying sites. It could cause the physical remotion of the oil layer and increase the oviposition.

Thus, our study demonstrated the deterrent activity of BGEO against the Mediterranean flour moth, *A. kuehniella* (Lepidoptera), and the cowpea weevil, *C. maculatus* (Coleoptera). It is essential to emphasize the relevance of these findings since this is the first phytochemical and biological studies from *B. gratioloides* oil. Therefore, the compounds from BGEO showed activity against pests from stored products, which can be used to develop repellents and deterrents of natural origin as an alternative to synthetic products, reducing the environmental impact.

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**Conflict of Interest**

All authors declare that they have no conflict of interest.

**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

**Author's Contributions**

NSC, JCPM, and DBS conceived and designed research. RCOA and TSY collected the plant. NSC, CFRO and LBS conducted experiments. NSC, CAC, JGA and DBS analyzed the chemical data. CFRO and MLRM analyzed the biological data. NSC and DBS wrote the manuscript. All authors read and approved the manuscript.

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# Capítulo 4

# 6 NEW SESQUITERPENES OBTAINED FROM BIOTRANSFORMATION OF A SESQUITERPENE FROM *Bacopa gratioloides* BY *Aspergillus niger*

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## Abstract

The sesquiterpene 7-epi Dihydroartemisinic acid (STP1), isolated from the essential oil of the aerial parts of *Bacopa gratioloides*, have showed cytotoxic effect against the murine melanoma cancer cells (B16F10-Nex2) and promoted inhibition of cell migration *in vitro*. Herein, we investigated the biotransformation of STP1 using *Aspergillus niger* (MW603826). LC-DAD-MS analysis showed that biotransformation of STP1 afforded seventeen new metabolites by hydroxylation (1, 3, 5, 7, 11, 12, 13, 15 and 16), hydroxylation and dehydrogenation (2, 4, 6, 9, 14 and 17) and hydroxylation and double bond reduction reactions (8 and 10). In addition, the compounds 11 and 13 were isolated and their structures were determined by spectroscopic techniques including two-dimensional NMR and HRMS. These compounds, obtained from regio and stereoselective hydroxylation reactions on non-functionalized hydrocarbons, would hardly be produced in common chemical reactions. Thus, in this work, we report for the first time the biotransformation of STP1 by *A. niger*, which proved to be an excellent biocatalyst for obtaining derivatives.

**Key words:** 7-epi Dihydroartemisinic acid, biotransformation, hydroxylation, *Aspergillus niger*.

## INTRODUCTION

Aromatic plants commonly present essential oils in their composition, which are complex mixtures of volatile compounds, such as monoterpenes, sesquiterpenes, and phenylpropanoids. The volatile compounds have presented over the years several pharmacological applications, such as antioxidant, anticancer, antiprotozoal, antimicrobial, and anti-inflammatory activities [1-5]. Sesquiterpenes are a subclass of terpenes that have been described to display a large range of biological and pharmaceutical activities that include effects on the central nervous system, antimicrobial, anti-inflammatory, and anti-tumor actions [6-8]. Sesquiterpenes have demonstrated therapeutic potential in decreasing the progression of cancer [8]. Costunolide, a sesquiterpene lactone from *Aucklandia lappa* DC., have showed effects against prostate (LNCaP, PC-3, DU-145) [9], ovarian (MPSC1(PT), A2780(PT), and SKOV3(PT)) [10], bladder (T24) [11] and leukemia (U937) cancer cells [12].

Sesquiterpenes of cadinene skeleton have also displayed important anti-cancer activities. Six new cadinene sesquiterpenoids, xenitorins from $CH_2Cl_2$ extracts of the Formosan soft coral *Xenia puerto-galerae* Roxas (family Xeniidae) showed significant cytotoxicity against A549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma), and P388 (mouse lymphocytic leukemia) cancer cells [13]. A recent study reported the isolation of a new muurolen sesquiterpene, 7-epi Dihydroartemisinic acid, from the essential oil of aerial parts of *Bacopa gratioloides* (Cham.) Edwall (Plantaginaceae), which displayed cytotoxic effects against murine melanome cancer cells (B16F10-Nex2) and it induced inhibition of cell migration *in vitro,* as well as cellular death by apoptosis. In addition, this sesquiterpene were evaluated on MRC5 healthy cell line (human lung fibroblasts), showing selectivity of 7-epi Dihydroartemisinic acid for B16F10-Nex2 cells [14].

Although many of the main constituents of essential oil are readily available in nature, they are also produced or modified by chemical transformation on a very large scale and demonstrate their ready availability. One of the techniques used to produce new analogues of bioactive compounds is biotransformation reactions by microorganisms. Biotransformation is a potential technology tool for sustainable development, besides it is according to the principles of green chemistry [15]. Through it, it is possible to carry out the molecular modification in a specific location of a target compound to obtain

distinct derivatives by biological catalysts including microorganisms such as fungi, bacteria, and isolated enzymes. The main advantages of biotransformation reactions are the regio- and stereospecificity of the reactions promoted by biocatalysts, in addition they are environmental friendly reactions of low cost, and promote commonly multiplicity of types of reactions such as hydroxylation, reduction, oxidation, and acetylation in a single step. Therefore, biotransformation reactions can be useful to industrial applications for obtention of new candidates and drugs [16-18].

Filamentous fungi, such as *Aspergillus niger*, have a great enzymatic apparatus capable of carrying out the most diverse reactions, including hydroxylation, oxidation, hydrogenation, hydrolysis, and methylation. The presence of oxygenases enzymes can catalyze regio- and stereoselective hydroxylation of a variety of non-functionalized hydrocarbon centers of a great variety of substrates. Sesquiterpenes are structurally components with non-funcionalized $sp^3$ hybridized carbons sites for oxidation reactions by microorganisms, which are hardly carried out by traditional chemical synthesis [17,19-21]. Chen et al. (2014) showed that the biotransformation reactions from the sesquiterpene curdione by *A. niger* yielded seven metabolites by regio- and stereoselective hydroxylations [21].

Therefore, here we aimed to explore structural modifications in the sesquiterpene 7-epi Dihydroartemisinic acid (STP1) isolated from essential oil of *B. gratioloides* by fungi-mediated biotransformation using *A. niger* fungus as biocatalyst.

## MATERIAL AND METHODS

### *Plant material and Extraction and purification of essential oil*

The obtainment and characterization of STP1 were carried out as described by Cassemiro et al [14].

### *Identification of microorganism*

The method here used followed the protocol proposed by Cassemiro et al (2021) [22]. The strain used in the biotransformation reactions was identified by morphology and molecular biology, and it provided from the library of Federal University of Mato Grosso

do Sul (UFMS). The *Aspergillus niger* was morphologically identified by MSc Clarice Rossato Marchetti (according to Bergey's Manual of Determinative Bacteriology). The ITS of strain was deposited at NCBI GenBank under the following accession number: *A. niger* (MW603826). The fungal strain was deposited in the library URM (University Recife Mycology) MMBF (Micoteca Mario Barreto Figueiredo), which is inserted in the World Data Centre for Microorganisms under number WDCM 942. *A. niger* was deposited in MMBF at number MMBF 02/2021.

***Biotransformation assay***

Liquid culture medium was composed by proteose peptone (0.500 g), glucose (0.609 g), $K_2HPO_4$ (0.075 g), $MgSO_4.7H_2O$ (0.075 g), and deionized water (50 mL). *A. niger* was inoculated in this medium, which was incubated at 30 $^oC$ under constant stirring (110 rpm) for 48 h. After this period, the STP1 (50 mg prepared in 200 μL of methanol) were added to the culture medium. The reaction was monitored every 12h until total consumption of the starting material. Aliquots of 400 μL of the medium were extracted with ethyl acetate (200 μL, three times) and analyzed by thin layer chromatography (TLC) (Silica gel, UVGF254, Sigma-Aldrich), using as eluent the solvents dichloromethane and acetone (9:1 v/v) added acetic acid (0.5%). The samples were also analyzed by liquid chromatography coupled to diode array detector and mass spectrometer (LC-DAD-MS). The reactions without sesquiterpene (contained only the culture medium and the strains) were used as blank and the controls were composed by the culture medium and sesquiterpene (without fungi). All fungi-mediated biotransformations were performed in duplicate. The blank and control samples were also analyzed by LC-DADMS to confirm the derivatives yielded from biotransformation reactions of sesquiterpene. After total consumption of the starting material (determined by TLC analyses), the mycelium was filtered, and the culture medium was extracted with ethyl acetate. The organic phase was dried over $Na_2SO_4$ and the solvent removed under reduced pressure, as described by Cassemiro et al [22].

***Purification of new analogs from STP1***

The extracts obtained from fungi-mediated biotransformation were firstly purified on a flash silica column (230-400 mesh) as stationary phase and mobile phase dichloromethane and acetone (9:1, v/v) added acetic acid (0.5%). The polarity of the mobile phase was gradually increased until it reached 100% acetone and at the end, it was changed to 100% methanol.

After obtaining 19 fractions from the chromatographic column, fraction 17 was subjected to a second purification by preparative TLC using the same initial mobile phase.

***LC-DAD-MS analyses***

The extracts of fungi-mediated biotransformation reactions were solubilized in methanol (2 mg/mL) and water deionized (7:3, v/v) and 1 µL was injected into the chromatography system LC-20AD UFLC (Shimadzu Corp.) coupled to a diode array detector and a mass spectrometer ESI-QTOF (MicrOTOF-Q III, Bruker Daltonics, Billerica, MA, USA). The mobile phase for the chromatographic analyses was water (Phase A) and acetonitrile (Phase B), both with 0.1% formic acid, and the elution gradient profile applied was the following: 0-2 min - 3% Phase B, 2-25 min - 3-25% Phase B, 25-40 min - 25-80% Phase B, 40-43 min - 80% B, followed by column washing and reconditioning (5 min). The chromatographic column was a Kinetex C18 (2.6 µm, 150 x 2.1 mm, Phenomenex), applying a flow rate of 0.3 mL/min and oven temperature of 50 °C. The analyses were monitored at 240-800 nm and acquired in negative ion mode (*m/z* 120-1200). MS/MS spectra were acquired by automatic method using a collision energy 45 to 65 eV and applying nitrogen as collision gas.

The data were processed by Data Analysis software version 4.2 (Bruker) and compounds were annotated based on spectral data (UV, MS and fragmentation profile), as well as some components were confirmed by injection of isolated analogs obtained here. The molecular formulas were determined based on the accurate mass considering errors up ±5 ppm and mSigma below 30.

### *NMR analyses*

The compounds isolated from fungi-mediated biotransformation reactions were dissolved in methanol-D4 and analyzed by nuclear magnetic resonance (NMR) spectrometer (DPX-500 Bruker, Germany) operating at 500 MHz for $^{1}H$ and 125 MHz for $^{13}C$. Chemical shifts ($\delta$) are given in parts per million (ppm) downfield from tetramethyl silane or deuterated solvent as the internal standard. The coupling constants (*J*) were expressed in Hertz (*Hz*).

## RESULTS

### *Identification of biotransformation products*

From LC-DAD-MS analysis, it was possible to observe seventeen analogs of STP1, such as hydroxylated (1, 3, 5, 7, 11-13, and 15-), hydroxylated and dehydrogenated (2, 4, 6, 9, 14, and 17) and hydroxylated and reduced on double bond analogs (8 and 10) (Figure 1, Table 1).

The ions from peaks 1, 3, 5, and 7 (*m/z* 267 $[M-H]^-$, $C_{15}H_{24}O_4$,) were observed added 32 *u* compared to STP1. In addition, the fragment ions at *m/z* 231 $[M-H-2xH_2O]^-$ and 203 $[M-H-2xH_2O-CO]^-$ were observed and suggested dihydroxylation on STP1. The analogs 11-13, and 15-16 exhibited the same ion at *m/z* 251 $[M-H]^-$ compatible with the molecular formula $C_{15}H_{24}O_3$, suggesting isomers. These analogs revealed one more oxygen atom than the precursor sesquiterpene STP1 ($C_{15}H_{24}O_2$) and exhibited the fragment ions *m/z* 233 relative to losses a water molecule that confirmed the hydroxyl group. Thus, they were identified as monohydroxylation analogs from STP1.

The metabolites 2, 4, 6, and 14 (*m/z* 265.1452 [M-H]-) revealed the same molecular formula $C_{15}H_{22}O_4$, indicating two hydrogens less and two hydroxylations on STP1 structure. The analog 14 exhibited the product ions at *m/z* 221 and 203 yielded from subsequent losses of $CO_2$ and water molecules. The losses of water molecule suggested the aliphatic hydroxyl groups in the structure [23]. The fragment ion at *m/z* 191 was compatible to $C_{12}H_{15}O_2^-$, indicating the losses of the carboxylated isopropyl unit. Thus, these data suggested dihydroxylated analogs with the formation of a double bond

(reduction). Compound 9 exhibited the molecular formula $C_{15}H_{20}O_4$ that revealed additionally two oxygens and less four hydrogens when compared to STP1. So, the analog 9 was putatively annotated as dihydroxylated and reduced with additional two double bonds in STP1. The analog 17 exhibited molecular formula $C_{15}H_{22}O_3$, indicating the addition of an oxygen and reduction of two hydrogens (double bond) from STP1. The fragment ion m/z 175 of 17 suggested no modifications on carboxylated isopropyl unit, since it was yielded from loss of 74 *u* ($C_3H_6O_2$). Thus, it was identified as a monohydroxylated product with the formation of a double bond.

Compounds 8 and 10, finally, showed an intense ion at *m/z* 269 $[M-H]^-$ that confirmed the molecular formula $C_{15}H_{26}O_4$ and indicated additionally two oxygens and two hydrogens on STP1 structure, suggesting the oxidation reactions. Compound 8 afforded the ions fragments at *m/z* 251 and 233, indicating two consecutive losses of water molecules and suggested the presence of aliphatic hydroxyls. Thus, compounds 8 and 10 were putatively annotated dihydroxylated and reduced analogs of STP1.

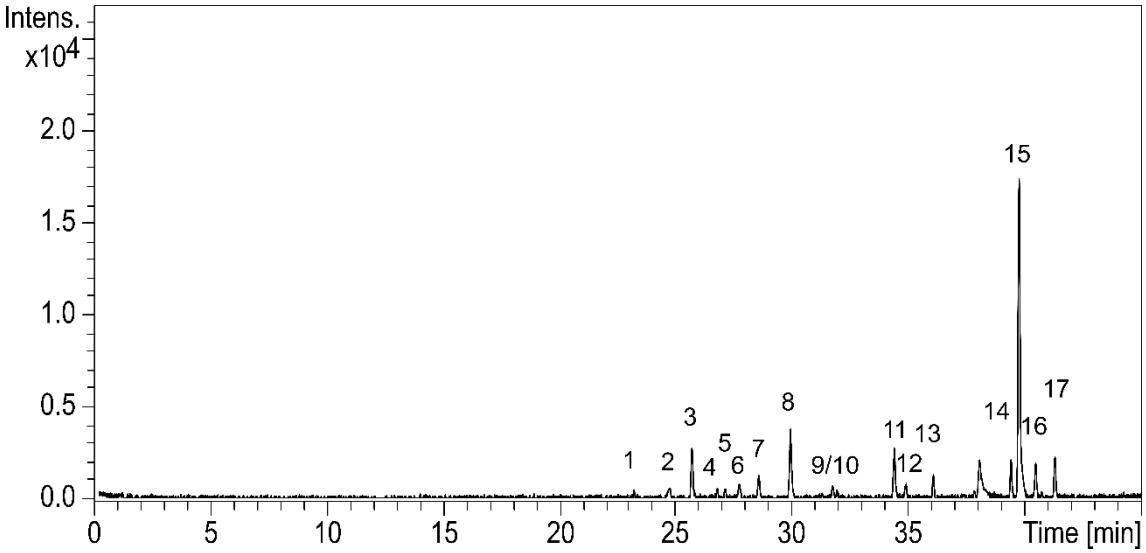


**Figure 1**. Base peak chromatogram by LC-DAD-MS from *A. niger*-mediated biotransformations of STP1.

**Table 1.** Compounds identified of biotransformation of STP1 by *A. niger* by LC-DAD-MS.

| Peak | RT (min) | MF | Negative mode (*m/z*) | | Compound | Reactions | Analyses |
|---|---|---|---|---|---|---|---|
| | | | MS $[M-H]^-$ | MS/MS $[M-H]^-$ | | | |
| 1 | 23.1 | $C_{15}H_{24}O_4$ | 267.1634 | - | Dihydroxy-STP1 | Dihydroxylation | LC-DAD-MS |
| 2 | 24.6 | $C_{15}H_{22}O_4$ | 265.1464 | - | Dihydroxy-dehydro-STP1 | Dihydroxylation and dehydrogenation | LC-DAD-MS |
| 3 | 25.6 | $C_{15}H_{24}O_4$ | 267.1648 | 205 | Dihydroxy-STP1 | Dihydroxylation | LC-DAD-MS |
| 4 | 26.7 | $C_{15}H_{22}O_4$ | 265.1469 | - | Dihydroxy-dehydro-STP1 | Dihydroxylation and dehydrogenation | LC-DAD-MS |
| 5 | 27 | $C_{15}H_{24}O_4$ | 267.1650 | - | Dihydroxy- STP1 | Dihydroxylation | LC-DAD-MS |
| 6 | 27.7 | $C_{15}H_{22}O_4$ | 265.1459 | - | Dihydroxy-dehydro-STP1 | Dihydroxylation and dehydrogenation | LC-DAD-MS |
| 7 | 28.5 | $C_{15}H_{24}O_4$ | 267.1622 | 231, 203 | Dihydroxy- STP1 | Dihydroxylation | LC-DAD-MS |
| 8 | 29.9 | $C_{15}H_{26}O_4$ | 269.1788 | 251, 233, 209, 193, 179 | Dihydroxy-dihydro-STP1 | Dihydroxylation and reduction | LC-DAD-MS |
| 9 | 31.7 | $C_{15}H_{20}O_4$ | 263.1320 | - | Dihydroxy-dehydro-STP1 | Dihydroxylation and dehydrogenation | LC-DAD-MS |
| 10 | 31.9 | $C_{15}H_{26}O_4$ | 269.1816 | - | Dihydroxy-dihydro-STP1 | Dihydroxylation and reduction | LC-DAD-MS |
| 11 | 34.3 | $C_{15}H_{24}O_3$ | 251.1672 | 233, 211, 205, 175, 163 | 3-Hydroxy- STP1 | Monohydroxylation | LC-DAD-MS NMR |
| 12 | 34.9 | $C_{15}H_{24}O_3$ | 251.1695 | | Hydroxy- STP1 | Monohydroxylation | LC-DAD-MS |
| 13 | 36.1 | $C_{15}H_{24}O_3$ | 251.1672 | 233, 215, 205, 191, 177, 160 | 10-Hydroxy- STP1 | Monohydroxylation | LC-DAD-MS NMR |
| 14 | 39.4 | $C_{15}H_{22}O_4$ | 265.1477 | 221, 203, 191 | Dihydroxy-dehydro-STP1 | Dihydroxylation and dehydrogenation | LC-DAD-MS |
| 15 | 39.7 | $C_{15}H_{24}O_3$ | 251.1683 | 233, 215, 205, 203 | Hydroxy- STP1 | Monohydroxylation | LC-DAD-MS |
| 16 | 41.1 | $C_{15}H_{24}O_3$ | 251.1699 | 233, 221, 205 | Hydroxy- STP1 | Monohydroxylation | LC-DAD-MS |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 17 | 41.3 | $C_{15}H_{22}O_3$ | 249.1510 | 175 | Dihydroxy-dehydro-STP1 | Monohydroxylation and dehydrogenation | LC-DAD-MS |

***Isolation and identification of compound 11 and 13***

The fungi-mediated biotransformation reaction from STP1 was subjected to purification by column chromatography using silica flash, resulting the isolated analog 11 (Figure 2). In addition, a fraction from this chromatographic column was subjected to preparative TLC and the metabolite 13 was obtained (Figure 2). The structures were established by NMR spectroscopy (1H-NMR, 13C-NMR, COSY, HSQC, HMBC and NOESY) and HRMS (Tables 2 and 3).

The metabolite 11 corresponds, reported previously here, exhibited an intense ion m/z 251.1677 [M-H]-, which confirmed the molecular formula $C_{15}H_{24}O_3$ and suggested an analog yielded by oxidation reaction. The $^{1}$H- and $^{13}$C-NMR (Table 2) showed oxymethyn signals at $\delta_H$ 3.86 (1H, *d,* 3.5) and $\delta_C$ 68.5. The other $^{1}$H and $^{13}$C chemical shifts of compound 11 were similar to those of its sesquiterpene precursor [14]. A doublet at $\delta_H$ 5.61 (integration to 1 H) and $\delta_C$ 128.9 indicated the presence of a mono-substituted double bond. Three methyl groups were observed; at $\delta_H$ 0.84 and $\delta_H$ 0.94 two doublets from methyl group 13 and 14, respectively, and at $\delta_H$ 1.70 a singlet from olefinic methyl group 15. An acetyl group signal at 182.3 ppm relative to the carboxylic carbon also was present. The COSY experiment showed spatial correlations between H-3 ($\delta_H$ 3.86), H-2 ($\delta_H$ 1.61) and H-5 ($\delta_H$ 5.61). Correlations between H-3 ($\delta_H$ 3.86) with C-4 ($\delta_C$ 136.4), C-15 ($\delta_C$ 21.1), C-5 ($\delta_C$ 128.9) and C-1 ($\delta_C$ 34.5), and were observed in HMBC spectrum. In NOESY spectra, correlations between H-6 ($\delta_H$ 1.76) with H-5 ($\delta_H$ 5.61) and H-13 ($\delta_H$ 0.94); H-1 ($\delta_H$ 1.92) with methyl group H-14 ($\delta_H$ 0.9), H-9 ($\delta_H$ 1.38) and H-5 ($\delta_H$ 5.45); and H-5 ($\delta_H$ 5.45) with methyl group H-13 ($\delta_H$ 0.94), H-11 ($\delta_H$ 2.62), H-6 ($\delta_H$ 1.76) and H-1 ($\delta_H$ 1.92) (Table 2). Thus, the structure of analog 11 was established as 3-hydroxy-7-epi Dihydroartemisinic acid.

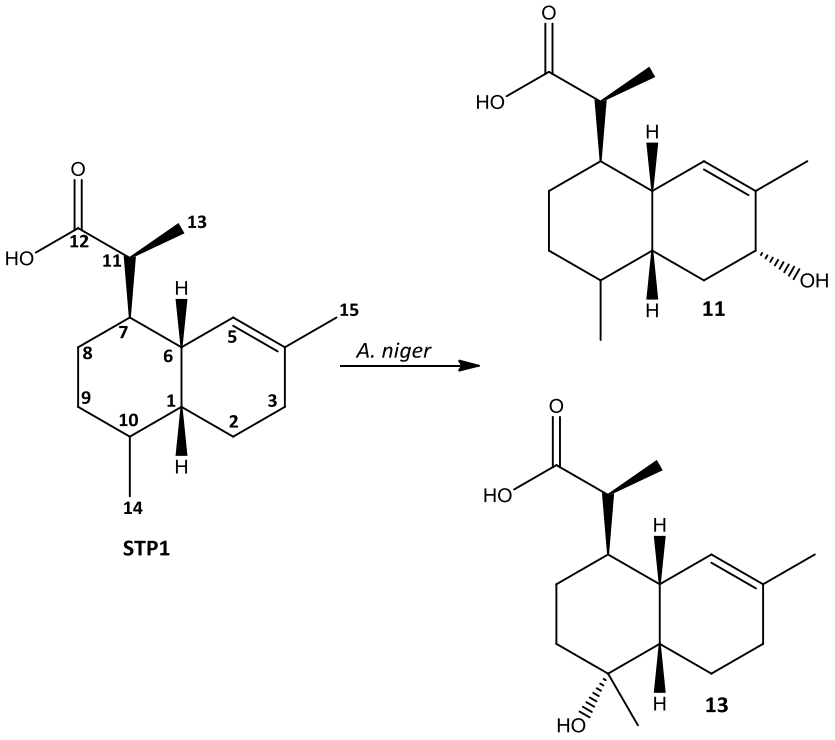


**Figure 2.** Compounds 11 and 13 isolated from STP1 biotransformation.

**Table 2.** $^{1}$H- (500 MHz, $CDCl_3$) and $^{13}$C- (125 MHz, $CDCl_3$) Chemical shift assignments of the compound 11

| H/C | δ $^{1}$H (int., mult., *J*)[a] | δ $^{13}$C | COSY | HMBC | NOESY |
|---|---|---|---|---|---|
| **1** | 1.92(1 H, *m*) | 34.5 | H-2; H-6 | C-2; C-3; C-6; C-10 | H-5; H-6; H-9; H-14 |
| **2** | 1.61 (2H, *m*) | 27.5 | H-1; H-3 | C-1; C-3; C-6 | H-3 |
| **3** | 3.86 (1H, *dl*, 2.7) | 68.5 | H-2; H-5 | C-1; C-4; C-5; C-15 | H-2 |
| **4** | - | 136.4 | - | - | - |
| **5** | 5.61 (1 H, *d*, 3.4) | 128.9 | H-3; H-6; H-15 | C-1; C-3; C-6; C-15 | H-1; H-6; H-11; H-13 |
| **6** | 1.76 (1 H, *m*) | 40.3 | H-1; H-5 | C-4. C-5; C-10 | H-13; H-5 |
| **7** | 1.77 (1 H, *m*) | 39.7 | H-9; H-11 | C-6 | H-9; H-11 |
| **8** | 1.09 (2 H, *m*) | 27.5 | H-9 | C-9 | H-9 |
| **9** | 1.28 (2 H, *m*) | 27.5 | H-7; H-8; H-10 | C-10; C-11 | H-1; H-7; H-8; H-14 |

| | | | | | |
|---|---|---|---|---|---|
| **10** | 1.57 (1 H, *m*) | 35.6 | H-9; H-14 | C-1; C-3; C-6 | H-8; H-14 |
| **11** | 2.62 (1H, *m*) | 40.5 | H-7; H-13 | C-6; C-8; C-13 | H-5; H-7; H-13 |
| **12** | - | 182 | - | - | - |
| **13** | 0.94 (3 H, *d*, *J*=6.9 Hz) | 9.3 | H-11 | C-11; C-12 | H-5; H-6; H-11 |
| **14** | 0.84 (3 H, *d*, *J*=6.9 Hz) | 19.3 | H-10 | C-10 | H-1; H-9 |
| **15** | 1.7 (3 H, *s*) | 21.1 | H-5 | C-3; C-4; C-5; C-6 | H-3; H-5 |

[a]500 MHz, 125 MHz, Metanol-D4

The compound 13, as reported previously, displayed an ion at *m/z* 251.1677 $[M-H]^-$ and confirmed the molecular formula $C_{15}H_{24}O_3$.The $^1H$ NMR showed three methyl groups, but these signals exhibited changed chemical shifts when compared with the analog 11 (Table 3). A methyl group presented as doublet, H-13 ($\delta_H$ 0.95). This indicated that hydroxylation occurred at C-10, which is directly linked to the methyl group 14 ($\delta_H$ 1.08), which was observed as a singlet. The olefinic methyl group was also showed as a singlet at $\delta_H$ 1,57. In the COSY experiment was observed spatial correlations between H-9 ($\delta_H$ 1.39) with C-8 ($\delta_H$ 1.03) and H-1 ($\delta_H$ 1.50); and C-3 ($\delta_H$ 1.93) with C-2 ($\delta_H$ 1.5) and C-5 ($\delta_H$ 5.43). The HMBC correlations between H-9 with C-10 ($\delta_C$ 72), C-8 ($\delta_C$ 21.4), C-7 ($\delta_C$ 41) and C-1 ($\delta_C$ 45.6), supported again a hydroxyl group at C-10. NOESY spectra showed correlation between H-1 with H-6 and H-14, showing that methyl group 14 kept the same relative configuration that its precursor STP1. The structure of new metabolite 13 was thus deduced as 10-hydroxy-7-epi Dihydroartemisinic acid.

**Table 3.** $^1H$- (500 MHz, $CDCl_3$) and $^{13}C$- (125 MHz, $CDCl_3$) Chemical shift assignments of the compound 13.

| H/C | δ $^{1}$H (int., mult., *J*)[a] | δ $^{13}$C | COSY | HMBC | NOESY |
|---|---|---|---|---|---|
| **1** | 1.50 (1 H, *m*) | 45.6 | H-6; H-7 | C-2; C-3; C-9; C-10; C-14 | H-6; H-14 |
| **2** | 1.50 (2 H, *m*) | 21 | H-3 | C-1; C-6; C-7 | H-3; H-7; H-8 |
| **3** | 1.93 (2 H, *m*) | 31 | H-2; H-5 | C-1; C-2 | H-2; H-15 |
| **4** | - | 134.8 | | - | |
| **5** | 5.43 (1 H, *dd*, 1.25, 5.4 ) | 123.8 | H-15; H-3; H-6 | C-1; C-3; C-6; C-15 | H-6; H-11; H-15 |
| **6** | 2.25(1 H, *m*) | 33.7 | H-1; H-7; H-5 | C-1; C-2 | H-1; H-5; H-7; H-11; H-13 |
| **7** | 1.80 (1 H, *m*) | 41 | H-1; H-6; H-8; H-11 | C-6; C-11 | H-2; H-6; H-11 |
| **8** | 1.03 (2H, *dd*, 3.3, 12.75) | 21.4 | H-7; H-9 | C-7; C-9; C-10 | H-2; H-7; H-9 |
| **9** | 1.39 (2H, *m*) | 34 | H-1; H-8 | C-1; C-7; C-8; C-10 | H-8; H-14 |
| **10** | - | 72 | - | - | |
| **11** | 2.66 (1H, *m*) | 39.5 | H-7; H-13 | C-7; C-8; C-12; C-13 | H-5; H-7; H-13 |
| **12** | - | 179.5 | - | - | |
| **13** | 0.95 (3 H, *d*, 7.1) | 8.6 | H-11 | C-7; C-11; C-12 | H-5; H-6; H-11 |
| **14** | 1,08 (3 H, *s*) | 28.3 | - | C-1; C-2; C-9; C-10 | H-1; H-9 |
| **15** | 1.57 (3 H, *s*) | 22.7 | H-5 | C-3; C-4; C-5; C-7 | H-3; H-5 |

[a]500 MHz, 125 MHz, Metanol-D4

## Discussion

In previous study, we isolated a new sesquiterpene STP1 in large quantities from the aerial parts of *B. gratioloides*. This sesquiterpene displayed cytotoxic effects against melanoma cells and it also promoted a marked inhibition of cell migration *in vitro* and death by apoptosis [14]. Therefore, in this work, we explored for the first time structural modifications of this sesquiterpene by fungi-mediated biotransformation reactions applying strains of the fungus *A. niger* as biocatalyst.

LC-DAD-MS analysis showed that biotransformation of sesquiterpene was efficient and seventeen new analogs of STP1 were yielded by hydroxylation and

dehydrogenation reactions that demonstrated the capacity of *A. niger* promotes both reactions, oxidation and reduction. *A. niger* is one of fungal species most frequently used for experimental and industrial scale biotransformations of various organic compounds and many studies have shown its ability to carry out these reactions. The presence of oxygenase enzymes in filamentous fungi such as *A. niger* make them capable of catalyzing region- and stereoselective hydroxylation of a variety of non-functionalized hydrocarbon centers of a great variety of substrates [17]. A recent work done by our research group showed the iridoid specioside biotransformation by *A. niger,* which was capable of performing several reactions and among the 19 metabolites obtained, which were obtained five hydroxylated products [22]. Enzymatic systems of *A. niger* MB and *A. niger* KB catalysed hydroxylation at C-4' in ring B of 5-hydroxyflavone affording 5,4'-dihydroxyflavone, which displayed higher antioxidant properties than the starting substrate [24]. In this work, all 17 metabolites from biotransformation are products of hydroxylation reactions. Some of them have undergone other reactions, but the metabolites 1, 3, 5, 7, 11, 12, 13, 15 and 16 are exclusively products of hydroxylation reactions. The compounds 11, 12, 13, 15 and 16 are isomers and were identified by LC-DAD-MS as monohydroxylated products. While the compounds 1, 3, 5 and 7 were identified as isomers of dihydroxylated products. These results corroborate the literature data showing the presence of oxygenases as part of the *A. niger* enzymatic apparatus, which was also shown to be present in the *A. niger* strain here used.

The isomers 8 and 10 showed, in addition to dihydroxylation, to be double bond reduction products. Several studies have also shown the ability of *A. niger* to carry out double bond reduction reactions and the applicability of the its metabolites [25, 26, 27]. Esmaeili et al (2011) showed the biotransformation of the terpenoid citral by *A. niger*-PTCC 5011, which yielded citronellol by reducing the C=C bond and the carbonyl group as one of the main products. Tons of citronellol are used worldwide in the manufacture of flavorings, extracts, foods and medicines [25]. Oxymetholone, an anabolic steroid used for treating anemia, was converted by *A. niger* ATCC 10549, in two products by reduction of the hydroxymethylidene and the carbonyl group. Both of these metabolites have been found to inhibit T-cell proliferation [28].

The metabolites 2, 4, 6 and 14 are isomers and indicated the dihydroxylation reaction and forming a double bond. Compound 9 exhibited two hydroxyl group and two double bonds more than STP1. The compound 17, in turn, can be a monohydroxylated

product with formation of a double bond. All these reactions have already been reported in several studies [26, 27]. Various *A. niger* strains have displayed the ability to dehydrogenate the C2, C3-bond of flavanones, for example. In the work done by Kostrzewa-Susłow et al. *A. niger* 13/5 transformed the substrate of the racemic mixture of 6-hydroxyflavanone into the dehydrogenation product, 6-hydroxyflavone [29].

The STP1 was isolated and studied chemical- and biological for our research group in a study previous and displayed anticancer effect against B16F10-Nex2 (murine melanoma) as well as a marked inhibition of cell migration *in vitro*. Thus, we reported by the first time the biotransformation of the sesquSTP1 by *A. niger*, which was capable of performing several reactions and yielding 17 news derivatives. Thus, these new metabolites obtained from biotransformation are promising molecules as anticancer candidates. In addition, *A. niger* proved to be an excellent biocatalyst in obtaining derivatives that would hardly be produced in common chemical reactions. Mainly the products obtained from regio- and stereoselective hydroxylation to non-functionalized hydrocarbons of STP1, such as compounds 11 and 13 isolated here.

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**Declarations**

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**Conflict of Interest**

All authors declare that they have no conflict of interest.

**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

**Author's Contributions**

NSC, JCPM, and DBS conceived and designed research. NSC and LBS conducted experiments. NSC and DBS analyzed the chemical data. NSC and DBS wrote the manuscript. All authors read and approved the manuscript.

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## 7 CONCLUSÃO GERAL

Este trabalho mostrou pela primeira vez o estudo fitoquímico e biológico do óleo da *B. gratioloides* e também suas atividades detergente alimentar e deterrente contra

insetos-praga de armazenamento de alimentos, a traça da farinha mediterrânea, *A. kuehniella*, e o gorgulho do feijão-caupi, *C. maculatus*, respectivamente. Dessa forma, os compostos do BGEO podem ser utilizados para desenvolver repelentes de origem natural como alternativa aos produtos sintéticos, reduzindo o impacto ambiental. Deste mesmo óleo foi isolado e identificado um sesquiterpeno inédito, ácido 7-epi Diidroartemisínico, o qual apresentou um excelente efeito anticâncer contra melanoma murino (B16F10-Nex2), além de promover marcada inibição da migração celular *in vitro* e morte por apoptose. Nenhum efeito tóxico foi observado para a linha de células saudáveis (fibroblasto humano MRC5). Posteriormente, este sesquiterpeno foi submetido a biotransformação usando *A. niger* como biocatalisador, a qual proporcionou 17 novos metabólitos por reações de hidroxilação, redução e desidrogenação. Além disso, dois compostos foram isolados e identificados como produtos de reações de hidroxilação régio- e estereosseletiva em hidrocarbonetos não funcionalizados do sesquiterpeno, os quais dificilmente seriam produzidos em reações químicas comuns.

O iridoide especiosídeo também foi submetido à biotransformação utilizando sete diferentes fungos, *A. niger, A. flavus, A. japonicus, A. terreus, A. niveus, P. crustosum e T. aurantiacus,* a qual resultou em um total de 19 diferentes análogos, 11 dos quais são inéditos. O especiosídeo não glicosilado foi o principal metabólito formado. Os outros análogos foram produzidos a partir de reações de hidrólise do éster, hidroxilação, metilação e hidrogenação. Assim, os fungos aplicados neste estudo mostraram a capacidade de realizar diversas reações e principalmente hidrólise glicosídica, a qual não foi possível por hidrólise ácida e nem básica neste trabalho.

Por fim, todos os fármacos alvos deste trabalho (meropenem, clindamicina, fluconazol, prednisolona e metilprednisolona) foram submetidos à biotransformação usando diferentes espécies de fungos e realizadas várias modificações de meio de

crescimento. Apesar disso, nenhum experimento levou a obtenção de nenhum derivado. Ao final dos ensaios os fármacos permaneceram inalterados. Exceto o meropenem que degradou no meio.

Assim, neste trabalho, demostramos a relevância da técnica de biotransformação e a habilidade dos fungos testados em realizar diversos tipos de reações régio- e estereoespecíficas rendendo compostos inéditos. Além disso, relatamos o potencial farmacológico do óleo extraído das partes aéreas da *B. gratioloides* e do novo sesquiterpeno isolado dele.

É importante enfatizar a relevância desses achados, visto que este é o primeiro estudo fitoquímico e biológico de *B. gratioloides* e de biotransformação do iridoide especiosideo e do sesquiterpeno ácido 7-epi Diidroartemisínico.

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