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EFEITO ANTITUMORAL DE 22*β*-HIDROXITINGENONA OBTIDA DE *Salacia impressifolia* (MIERS) A.C. (CELASTRACEAE) CONTRA CÉLULAS DE MELANOMA HUMANO

ELENN SUZANY PEREIRA ARANHA

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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Rede BIONORTE, na Universidade Federal do Amazonas, como requisito parcial para a obtenção do título de Doutora em Biodiversidade e Biotecnologia, área de concentração Biotecnologia.

Orientadora: Profª Dra. Marne Carvalho de Vasconcellos

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ELENN SUZANY PEREIRA ARANHA

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Aos meus pais, Sérgio e Elenilda.

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RESUMO

O melanoma é um tipo de câncer de pele com elevada capacidade metastática e significativa resistência tumoral aos tratamentos disponíveis. A busca por novas opções terapêuticas para o melanoma continua sendo um dos focos de investigações científicas em torno deste câncer, incluindo fontes oriundas de produtos naturais, como, por exemplo, a 22*β*-hidroxitingenona (22-HTG), um triterpeno quinonametídeo obtido da espécie *Salacia impressiofolia*. A presente tese teve como objetivo investigar o efeito da substância 22-HTG em células de melanoma. Inicialmente, foi avaliado o efeito de 22-HTG em propriedades relacionadas a progressão tumoral, como proliferação, migração e invasão celular, além de mecanismos de reprogramação do metabolismo energético, através da expressão gênica e atividade enzimática da lactato desidrogenase (LDHA). Posteriormente, foi investigado o mecanismo de morte celular induzido por 22-HTG por meio da análise das alterações morfológicas, ciclo celular, ensaio de morte celular pela marcação com anexina V-FITC/iodeto de propidio (Anexina/IP), potencial de membrana mitocondrial, produção de espécies reativas de oxigênio (EROs) utilizando citometria de fluxo e ainda coloração com laranja de acridina/ brometo de etídio (AO/BE) por microscopia de fluorescência. A expressão dos genes BRAF, NRAS e KRAS foi avaliada por RT-qPCR e também foram realizadas análises *in silico* por docking molecular. O potencial anti-invasivo de 22-HTG foi avaliado usando um modelo tridimensional (3D) de pele humana reconstruída com células de melanoma. Com os resultados obtidos foi possível identificar o efeito citotóxico de 22-HTG na linhagem de melanoma SKMEL28, com CI_{50} 4,35; 3,72 e 3,29 µM após 24, 48 e 72 horas de exposição. Foi demonstrado que 22-HTG reduziu a viabilidade celular, inibiu da formação de novas colônias, reduziu a expressão gênica de LDHA, e ainda diminuiu a migração, invasão de células de melanoma e a atividade de metaloproteinases (MMP-2 e MMP-9). Quanto a investigação da morte celular, o tratamento com 22-HTG causou alterações morfológicas em células de melanoma, como redução do volume celular, condensação da cromatina e fragmentação nuclear, características de apoptose. A continuação da investigação mostrou aumento de células marcadas com AO/BE e a apoptose foi confirmada com a marcação por Anexina/IP. A exposição a 22-HTG provocou redução do potencial de membrana mitocondrial, o que não estava relacionado ao aumento dos níveis de EROs. Usando o modelo 3D de pele humana reconstruída, 22-HTG reduziu a capacidade das células de melanoma invadirem a derme, relevando um potencial antimetastático. A expressão dos genes BRAF, NRAS e KRAS, alvos moleculares importantes no melanoma, foi reduzida pela presença de 22-HTG e o docking molecular demonstrou forte afinidade de ligação entre a substância testada e as proteínas BRAF e NRAS, o que pode ajudar a explicar os eventos celulares observados. A partir dos dados oriundos da tese, foi possível concluir que 22-HTG possui efeito anti-tumoral em células de melanoma, com a indução de apoptose e redução de eventos como proliferação, migração, invasão, a ação proteolítica de MMPs. Esse estudo fornece novas informações para trabalhos futuros acerca do uso de 22-HTG para o tratamento do melanoma.

Palavras-chave: Melanoma, Citotoxicidade, Invasão, Apoptose, Triterpeno quinonametídeo.

ABSTRACT

Melanoma is a type of skin cancer with high metastatic ability and significant tumor resistance to conventional therapeutic. The search for new therapeutic options to melanoma remains one of the focus of scientific research around this type of cancer, including sources from natural products, such as 22*β*-hydroxytingenone, a quinonemethide triterpenes obtained from *Salacia impressiofolia*. The present thesis aimed to investigate the effect of the substance 22*β*-hydroxytingenone in melanoma cells. Initially, the effect of 22*β*-hydroxytingenone on properties related to tumor progression, such as proliferation, migration, cellular invasion, and mechanisms of reprogramming of energetic metabolism, through the expression and enzymatic activity of lactate dehydrogenase (LDHA), was evaluated. Subsequently, the mechanism of cell death induced by 22-HTG was investigated through the analysis of morphological changes, following cell cycle, annexin V-FITC/propidium iodide assays (Annexin/PI), mitochondrial membrane potential, production of reactive oxygen species (ROS) by flow cytometry, and fluorescence microscopy with acridine orange/ ethidium bromide assay (AO/BE) staining. RT-qPCR was performed to evaluate the expression of BRAF, NRAS, and NRAS genes, and docking molecular was executed. The anti-invasiveness potential of 22-HTG was evaluated in a three-dimensional (3D) model of reconstructed human skin. With the results obtained, it was possible to identify the cytotoxic effect of 22-HTG in SKMEL28 melanoma cell line, with IC_{50} values of 4.35, 3.72, and 3.29 μM after 24, 48, and 72 h of incubation, respectively. It has been demonstrated that 22-HTG reduced cell viability, inhibited new colony formation, reduced LDHA gene expression, decreased migration and invasion of melanoma cells, and activity of metalloproteinases (MMP-2 and MMP-9). Regarding the investigation of cell death, treatment with 22-HTG caused morphological changes, as cell shrinkage, chromatin condensation, and nuclear fragmentation. Further investigation showed an increase in AO/BE-labelled cells and apoptosis was confirmed with Annexin/IP-labeling. Exposure to 22-HTG led to reduced mitochondrial membrane potential, which is not involved by increased oxidative stress. Using the 3D model of reconstructed human skin, 22-HTG reduced the ability of melanoma cells to invade the dermis, highlighting antimetastatic potential. Expression of *BRAF*, *NRAS*, and *KRAS*, important biomarkers in melanoma development, was reduced by 22-HTG treatment and showed a strong binding affinity with BRAF and NRAS proteins in molecular docking, which may help explain the observed cellular events. According to the data from the thesis, it was possible to conclude that 22-HTG has an anti-tumor effect on melanoma cells, with the induction of apoptosis and reduction of events such as proliferation, migration, invasion, proteolytic action of MMPs. This study provides new insights for future work on investigating the utilization of 22-HTG in malignant melanoma treatment.

Keywords: Melanoma, Cytotoxicity, Invasion, Apoptosis, Quinonemethide triterpenes.

LISTA DE FIGURAS

CAPÍTULO II

CAPÍTULO III

LISTA DE TABELAS

INTRODUÇÃO

CAPÍTULO II

CAPÍTULO III

DADOS SUPLEMENTARES

SUMÁRIO

1. INTRODUÇÃO

O melanoma é a forma mais invasiva entre os tipos de câncer de pele, com elevada taxa de mortalidade entre os indivíduos acometidos pela doença, e sua incidência vem aumentando de forma rápida no mundo todo (WANG et al., 2014). Além disso, células presentes nesse tipo de tumor apresentam alta resistência aos tratamentos existentes, o que contribui de forma negativa com a condição dos pacientes com essa doença (LIU et al., 2017b).

A alta capacidade de invasão de células do melanoma pode favorecer as metástases, processo no qual as células tumorais podem disseminar no organismo e atingir outros locais longe do tumor primário, o que é considerado o aspecto mais grave e letal de todos os tipos de câncer (WANG, et al., 2014). Entre diversos fatores que contribuem para o desenvolvimento de metástase, o aumento da expressão de Metaloproteinases (MMPs) está entre as principais alterações moleculares existentes, as quais levam a degradação da matriz extracelular e de componentes da membrana basal, favorecendo processos de migração, invasão e angiogênese, o que em conjunto contribuem para a progressão metastática (PITTAYAPRUEK et al., 2016; WANG; ZHAI; DU, 2017). No melanoma, existe elevado potencial metastático, o que aumenta o risco de morte entre os pacientes, principalmente quando detectado tardiamente (STUEVEN et al., 2017).

O melanoma em estágio avançado permanece um desafio clínico (GUO et al., 2019). Apesar dos recentes avanços nas terapias alvo direcionadas e baseadas no sistema imunológico (imunoterapia), existe elevado potencial de desenvolvimento de resistência a essas terapias e ainda pode ocorrer a ativação exacerbada do sistema imunológico, o que no longo prazo leva a um prognóstico ruim (BERNING et al., 2019; KOZAR et al., 2019). Além disso, pacientes com melanoma que não possuem mutações *BRAF* continuam sem opções de tratamento adequado e eficaz (PONTI et al., 2017). Assim, novas alternativas terapêuticas continuam sendo necessárias para diminuir os obstáculos do tratamento para o melanoma e melhorar a qualidade de vida dos pacientes com essa doença (MENEZES et al., 2018).

Existe considerável interesse científico em novos agentes anticâncer obtidos de fontes naturais, principalmente plantas, por constituírem uma fonte de diversas moléculas, muitas dessas frequentemente identificadas com ação antitumoral, o que é considerado de grande valor em atividades biológicas (SAMPATH et al., 2017). Nesse contexto, triterpenos quinonametídeos constituem uma classe de substância com diferentes atividades biológicas já identificadas, os quais

são considerados promissores agente anticâncer (SALMINEN et al., 2008), entre os quais podemos citar o celastrol e pristiminerina como importantes representantes dessa classe química. A substância 22*β*-hidroxitingenona pertence a classe dos triterpenos quinonametídeos e foi isolada a partir do tronco e galhos da espécie *Salacia impressifolia* (Miers) A. C. Smith., uma espécie amazônica (DA SILVA et al., 2016). Recentes estudos demonstraram o efeito do extrato e frações da casca do caule de *S. impressifolia* contra leucemia, sendo essa atividade atribuída a presença de triterpenos quinonametídeos (RODRIGUES et al., 2019). Essas informações geram expectativas positivas quanto o efeito de 22*β*-hidroxitingenona contra células de melanoma humano.

Através de estudos prévios realizados no Laboratório de Atividade Biológica/ Faculdade de Ciências Farmaceuticas/ Universidade Federal do Amazonas foi identificado o potencial citotóxico de 22*β*-hidroxitingenona usando um pequeno painel de células tumorais (Tabela 1). Outros estudos divulgados na literatura tem demonstrado o efeito citotóxico de 22*β*-hidroxitingenona contra células de câncer de mama, (CEVATEMRE et al., 2016; RODRIGUES et al., 2019) leucemia, carcinoma de coloretal, hepatocelular e células escamosas da boca (RODRIGUES et al., 2019). No entanto, o efeito de 22*β*-hidroxitingenona contra células de melanoma nunca foi estudado anteriormente.

Diante do exposto e estimulados pela possibilidade de apresentar contribuições científicas consistentes que possam inovar ou melhorar as opções terapêuticas para o melanoma, pretendeu-se com este projeto avaliar o potencial biológico da substância 22*β*-hidroxitingenona em relação ao seu efeito contra células de melanoma humano, o que de maneira geral poderá contribuir com informações acerca de novas fontes farmacológicas que possam interferir no desenvolvimento desse tipo de câncer.

Tabela 1- Valores de CI_{50} obtidos pelo ensaio Alamar blue após 72 horas de exposição à 22*β*-hidroxitingenona (22-HTG).

	CI_{50} µM (Intervalo de confiança)[a]	
Linhagem celular[c]	**22-HTG**	**Doxorrubicina[b]**
MRC5	2,61 (2,29-2,95)	0,14 (0,10 - 0,17)
SK-MEL-19	1.98 (1.9 – 2.16)	0.77 (0.62 – 0.95)
SK-MEL-28	3,2 (3,05- 3,37)	0,22 (0,05 - 0,88)
DU 145	6,94 (6,67- 7,38)	0,39 (0,32 – 0,48)
MCF7	2,75 (2,5- 2,98)	0,84 (0,50 - 1,43)
HCT-116	2,25 (2,04- 2,49)	0,52 (0,27 - 0,97)
MES-SA/DX	8,02 (7,6- 8,79)	0,42 (0,20 – 0,87)

[a]Dados são representados como concentração que reduz 50% da viabilidade celular (CI_{50}) e intervalo de confiança de 95%.
[b] Doxorrubicina foi utilizada como controle positivo.
[c] Linhagens tumorais: SK-MEL-19- melanoma; SK-MEL-28- melanoma; DU 145- carcinoma de próstata; MCF7- adenocarcinoma de mama; HCT-116- carcinoma de coloretal; MÊS-AS/DX- sarcoma uterino resistente a doxorrubicina. Linhagem não tumoral: MRC5- fibroblasto.

2. CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

Melanoma: uma abordagem sobre o uso de produtos naturais como potenciais agentes quimioterapêuticos [1]

[1]Este capítulo está formatado na forma de artigo e deverá ser submetido para publicação na revista *"Scientia Amazonia"* (ISSN 2238-1910).

Melanoma: uma abordagem sobre o uso de produtos naturais como potenciais agentes quimioterapêuticos

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Resumo

Apesar das baixas taxas de incidência do melanoma no mundo, existe elevada letalidade entre os pacientes com esse tipo de câncer de pele. Por esse motivo, a procura por novas opções terapêuticas de combate ao câncer melanoma continua sendo um dos focos de investigações científicas em torno desse tipo de câncer, incluindo fontes oriundas de produtos naturais. Compostos de origem natural tem sido amplamente estudados por seus efeitos anti-melanoma, com ação inibitória relevante no crescimento tumoral, indução de apoptose, supressão de angiogênese e metástase. Essa revisão aborda algumas características relacionadas a progressão do melanoma, o desenvolvimento de metástases, o que leva a malignidade da doença, o uso produtos naturais como recurso pra identificação de novas opções terapêuticas, com destaque para o uso de triterpenos quinonametídeos, como uma classe química promissora por sua ação anticâncer e potencial ação no melanoma.

Palavras-chave: Câncer de pele, Invasão, Triterpeno quinonametídeo, Produto natural.

Melanoma: an approach to the use of natural products as potential chemotherapeutic agents

Abstract

Despite the low incidence rates of melanoma in the world, there is high lethality among patients with this type of skin cancer. Thus, the search for new therapeutic options to combat melanoma cancer remains one of the focus of scientific investigations around this type of cancer, including sources derived from natural products. Compounds of natural origin have been extensively studied for their anti-melanoma effects, including tumor growth inhibition, apoptosis induction, angiogenesis and metastasis suppression. This review addresses some characteristics related to melanoma progression, development of metastases, which leads to disease malignancy, the use of natural products as a resource to identify new therapeutic options, with focus on the use of quinonemethide triterpene, as a promising chemical class for its anticancer action and potential action in melanoma.

Keywords: Skin cancer, Invasion, Quinonemethide triterpene, Natural product.

1. Introdução

Melanoma é um tipo de câncer de pele agressivo, responsável por muitas mortes de pacientes em estágios avançados. Entre as principais fatores que contribuem para o desenvolvimento da doença se destacam a predisposição genética e a exposição à radiação ultravioleta, o que no longo prazo contribuem para transformação maligna de melanócitos (Swetter et al. 2019).

As lesões melanocíticas iniciais são curáveis por excisão, no entanto com o avanço da doença para malignidade, o melanoma torna-se invasivo e metastático, o que leva ao prognóstico ruim da doença (Zeng et al. 2018). A invasão é uma característica preocupante no melanoma, e os fatores envolvidos na aquisição ou na manutenção de um fenótipo invasivo são alvos atraentes para a terapia (Bettum et al. 2015). Apesar do progresso significativo para o tratamento do melanoma na última década, com o uso de inibidores moleculares específicos e imunoterapia, existe a probabilidade elevada do desenvolvimento de resistência a esses tratamento. Sendo assim, o entendimento dos mecanismos envolvidos na progressão do melanoma ainda são necessários, buscando identificar biomarcadores, vias e novos alvos terapêuticos (Yang et al. 2019).

Paralelo a necessidade de melhorar o conhecimento sobre a biologia tumoral do melanoma, é necessário investigar e identificar novos compostos que sejam capazes de inibir as etapas de progressão desse tipo de câncer de pele, principalmente a fim de evitar o fenótipo metastático (Li et al. 2019). Produtos naturais compreendem grande parte dos agentes farmacêuticos atuais, principalmente nas áreas de antibióticos e terapias contra o câncer. O uso de agentes sintéticos ou naturais para inibir, retardar ou reverter o processo carcinogênico continua sendo uma abordagem relevante na busca do alívio para esses doenças (Cragg and Pezzuto 2016).

2. Metodologia

Para a elaboração do presente artigo foram consultados os seguintes sítios de busca de bancos de dados: *Pubmed, Scielo, ScienceDirect* e *Web of Science* no período de 2002 a 2020. As palavras chaves utilizadas foram: "*melanoma*" associada com as palavras "*natural product*", "*quinonemethide triterpenes*", "*cancer*", "*invasion*" e "*skin cancer*". Para os dados epidemiológicos foram consultados os sites da Organização Mundial De Saúde e Instituto Nacional Do Câncer.

3. Revisão bibliográfica

3.1. Câncer de pele e melanoma

O câncer de pele pode apresentar tumores de diferentes linhagens, nomeados de acordo com a célula de origem e seu comportamento. Os três tipos mais comuns são os carcinomas basocelulares, os

carcinomas espinocelulares – tipos não melanoma - e os melanomas cutâneos, os quais são também referidos como melanoma maligno da pele ou apenas melanoma (INCA, 2017; NAVES et al., 2017).

Os carcinomas basocelulares possuem crescimento lento, com capacidade de invasão mínima, ao contrário dos carcinomas espinocelulares, com crescimento rápido, podendo evoluir e formar tumores com capacidade de invasão, o que geralmente leva às metástases (Gloster and Neal 2006). Apesar de ser menos frequente que outros tipos de tumores, o melanoma possui letalidade elevada, sendo considerado a forma mais invasiva dos tipos de câncer de pele, o que é relacionado ao alto potencial de ocasionar metástases. Por esse motivo, é considerado um problema de saúde pública em muitos países (Tuong, Cheng, and Armstrong 2012; Rizzi et al. 2017), principalmente em populações com pele branca, com aproximadamente 80% das mortes relacionadas a esse tipo de câncer (Paluncic et al. 2016).

O câncer de pele é o tipo de câncer mais comum no mundo (Skin Cancer Foundation, 2020). Dados mais recentes disponibilizados pela Organização Mundial de Saúde mostram o aumento global na incidência do câncer de pele, com a expectativa de aproximadamente 1 milhão de novos casos de câncer de pele do tipo não-melanoma e 300 mil para o melanoma no ano de 2020 (WHO, 2020). Apesar da baixa incidência, o melanoma é responsável pela grande maioria das mortes por câncer de pele. Em 2020, são estimados aproximadamente 100 mil novos casos de melanoma nos EUA e 6.850 pessoas morrerão da doença (American Cancer Society, 2020). No Brasil, Instituto Nacional do Câncer (INCA) estimou para o biênio 2020-2022 a ocorrência de aproximadamente 625 mil novos casos de câncer, e assim como no mundo todo o câncer de pele continua entre os mais incidentes, com a estimativa de aproximadamente 177 mil novos casos do tipo não melanoma e 8.450 para o melanoma nesse mesmo período, o que no total corresponde a quase 30% de todos os tumores registrados no país (INCA, 2020).

Em homeostase, os melanócitos são células especializadas na produção e na transferência de melanina para outros componentes celulares presentes na camada basal da epiderme, como os queratinócitos. A melanina transferida é acumulada próximo ao núcleo celular e confere proteção aos danos provocados pela radiação ultravioleta (UV) (Tuong, Cheng, and Armstrong 2012). O melanoma é uma doença multifatorial, baseada na interação entre exposição ambiental e susceptibilidade genética. No entanto, a exposição à radiação UV está entre os principais fatores de risco associado ao desenvolvimento do melanoma, principalmente devido ao seu efeito genotóxico, provocando danos ao DNA. Sendo assim, o excesso de exposição à radiação UV pode provocar a transformação dos melanócitos em células tumorais e ao longo do tempo favorecer o surgimento do melanoma (Rastrelli et al. 2014; R. S. Moreira et al. 2020).

Assim como nos diferentes tipos de turmor, a tumorigênese do melanoma é um processo complexo, o qual comumente ocorre na pele, mas pode acometer também a mucosa oral e nasal, intestinos, olhos e trato urogenital (H. Liu et al. 2017). A progressão do câncer de pele ocorre a partir da perda do controle da

proliferação celular, levando a formação de uma lesão hiperplásica, na maioria dos casos essa lesão não progride devido a senescência celular. No entanto, algumas células podem se manter viáveis, ultrapassar essa barreira proliferativa e ao longo do tempo progredir para o melanoma (Miller and Mihm 2006).

De acordo com a localização e estágio de progressão, a formação do melanoma envolve etapas distintas: (1) nevos melanocíticos com hiperplasia melanocítica (sem alterações displásicas); (2) nevos melanocíticos com atipia nuclear melanocítica, displasia melanocítica; (3) fase de crescimento radial; (4) fase de crescimento vertical e (5) melanoma metastático, as quais podem ser distinguíveis devido a produção de melanina (R. S. Moreira et al. 2020). A compreensão de cada etapa desse complexo processo carcinogênico é fundamental para limitar e direcionar alvos terapêuticos (De Luca et al. 2017).

Na maioria dos casos, o processo de desenvolvimento do melanoma ocorre a partir do surgimento de lesões precursoras distintas (na forma de nevos melanocíticos benignos e/ou intermediárias, ou ainda nevos displásicos), as quais podem sofrer uma série de lesões cada vez mais malignas e evoluir para um espalhamento superficial, caracterizando a fase de crescimento radial, ainda restrita a epiderme e com baixo potencial invasivo (melanoma *in situ*). Com o passar do tempo e continuação do estímulo carcinogênico, as células podem adquirir a capacidade de invadir a derme, o que caracteriza a fase de crescimento vertical (também denominado melanoma invasivo), e, por fim, chegar ao melanoma metastático, com diferentes graus de agressividade (Miller and Mihm 2006; R. S. Moreira et al. 2020; Savoia et al. 2019).

O melanoma é um tipo de câncer bastante associado a mutações genéticas, com ativação e desregulação de vias de sinalização celular (Leonardi et al. 2018). As alterações moleculares levam a transformação maligna do melanócito e são fortemente associadas ao elevado potencial metastático do melanoma (Chin 2003; Miller and Mihm 2006). Mutações BRAF- um importante iniciador da progressão da doença- ocorrem na maioria dos casos do melanoma e levam a ativação constitutiva da via das proteínas quinases ativadas por mitógenos (*Mitogen-activated protein kinases*- MAPK), também denominada RAS/RAF/MEK/ERK (Massaro et al. 2017). Essa via de sinalização celular representa uma das mais comuns no melanoma, com a ocorrência em cerca de 90% dos casos (Leonardi et al. 2018).

A via MAPK está envolvida na transdução de sinais extracelulares para o núcleo, regulando funções celulares importantes, como proliferação, sobrevivência, migração e invasão celular (Tran et al. 2016; Hong and Ahn 2017; Lim, Baek, and Jung 2019). É uma via bastante estudada no melanoma devido a mutação oncogênica em BRAF e NRAS (R. S. Moreira et al. 2020). Mutação do gene BRAF podem levar a ativação constitutiva da via MAPK, sendo a mutação BRAF V600E detectada na maioria dos pacientes com melanoma avançado, o que provoca a hiperativação das quinases MEK e ERK, levando ao rápido crescimento tumoral, capacidade metastática aumentada, inibição da apoptose e resistência terapêutica significativa, etapas envolvidas no desenvolvimento do melanoma (Szabo et al. 2016; Strickland et al.

2015). A busca por novas opções terapêuticas geralmente visam evitar ou controlar algumas dessas propriedades do processo carcinogênico, que permitam a ativação de morte celular e controle da progressão tumoral (Lim, Baek, and Jung 2019).

Apesar dos consideráveis avanços na terapêutica do câncer, a partir da compreensão dos fundamentos genéticos do melanoma e dos progressos obtidos em relação ao tratamento da doença, a heterogeneidade das células presentes no microambiente tumoral acaba levando desenvolvimento de resistência, e no caso da imunoterapia são relatados problemas como superativação do sistema imunológico. Por esse motivo, ainda torna-se necessário a busca por intervenções eficientes e que melhorem a qualidade de vida dos pacientes, principalmente evitando a progressão para o fenótipo metastático (Schadendorf et al. 2018).

3.2. Invasão e metástase

O desenvolvimento de metástases é uma característica preocupante nos diferentes tipos de câncer. Esse processo inclui um série de etapas inter-relacionadas, nas quais as células de um tumor primário chegam ao sistema circulatório e alcançam novos sítios em lugares distintos (WANG, et al., 2014). O sucesso da formação de tumores secundários, a partir da disseminação celular, depende da capacidade de adesão, degradação dos componentes da matriz extracelular, bem como da membrana basal, e a partir disso a invasão de outros tecidos próximos, o que pode levar a formação de metástases (De Luca et al. 2017).

Pacientes com melanoma em estágios avançados possuem sobrevida reduzida, o que pode depender de variáveis como, por exemplo, local de metástases, sexo e idade (Korn et al. 2008). Quando em fase inicial, pode ocorrer a remoção cirúrgica da lesão do melanoma, o que pode resultar em até 90% de chance de cura. No entanto, após a excisão, mesmo com margens de segurança suficientes, ainda pode haver recorrência da doença, o que pode ocorrer através do crescimento de um novo tumor primário ou o surgimento de metástase, cada um com um prognóstico distinto (Posch et al. 2016).

Durante o processo metastático, o controle da integridade da matriz extracelular é fundamental para a manutenção da estrutura do tecido, no entanto, em um ambiente tumoral a desregulação da atividade proteolítica de MMPs pode favorecer a metástases de células do tumor (Ghosh et al. 2014). O desenvolvimento de metástases ocorre através de diferentes processos, que juntos alteram mecanismos celulares e moleculares. Nesse contexto, as MMPs são consideradas umas das principais enzimas envolvidas no desenvolvimento de metástases de células tumorais, pois acabam comprometendo a organização do tecido, com a degradação de componentes da matriz extracelular, o que pode interferir na capacidade adesiva entre as células e favorecer a migração e invasão celular (Zhenhua Wang et al. 2014; Chang et al. 2014).

Entre as MMPs, diferentes estudos correlacionam o aumento da expressão de MMP-2 e MMP-9 ao favorecimento da invasão e metástases de células tumorais (Chang et al. 2014; Velinov et al. 2010; Yan Liu et al. 2016; Kumar et al. 2010). Durante o processo de invasão de células tumorais, a enzima MMP-2 participa da degradação de colágeno tipo IV, encontrado na membrana basal dos tecidos, além de potencializar a invasão, angiogênese e metástase. A enzima MMP-9, além de degradar colágeno IV, também participa na clivagem dos componentes da matriz extracelular, estimula a angiogênese tumoral através da manutenção de fatores de crescimento endotelial (VEGF) (Yelken et al. 2017; Lee et al. 2007).

A alta expressão de MMPs é encontrada durante a progressão do câncer devido a mudanças moleculares na transcrição gênica, o que pode ser encontrado em pacientes com diferentes tipos de câncer, levando a um prognóstico ruim. Alguns autores sugerem que o aumento na expressão de MMPs pode ser utilizado como um marcador para o diagnóstico de alterações tumorais malignas (Yelken et al. 2017). Assim, a expressão e ativação de MMPs pode ser um alvo importante para estudos que buscam inibir a expressão e ativação dessas enzimas, e consequentemente evitar as metástases (Gong, Chippada-Venkata, and Oh 2014).

Em melanomas, o aumento da expressão de diferentes MMPs é associado a progressão da doença, sendo as MMP-2 e MMP-9 identificadas como as principais MMPs envolvidas em diferentes estágios do processo invasivo (Hofmann et al. 2000; Belter, Haase-Kohn, and Pietzsch 2017) e associadas à degradação de diferentes tipos de colágeno e fibronectina. No melanoma, o aumento da atividade de MMP2 é considerado como um fator prognóstico da forma invasiva (Candrea et al. 2014).

O melanoma é um excelente modelo para investigar as bases mecanicistas de metástase. A compreensão dos mecanismos envolvidos na disseminação metastática pode revelar alvos importantes na migração e invasão de células cancerígenas, evitando metástases. O melanoma reflete uma natureza altamente agressiva, e os mecanismos celulares e moleculares responsáveis por esse fenótipo ainda não são totalmente compreendidos (Hanniford et al. 2020). Por esse motivo, é necessário continuar estudando a biologia tumoral do melanoma, o que poderá ajudar no desenvolvimento de melhores opções terapêuticas para os diferentes tipos de pacientes, principalmente para aqueles em estágios iniciais, evitando o risco de metástases, o que é considerada a principal causa da elevada mortalidade dos pacientes com câncer (Guo et al. 2015; Ji et al. 2015).

3.3. Produtos naturais e terapia anticâncer

Os avanços na compreensão da biologia do melanoma levaram ao desenvolvimento de novas opções terapêuticas. O uso de terapias alvo especifico, como vemurfenibe e dabrafenibe- inibidores da via de sinalização MAPK e usados no tratamento de pacientes com a mutação BRAF V600E- foi sem dúvida

um progresso para o tratamento do melanoma, aumentando a sobrevida dos pacientes em estágios avançados da doença (Han and Parker 2017; Jang and Atkins 2013). Além das abordagens comumente utilizadas, o desenvolvimento da terapia de controle imunológico, a imunoterapia, com o uso de anticorpos monoclonais que melhoram a resposta do sistema imune antitumoral, tem sido um dos principais avanços na terapêutica do melanoma (Peterson et al. 2018).

No melanoma, o tratamento usando a terapia alvo específica e imunoterapia, pode levar ao desenvolvimento de resistência tumoral ou ainda pode causar o estímulo excessivo do sistema imunológico (Spain, Julve, and Larkin 2016; Berning et al. 2019). Além disso, o tratamento existente com a terapia direcionada é apenas para pacientes com a mutação BRAF V600E, não existindo ainda alternativas para os pacientes que possuem outros tipos de mutações (Hong and Ahn 2017). Sendo assim, mesmo com os recentes avanços na terapia anticâncer, ainda existe interesse em novos fármacos que possam ajudar no tratamento do melanoma (Yu-xi Liu et al. 2019).

Entre as pesquisas científicas existe grande interesse em recursos de origem natural, um vez que esses podem ser fonte promissora de princípios ativos para o tratamento de doenças, os quais podem inclusive ser utilizados em combinação na terapêutica já existente para potencializar o efeito farmacológico e também reduzir a toxicidade (Oprean et al. 2018). Entre as pesquisas que buscam estratégias terapêuticas, é crescente o interesse científico em agentes ativos anticâncer de fontes naturais, principalmente plantas (Q. Zhu et al. 2019), uma vez que essas moléculas ativas podem afetar diferentes etapas do processo carcinogênico, como o controle da proliferação e diferenciação celular, reverter disfunções epigenéticas relacionadas ao câncer, prevenir metástases, reduzir a tumorigênese e aumentar a eficácia do rádio e quimioterapia (Barroso et al. 2019).

Devido a vasta diversidade química e potencial ação anticâncer, muitos pesquisadores estão focando suas investigações sobre o atividade biológica de produtos naturais, mesmo que estes compostos não sirvam diretamente como novas drogas, mas possam fornecer informações para desenvolvimento de um importante agente contra o câncer (Khazir et al. 2014; Shah et al. 2013), o qual pode ser de interesse clínico e chamar a atenção da indústria farmacêutica (Seca and Pinto 2018). Dados do Departamento de Saúde e Serviços Humanos dos Estados Unidos (*Food and Drug Administration*) mostram que 40% das moléculas aprovadas para uso terapêutico são oriundas de produtos naturais ou foram desenvolvidas a partir da estrutura química desses compostos, e desse total 74% são usados no tratamento do câncer (Seca and Pinto 2018). Medicamentos antineoplásicos como paclitaxel, vimblastina ou vincristina, irinotecano, camptotecina, são exemplos importantes de medicamentos obtidos de produtos naturais derivados

de plantas e que são utilizados clinicamente no tratamento de diferentes tipos de câncer (Newman and Cragg 2020).

Recentemente, Fontana et al. (2019) fizeram uma abordagem sobre os mecanismos e a aplicação de muitos produtos naturais e destacaram o papel desses compostos na prevenção e tratamento do melanoma. Outros autores publicaram uma revisão na qual abordam evidências da atividade de produtos naturais no melanoma e demonstram propriedades interessantes e promissoras de compostos obtidos de plantas para futuros estudos clínicos (AlQathama and Prieto 2015). Essas evidências destacam que compostos naturais exercem um papel fundamental na descoberta e no desenvolvimento de novos agentes anticâncer e esses podem ser promissores para investigação anti-melanoma (de Sousa et al. 2019).

3.4. Triterpenos quinonametídeos

Os terpenóides estão entre as maiores classes de produtos naturais que constituem uma fonte de compostos com atividades biológicas promissoras, podendo interferir em vários estágios da formação de tumores, com a inibição da proliferação e morte celular, além de inibir processos como angiogênese e metástases (Gali-Muhtasib et al. 2015). Entre todos os terpenóides, os triterpenos quinonametídeos são considerados importantes substâncias de origem natural com atividades biológicas comprovadas, incluindo efeito anticâncer (Shanmugam et al. 2012; Inácio et al. 2017).

Triterpenos quinonametídeos são metabólitos secundários de plantas, restritos à família Celastraceae e, portanto, considerados marcadores químicos dessa família (Taddeo et al. 2019). Entre os grupos botânicos importantes para obtenção de quinonametídeos, pode-se citar as espécies do gênero *Maytenus* como uma fonte importante desses compostos (Taddeo et al. 2019), mas também ocorrem nos gêneros *Peritassa* (Rodrigues-Filho et al. 2002), *Cheiloclinium* (Haroldo Jeller et al. 2004), *Glyptopetalum* (Sotanaphun et al. 1998) e *Salacia* (Rodrigues et al. 2019).

O gênero *Salacia* possui importância química e biológica, pois é considerado uma fonte de compostos ativos com ação em diferentes processos patológicos humanos. Muitos dos efeitos biológicos já identificados para espécies de *Salacia* têm sido relacionados a presença de triterpenos quinonametídeo (Shanmugam et al. 2012; Coppede et al. 2014), dentre os quais se destacam a atividade antidiabética (Stohs and Ray 2015), antioxidante (Savariraj Sahayam, Brindha, and Logamanian 2014) e relacionadas a obesidade e distúrbios metabólicos (Shimada et al. 2014), mas

também já foi identificada a atividade antiviral (Ferreira et al. 2018) e citotóxica contra células tumorais (Musini, Rao, and Giri 2015).

Espécies do gênero *Salacia* são uma fonte de compostos quinonametideos, como, por exemplo, a identificação de 11 *β*- hidroxipristimerina obtido da *Salacia crassifolia* (Espindola et al. 2018). Um estudo realizado por da Silva et al. (2016) investigou os constituintes químicos presentes na espécie *Salacia impressifolia* (Miers) A. C. Sm. coletada na região Amazônica, onde é popularmente conhecida como “miraruíra” ou "cipó-miraruíra (Lorenzi e Matos, 2002). Os autores descreveram a presença de diferentes triterpenos, com destaque para triterpenos quinonametídeos, relacionando esse espécie como uma fonte promissora de compostos biologicamente ativos (da Silva et al. 2016). Em um recente estudo realizado por Rodrigues et al. (2019) foi demonstrado o efeito do extrato e frações obtidos da casca do caule de *S. impressifolia* contra células de leucemia in vivo. Nesse estudo, o potencial redutor da massa tumoral foi atribuído à presença de triterpenos quinonametídeos.

Moléculas com estrutura química quinonametídeo apresentam efeitos biológicos bem estabelecidos, sendo a atividade antitumoral uma das principais atividades atribuídas a esses metabólitos, com supressão da iniciação, promoção e metástases em vários tipos de câncer (Kim et al. 2011; Zhongye Wang, Zhai, and Du 2017; Yadav et al. 2010). Pristimerina e celastrol são os principais representantes da classe quinonametídeo (Hernandes et al. 2020), existindo diversos estudos demonstrando os seus efeitos biológicos in vitro e in vivo, com destaque para atividade contra o câncer (H. Moreira et al. 2019; Chen et al. 2020; B. Zhu and Wei 2020; Zhao et al. 2019).

Informações relevantes vem sendo geradas a respeito do composto 22*β*-hidroxitingenona, um triterpeno quinonametídeo ainda pouco estudado, mas com a ação contra diferentes células tumorais descrita por alguns autores (Cevatemre et al. 2016; Rodrigues et al. 2019). Recentemente, um trabalho demonstrou o potencial efeito 22*β*-hidroxitingenona contra a proliferação e invasão células de melanoma humano (Aranha et al. 2020). Os autores identificaram redução da invasão celular através de modelo in vitro, incluindo efeito inibitório da ação de MMPs. Essas informações geram expectativas positivas acerca do potencial anticâncer da substância 22*β*-hidroxitingenona em modelos de melanoma, para o qual possa ser identificado efeitos sobre os mecanismos moleculares que regulam o processo carcinogênico, evitando a progressão tumoral e os aspectos invasivos do melanoma.

4. Considerações finais

Nesta revisão foram abordados temas referentes a progressão do melanoma, o processo invasivo que leva ao fenótipo metastático, o qual é responsável pelo prognóstico ruim da doença. A presente revisão também contempla o uso de produtos naturais, mencionando a importância de substâncias com estrutura quinonametídeo contra células tumorais, sendo, portanto, consideradas promissoras para investigação anticâncer. Com a abordagem realizada, espera-se contribuir para o conhecimento científico e ajudar na disseminação de informações sobre os temas investigados.

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Divulgação

"Este artigo de revisão é inédito. Os autores e revisores não relataram qualquer conflito de interesse durante a sua avaliação. Logo, a revista *Scientia Amazonia* detém os direitos autorais, tem a aprovação e a permissão dos autores para divulgação, desta revisão, por meio eletrônico".

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3. OBJETIVOS

3.1. Objetivo geral

Avaliar o efeito antitumoral do triterpeno quinonametídeo (22*β*-hidroxitingenona) contra células de melanoma humano.

3.2. Objetivos específicos

- Avaliar o efeito citotóxico de 22*β*-hidroxitingenona em células de melanoma humano e o seu efeito em parâmetros relacionados a progressão tumoral.
- Investigar a indução de morte celular provocada pela exposição a 22*β*-hidroxitingenona.
- Avaliar o efeito de 22*β*-hidroxitingenona na invasão de células de melanoma em um modelo de cultura de células 3D (pele artificial).

4. APRESENTAÇÃO DOS CAPÍTULOS

O desenvolvimento da presente tese está apresentada em 2 capítulos, onde são mencionados os materiais, as metodologias, os resultados e discussão, de acordo com a apresentação abaixo:

4.1. Capítulo II: *"22β-hydroxytingenone reduces proliferation and invasion of human melanoma cells"*. Esse capítulo está formatado na forma de artigo, o qual foi submetido à revista *Toxicology in vitro* (ISSN 0887-2333) em setembro de 2019 e aceito para publicação em maio de 2020.

4.2. Capítulo III: *"22β-hydroxytingenone induces apoptosis and suppresses invasiveness of melanoma cells by inhibiting MMP-9 activity and MAPK signaling".* Esse capítulo está formatado na forma de artigo e submetido na revista *"Journal of etnhopharmacology"*.

5. CAPÍTULO II

22*β*-hidroxitingenona reduz a proliferação e invasão de células de melanoma humano[2]

Elenn Suzany Pereira Aranha, Emerson Lucena da Silva, Felipe Pantoja Mesquita, Leilane Bentes de Sousa, Felipe Moura Araújo da Silva, Waldireny C. Rocha, Emerson Silva Lima, Hector Henrique Ferreira Koolen, Maria Elisabete Amaral de Moraes, Raquel Carvalho Montenegro, Marne Carvalho de Vasconcellos

Este capítulo apresenta o potencial citotóxico da substância 22*β*-hidroxitingenona (22-HTG) contra células de melanoma humano, evidenciando seu potencial anticâncer em diferentes propriedades das células tumorais, as quais são fundamentais para a progressão do tumor, como a proliferação descontrolada, migração e invasão celular, atividade de metaloproteinases, metabolismo energético e expressão de genes considerados marcadores moleculares no melanoma. Todos os experimentos foram realizados a partir de concentrações sub-tóxicas de 22-HTG, garantindo a interferência na proliferação celular, mas sem causar morte celular expressiva. A exposição a 22-HTG causou redução de todos os parâmetros relacionados a progressão tumoral analisados nesse estudo, e portanto, pode ser considerada promissora para continuidade da investigação contra o melanoma.

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22β-hydroxytingenone reduces proliferation and invasion of human melanoma cells

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ABSTRACT

Melanoma is a skin cancer with high invasive potential and high lethality. Considering that quinonemethide triterpenes are described as promising anticancer agents, the aim of this study was to evaluate the effect of 22β-hydroxytingenone (22-HTG) against human melanoma cells. Alamar blue assay was performed in order to evaluate its cytotoxic effect. Thus, subtoxic concentrations (1.0, 2.0, and 2.5 μM) were used to evaluate the effect of this compound on proliferation, migration, metabolism, and invasion. IC_{50} value against SK-MEL-28 cell line was 4.35, 3.72, and 3.29 μM after 24, 48, and 72 h of incubation, respectively. 22-HTG reduced proliferation, migration and invasion by melanoma cells, with decreased activity of metalloproteinases (MMP-2 and MMP-9). Futhermore, 22-HTG decreased expression of lactate dehydrogenase (LDHA), an enzyme associated with cell metabolism. However, the small reduction in LDHA enzyme activity must have occurred by the cytotoxic effect of 22-HTG. According to the results, 22-HTG interferes with important characteristics of cancer, with anti-proliferative, and anti-invasive effect against melanoma cells. The data suggest that 22-HTG is an effective substance against melanoma cells and it should be considered as a potential anticancer agent.

1. Introduction

Melanoma is originated from the malignant transformation of melanocytes, which, despite being less frequent than other types of tumors, has a high lethality and is considered the most invasive form of skin cancer, with high metastatic potential, becoming a public health problem in many countries (Paluncic et al., 2016; Rizzi et al., 2017; Villanueva et al., 2010). Despite the advances in targeted therapies with specific inhibitors and immunotherapies, patients' survivability is still reduced (Liu et al., 2019). Thus, the combination of these factors makes the study of this tumor challenging (Brys et al., 2016).

There is a considerable scientific interest in the pursuit for new anticancer agents from natural sources, mainly plants, since they are a source of several molecules (Mi et al., 2014). It is well described in the literature many biological effects of molecules obtained from plants, including antitumor properties (Dutra et al., 2016). In the past few years, several small-molecules obtained from natural products have been identified and characterized as potential antitumor agents or served as template for modelling, such as camptothecin and podophyllotoxin/etoposide, contributing for the improvement of treatment strategies of different types of cancer (Cragg and Pezzuto, 2016).

Among chemical classes that stand out for having substances with biological activity are terpenoids, phenolic compounds, flavonoids, and alkaloids, which are mentioned as causing changes in cell metabolism (Sampath et al., 2017). Terpenes are a class of structurally diverse chemical substances with important biological functions, such as paclitaxel, a diterpene isolated from *Taxus brevifolia* and commercially known as Taxol®, a drug used for cancer treatment (Yoshida et al., 2008).

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In the Amazon region, plants are commonly used for the treatment of different diseases (da Rocha et al., 2019), and several compounds extracted from Brazilian plants are described to be cytotoxic against cancer cells (Galúcio et al., 2019). 22β-hydroxytingenone (22-HTG) is a quinonemethide triterpene isolated from *Salacia impressifolia* (Miers) AC Smith (da Silva et al., 2016), a plant found in the Amazon region presenting few studies on its ethnopharmacological potential.

Quinonemethide triterpenes are considered promising molecules for anticancer research (Coppede et al., 2014; Shanmugam et al., 2012). Several studies have described the effect of these compounds on tumor cells, including the suppression of breast cancer cell invasion and migration (Kim et al., 2011), the inhibition of chondrosarcoma cell proliferation, migration and invasion (Wu et al., 2017), and suppression of colon and pancreatic cancer cell invasion and metastasis (Yadav et al., 2010). However, there are few studies on the biological activities of 22-HTG. Thus, the present study aimed to evaluate the potential of 22-HTG against human melanoma cells, seeking to identify its effect on cell proliferation and on parameters associated with tumor progression.

2. Materials and methods

2.1. Plant material

Trunk and twigs of *S. impressifolia* were collected at Adolpho Ducke Reserve (located at Km 26 of the AM-010 highway, in the municipality of Manaus, Amazonas state, Brazil). A voucher specimen (#4699) was deposited in the herbarium of the National Institute for Amazonian Research (INPA), Brazil.

2.2. Extraction and compound isolation

The dried and powdered materials (trunk and twigs) of *S. impressifolia* (300 g) were macerated over 3 days (ca. 27 °C), three times with ethanol (0.5 L, each time). The obtained extract was evaporated at reduced pressure to afford 1.76 g of crude extract. This resulting ethanol extract was dissolved in ethanol:water (1:3, v:v, 1 L), and partitioned with dichloromethane in triplicate (0.5 L, each time). A part of the resulting dichloromethane extract (496 mg) was subjected to silica gel column chromatography (CC) eluted with gradient systems of cyclohexane-dichloromethane, dichloromethane-ethyl acetate, and ethyl acetate-methanol affording 17 fractions. Fraction 7 (Fr.7, 78.3 mg) eluted with cyclohexane-dichloromethane 2:8 (v/v) displayed high cytotoxicity and, therefore, was chosen for the purification step. An aliquot (75 mg) of Fr.7 was subjected to preparative HPLC using a C18 column (30 mm × 250 mm, 5 µm) with a flowrate of 7.5 mL/min (80% MeOH at isocratic elution mode), and with UV detection at 215 and 420 nm, respectively. Three injections (25 mg, each) were carried onto the column in 500 µL of dimethyl sulfoxide (DMSO) to give 9.1 mg of pure 22-HTG (Fig. 1). The authenticity of this compound was checked by nuclear magnetic resonance spectroscopy in comparison with a previously identified standard fully characterized and described in previous work (da Silva et al., 2016).

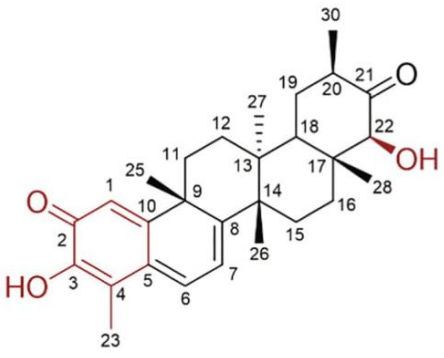

Fig. 1. Chemical structure of 22β-hydroxytingenone.

For all the tests, 22-HTG was dissolved in DMSO and the final concentrations never exceeded 0.2% (v/v). This concentration of DMSO has no influence on cell proliferation (Boncler et al., 2014).

2.2.1. 22-HTG spectral data

Red amorphous powder; UV (MeOH) lmax (log ε) 208 (1.40), 220 (0.60), 241 (0.56), 420 (0.50); HRESIMS obsd m/z 437.2668 $[M + H]^+$, calcd for $C_{28}H_{37}O_4$, 437.2691; H-NMR ($CDCl_3$): δ = 7.04 (1H, dd, J = 8.0 and 1.6 Hz, H-6), 6.54 (1H, s, H-1), 6.39 (1H, d, J = 8.0 Hz, H-7), 4.54 (1H, d, J = 3.6 Hz, H-22), 3.70 (1H, d, J = 6.0 Hz, 22-OH), 2.67 (1H, t, J = 6.7 Hz, H-20), 2.22 (3H, s, H-23), 2.04 (1H, m, H-18), 1.52 (3H, s, H-25), 1.37 (3H, s, H-26), 1.07 (3H, d, J = 6.3 Hz, H-30), 0.98 (3H, s, H-27) and 0.87 (3H, s, H-28); ^{13}C NMR ($CDC1_3$): δ = 119.7 (C-1), 178.2 (C-2), 168.3 (C-3), 164.5 (C-4), 127.5 (C5), 133.6 (C-6), 118.0 (C-7), 117.1 (C-8), 42.4 (C-9), 145.9 (C10), 33.8 (C-11), 29.7 (C-12), 40.4 (C-13), 44.1 (C-14), 28.1 (C-15), 29.7 (C-16), 44.7 (C-17), 44.8 (C-18), 31.9 (C-19), 40.7 (C-20), 213.4 (C-21), 76.3 (C-22), 10.1 (C-23), 39.0 (C-25), 21.4 (C-26), 24.8 (C-27), 20.3 (C-28), 14.3 (C-30).

2.3. Cell lines and culture conditions

Human melanoma cell (SK-MEL-28) and human fibrosarcoma (HT1080) were kindly provided by Sylvia Stuch-Engler (Department of Pharmaceutical Sciences, School of Pharmaceutical Sciences, University of Sao Paulo). Cells were grown in 75 cm^2 culture flasks, containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco™ #12100046), supplemented with 10% bovine fetal serum (FBS, Gibco™ #12657029) and 1.0% penicillin-streptomycin (Pen Strep, Gibco™ #15070063), and maintained at 37 °C in a humidified atmosphere of 5%. Cells were passaged after reaching 90% confluence, detached with 0.05% Trypsin-EDTA (Gibco™ #25300062) and continuously cultured from the passage no. 20 until passage no. 50, for all experiments.

2.4. Cell viability assay

The 50% inhibitory concentration (IC_{50}) of SK-MEL-28 cell line was determined by Alamar blue assay (resazurin 7-Hydroxy- 3H-phenoxazin-3-one 10-oxide) (Ansar Ahmed et al., 1994), wich is a fluorometric method for the detection of cell viability based on the reduction of resazurin (oxidized form) to resofurin (reduced form) (Zachari et al., 2014). For the assay, a total of 0.5 × 10^4 cells was plated in 96-well plate and allowed to attach for 24 h. 22-HTG (46–0.71 µM) was then added to each well with a volume of 100 µL/well and after 24, 48, and 72 h of 22-HTG exposure, 10 µL of 0.02% Alamar blue solution in DMEM were added to each well. Fluorescence was measured by using 465 nm excitation and 540 nm emission in microplate reader (DTX 800 Beckman Coulter Multimode Detector).

Cell viability was assessed by trypan blue exclusion assay (Strober, 2001). In 24-well plate, a total of 0.5 × 10^5 cells was plated for an attachment period of 24 h. This step was followed by the addition of 1.0, 2.0, and 2.5 µM of 22-HTG with a volume of 1.0 mL/well. After incubating for 24 and 48 h, SK-MEL-28 cells were harvested to count live versus dead cells on a hemocytometer chamber using 0.4% trypan blue solution.

2.5. Clonogenic survival assay

To evaluate the ability of a single cell to grow into a colony, a clonogenic assay was performed (Plumb, 1999). SK-MEL-28 cells were seeded at a density of 0.3 × 10^5 in 6-well plates and allowed to attach for 24 h. After the treatment with 22-HTG (1.0, 2.0, and 2.5 µM), the colony growth was observed for 10 days, and during this time the cell culture plate remained under ideal growing conditions. Colonies were

fixed with methanol (Sigma-Aldrich, MO), stained with crystal violet (0.2% *w/v*), and washed with distilled water. The number of colonies (> 50 cells) was counted and photographed for subsequent analysis.

2.6. Lactate dehydrogenase (LDHA) activity

LDHA is involved in anaerobic glycolysis, known as Warburg effect, where cancer cells use glycolysis rather than oxidative phosphorylation even in the presence of oxygen (Bettum et al., 2015; Gao et al., 2016). To estimate intracellular LDHA activity, SK-MEL-28 cells were seeded at a concentration of 4×10^4 cells/well in 24-well-plate and, after 24 h of cell attachment, 22-HTG (1.0, 2.0, and 2.5 μM) was added. After 24 h of treatment, cells were trypsinized, collected, and centrifuged (400 x*g* for 5 min at 4 °C), then cellular pellet was resuspended in Triton X-100 (0.5%), and incubated at 37 °C, for 30 min, for membrane fragmentation. Next, LDHA activity was measured by commercial biochemical test (Liquiform®, Brazil) following the manufacturer's manual. To estimate enzyme activity (U/L), absorbance was measured using Beckman Coulter Microplate Reader DTX 880 (340 nm) (Landi-Librandi et al., 2012).

2.7. Wound-healing assay

In order to evaluate the ability of 22-HTG to inhibit cell migration, the scratch wound-healing assay was performed as previously described (Liang et al., 2007). Briefly, SK-MEL-28 cells were seeded at a density of 0.5×10^6 in 6-well plates, allowed to attach for 24 h, to create a confluent monolayer. Using a 10 μL tip, a scratch was performed on the confluent cell monolayer, and then 22-HTG (1.0, 2.0, and 2.5 μM) was added. Gap distances were monitored and photographed, using microscopy (Carl Zeiss Microscopy GmbH/ Axiocam ERc 5 s), over 0, 3, 6, 12, and 24 h for subsequent evaluation using the software Zen software Zeiss.

2.8. Determination of MMP activity by gelatin zymography

The HT1080 cell line was used since it produces MMP-2 and MMP-9 enzymes with an important role in metastatic cancer (Takahashi et al., 2013). Cells (20×10^4 cells/well) were seeded on a 24-well culture plate and allowed to attach cell for 24 h. Afterwards, 22-HTG (1.0, 2.0, and 2.5 μM) was added, using a serum-free culture medium, and further incubated for 12 and 24 h. After incubation, the medium was collected and centrifugated at $425 \times g$ for 10 min at 4 °C to remove cell debris. Total protein content was normalized by Bradford's method and a total of 30 μg of protein containing the conditioned media was applied on 10% polyacrylamide gels containing gelatin (1%). After electrophoresis, polyacrylamide gels were washed with 2.5% Triton X-100 at room temperature to remove sodium dodecyl sulfate. Gels were then incubated at 37 °C, overnight, in a buffer containing 5.0 mM $CaCl_2$, 50 mM Tris–HCl pH 8.5 and 5.0 μM $ZnCl_2$ to digest gelatin by MMP. The gels were stained with 1% Brilliant Blue R in 45% methanol and 10% glacial acetic acid. After 30 min, the gels were destained in the same solution without the Coomassie blue dye. Proteolytic activity was detected as clear zones against the background stain of undigested substrate and then intensities of the bands were estimated using ImageJ Software®.

2.9. Matrigel invasion assay

In vitro invasion assay was performed according to McEwan et al. (1987), using modified Boyden chamber with filter inserts (8-μm pores) for 6-well plates (BD Biosciences, San Jose, CA, USA). The upper surfaces of the membranes were coated with Matrigel® and placed into 6-well tissue culture plates. Initially, SK-MEL-28 cells were treated with 22-HTG (2.0 μM) for 24 h. After treatment, cells (3×10^5/mL) were added to each insert with serum-free culture medium and the lower chamber was filled with 2 mL of medium containing 10% BFS. Cells were then allowed to invade towards the bottom side of the membrane at 37 °C and 5% CO_2, for 24 h. The cells that passed through the membrane were fixed in methanol, stained with crystal violet (0.2%) and non-invading cells were removed using a cotton swab. Number of invasive cells were then counted in ten random fields of the insert using ImageJ Software®.

2.10. mRNA isolation and expression analysis by qRT-PCR

SK-MEL-28 cells were plated at 4×10^4 cells/well in 12-well plates and allowed to attach for 24 h. Afterwards, the medium was replaced with 22-HTG (1.0, 2.0, and 2.5 μM) and incubated for further 24 h. Then, cells were trypsinized, collected, and used for total RNA extraction using TRIzol® reagent (Life Technologies, USA). RNA integrity was verified by electrophoresis and concentration and quality of the RNA were determine using NanoDrop (Thermo Scientific). RNA concentration was normalized to 20 ng/μL and the reverse-transcription was performed using High-Capacity cDNA kit, according to the manufacturer's protocol (Life Technologies, USA).

Quantitative real-time PCR (qRT-PCR) was performed by Fast SyberGreen kit (Applied Biosystems, USA). Expression levels of LDHA and Vimentin (VIM) were determined in triplicate using stable expressed Actin Beta (ACTB) gene as the endogenous control. Primer efficiency was determined for all genes. PCR primers sequence of each gene was design using OligoPerfect Designer (Invitrogen) and are described in Table 1.

Table 1
Sequence of oligonucleotides used for qRT-PCR.

Gene	Sequence (5′- 3′)	NCBI reference sequence
LDHA	F- TCTGGATTCAGCCCGATT R- TACAGGCACACTGGAATCTC	NM_005566.3
VIM	F- CCAGCTAACCAACGACAAAG R- CCTCTCTCTGAAGCATCTCC	NM_003380.4
ACTB	F- CTGGAACGGTGAAGGTGACA R- AAGGGACTTCCTGTAACAACGCA	NM_001101.5

All requirements proposed in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments - MIQE Guidelines were followed (Bustin et al., 2009). The expression level was calculated using $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), considering negative control samples as a calibrator of the experiments.

2.11. Statistical analysis

All data were presented as the mean ± standard deviation (SD) of three independent experiments performed in triplicate. Cell viability was expressed as IC_{50} and was obtained using nonlinear regressions based on three replicates per concentration. Statistic differences ($p < .05$) were evaluated by comparing with the negative control

(DMSO 0.2%) by analysis of variance (ANOVA) followed by Tukey's or Bonferroni's post-test using GraphPad Prism 6.0 for Windows (Institute Software for Science, San Diego, CA).

3. Results

3.1. Cytotoxic and cytostatic effect of 22-HTG

The cytotoxic effect of 22-HTG against SK-MEL-28 cell line was assessed by Alamar blue assay and trypan blue exclusion test. 22-HTG reduces cell viability of melanoma cells at different concentrations (Fig. 2-A), with IC_{50} of 4.35, 3.72, and 3.29 μM after 24, 48 and 72 h of incubation, respectively (Table 2). Concentrations below the IC_{50} (1.0, 2.0, and 2.5 μM) were chosen and used in subsequent experiments to evaluate changes in tumor progression associated parameters.

Table 2
IC_{50} values in SK-MEL-28 cells after exposure to 22-HTG using the Alamar blue assay. Doxorubicin was used as positive control. Data are presented as half maximal inhibitory concentration (IC_{50}) value and 95% confidence intervals (CI 95%) of three independent experiments performed in triplicate.

Time	IC_{50} μM (CI 95%)		
	24 h	48 h	72 h
22-HTG	4.35 (2.17–8.68)	3.72 (3.0–4.59)	3.29 (2.96–3.65)
Doxorubicin	2.13 (1.7–2.67)	0.27 (0.17–0.42)	0.22 (0.05–0.88)

As demonstrated in Fig. 2-B and 2-C, 22-HTG decreases the viability of SK-MEL-28 cells after 24 and 48 h of incubation, indicating the continuous cytotoxic effects. All concentrations reduced cell viability at both incubation periods, thus 24 h incubation was set as the maximum exposure time to 22-HTG. Futhermore, 22-HTG inhibited colony formation of melanoma cells at concentrations of 1.0, 2.0, and 2.5 μM, as shown in Fig. 2-D. The lower concentration of the substance (0.25 μM) was also evaluated, but no inhibitory effects were observed (Fig. 2-D). According to the result, 22-HTG has cytotoxic effect and it interferes with cell survival for a long period (10 days), reducing proliferative capacity and formation of new colonies by human melanoma cells.

3.2. LDHA activity and expression

Cancer cells display increased glucose consumption during proliferation, which makes it necessary to consistently maintain the glycolytic pathway through the regeneration of NAD. This process has been attributed to LDHA (Hanse et al., 2017), which converts pyruvate generated by glycolysis to lactate (Huang et al., 2016). Gene expression of LDHA was decreased ($p < .05$) by treatment with 22-HTG (Fig. 3-B), however the reduction in enzyme activity of LDHA occurred only at the highest concentration of 22-HTG tested, which must have occurred due to interference in cell viability (Fig. 3-A). The positive control, rotenone (1.0 μM), a specific inhibitor of electron transporter chain, significantly increased LDHA activity, as expected, due to metabolic route deviation.

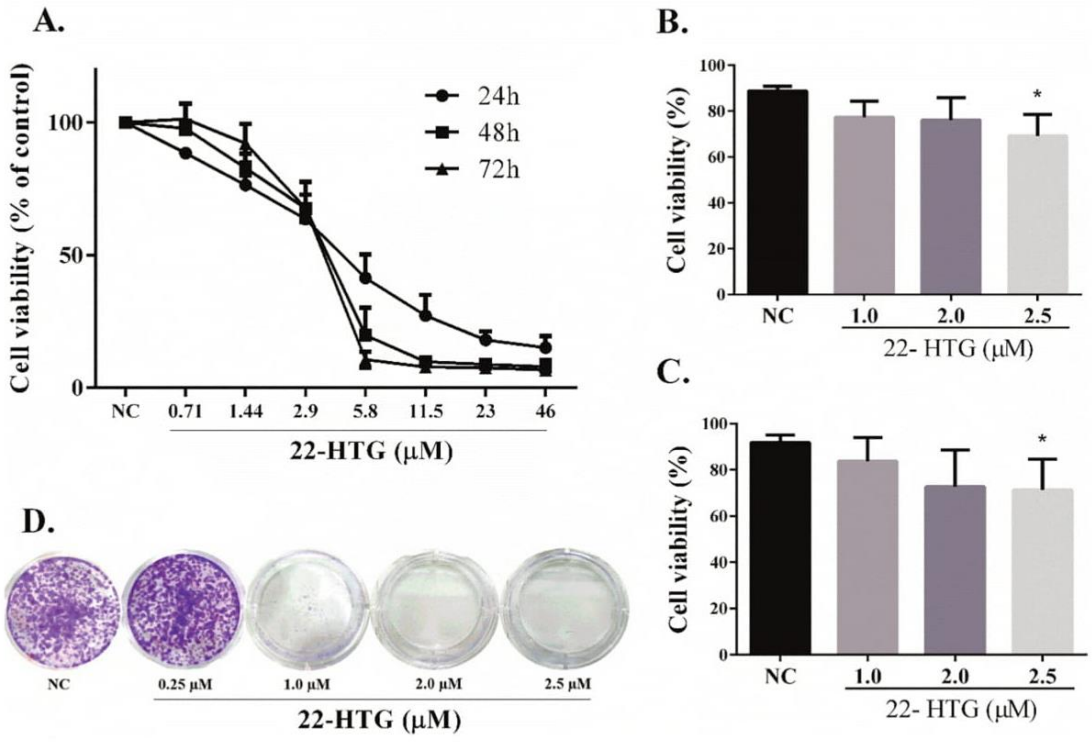

Fig. 2. (A) Cell viability of SK-MEL-28 cells after 24, 48 and 72 h of treatment with 22-HTG by the Alamar blue assay; Cell viability by trypan blue exclusion assay using subtoxic concentrations of 22-HTG after 24 h (B) and 48 h (C) of exposure; (D) Effect of 22-HTG on cell survival and growth of SK-MEL-28 colonies. The cells remained in the presence of the substance for a period of 10 days, and after this time they were fixed with methanol and stained with crystal violet. The images and values represent results from three independent experiments, mean (±) standard deviation. *$p < .05$ was considered significant when compared to the negative control (NC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

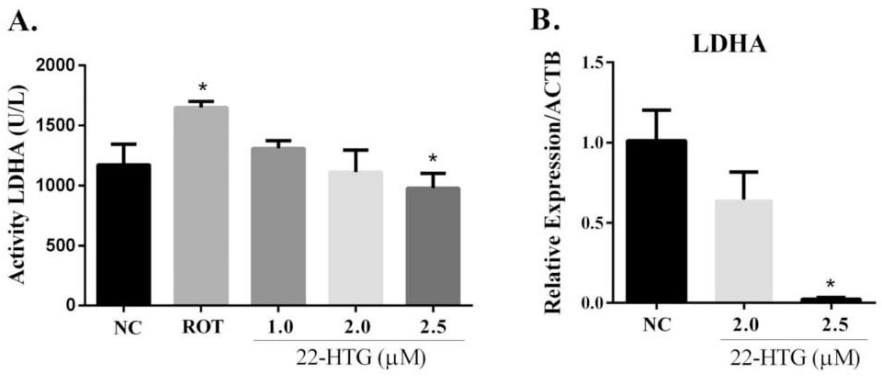

Fig. 3. (A) LDHA activity in SK-MEL-28 cell line after treatment with 22-HTG for 24 h. For this assay, Rotenone (ROT), at 1.0 μM, was used as positive control. (B) Relative expression of LDHA by SK-MEL-28 cell line, calculated by $2^{-\Delta\Delta Ct}$ method. ACTB gene was used as internal control. Data of all experiments represent the mean (±) standard deviation of three independent experiments. *p < .05 was considered significant when compared to the negative control (NC).

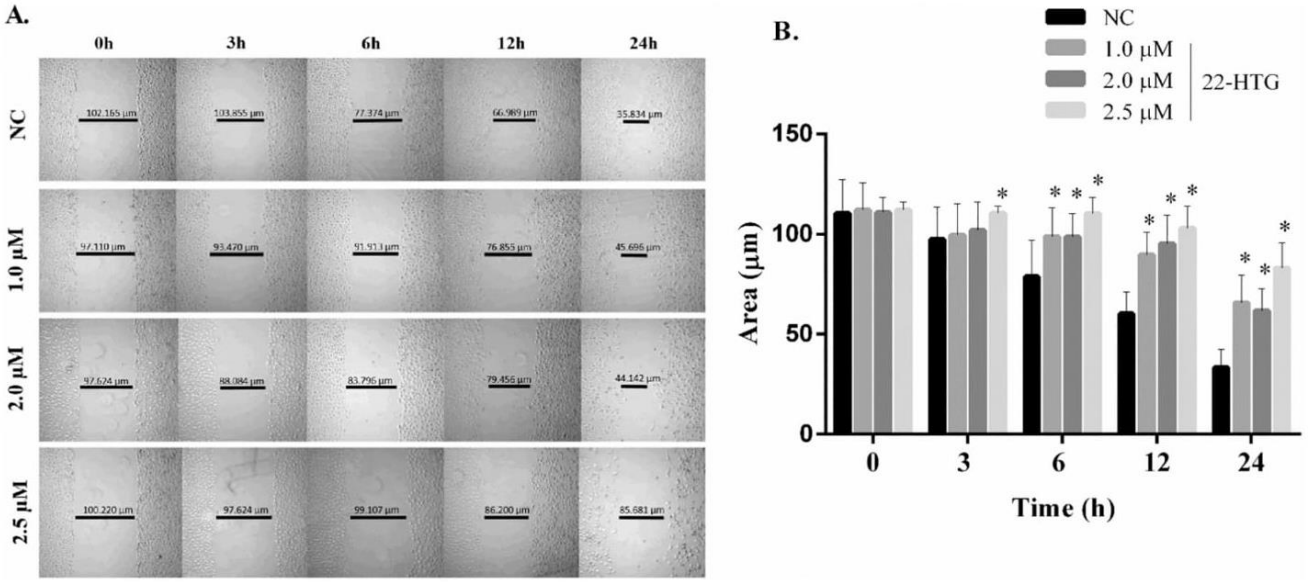

Fig. 4. (A) Effect of 22-HTG on migration by SK-MEL-28 cell line. Images of the cell migration area using an optical microscope at a magnification of 100×. (B) Representation of cell migration at different time points and concentrations. The values represent the mean of the area (±) standard deviation of three independent experiments. *p < .05 was considered significant when compared to the negative control (NC).

3.3. 22-HTG inhibits migration and invasion of melanoma cells, and decrease of MMP activity

As shown in Fig. 4-A, during the scratch woud analysis, cells in the negative control (DMSO 0.2%) showed migration signals starting at 3 h of incubation, while for 22-HTG treated cells, migration was observed starting at 6 h, then 22-HTG reduced the migration ($p < .05$), and the effect is observed for up to 24 h (Fig. 4-B). The effect of 22-HTG on melanoma cell invasion is shown in Fig. 5-C. 22-HTG reduced ($p < .05$) invasion by cells at the concentration of 2.0 μM, compared to negative control (DMSO 0.2%). Although 22-HTG a reduced vimentin expression, this effect was not statistically significant.

The activity of metalloproteinases was determined by the zymographic method and is demonstrated by the clear bands formed after degradation of the substrate (gelatin) by MMP-2 and MMP-9 (Fig. 5- A and B). According to the analysis of the results, 22-HTG reduced ($p < .05$) the activity of MMP-2 and MMP-9, with a greater effect on MMP-9. MMP-2 was inhibited only at the highest concentration tested.

4. Discussion

Hanahan and Weinberg (2011) have described some biological changes that are fundamental for the development of malignant cells, such as maintenance of proliferative signals, resistance to cell death, invasiveness, metastasis, and reprogramming energy metabolism. Pentacyclic triterpenoids, as 22-HTG, are chemical compounds obtained from natural products, which, in turn, are a source of compounds with promising biological activities, including those interfering with several of these hallmarks (Gali-Muhtasib et al., 2015; Salvador et al., 2017). This research describes, for the first time, the effects of 22-HTG on cell proliferation, metabolism, migration, and invasiveness of human melanoma cells.

The uncontrolled proliferation is a characteristic found in all tumor cells, for this reason, it is fundamental to identify substances that can reduce the rate of cell proliferation, and then investigate other mechanisms related to other characteristics that lead to the progression of cancer (Jain and Jain, 2011). Celastrol and pristimerin, well-known quinonemethide triterpenes, have shown anti-proliferative and anti-migration activity against several tumor cell lines (Yousef et al., 2018; Zhu et al., 2010), which corrobore with results obtained in the present study, with inhibition of cell proliferation and cell invasion by exposure to 22-HTG.

Recently, Rodrigues et al. (2019) demonstrated that 22-HTG presents cytotoxic properties against different cell lines. The authors mentioned anti-leukemia activity and attributed this effect to quinone methide triterpenes (Rodrigues et al., 2019). Other studies also

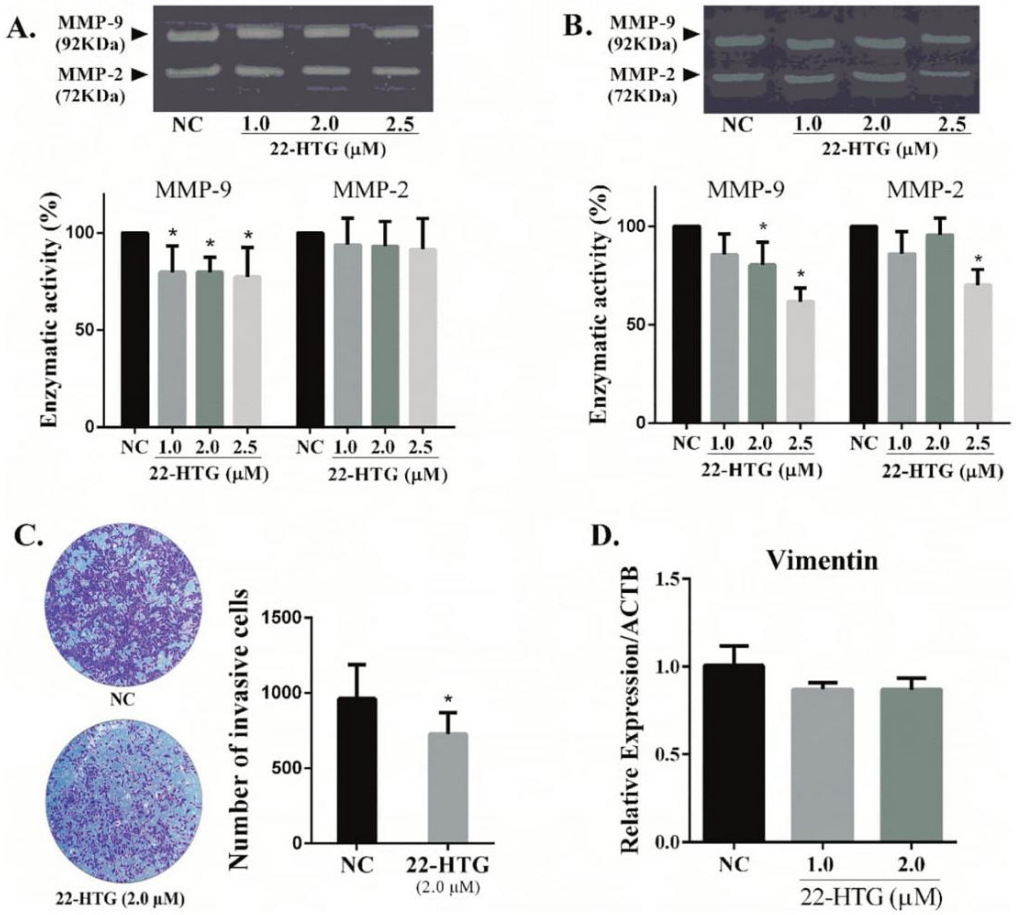

Fig. 5. Proteolytic action of MMP-2 and MMP-9 and cell invasion after treatment with 22-HTG. The activity of the enzymes appeared through the light bands, indicating degradation of the substrate, after 12 h (A) and 24 h (B) of incubation, using HT1080 cells (IC_{50} 8.73 μM and 3.42 μM, respectively). (C) Effect of 22-HTG on invasion by SK-MEL-28 cells after 24 h of treatment using matrigel invasion assay. (D) Relative expression of Vimentin (VIM) by SK-MEL-28 cell line, calculated according $2^{-\Delta\Delta Ct}$ method. ACTB gene was used as internal control. Data of all experiments represent the mean (±) standard deviation of three independent experiments. *p < .05 was considered significant when compared to the negative control (NC).

identified biological effects related to 22-HTG, such as antibacterial and antiparasitic action (Maregesi et al., 2010) and cytotoxicity against breast cancer cells (Cevatemre et al., 2016).

Reprogramming cell metabolism in cancer cells is another hallmark characterized by the modification in metabolic enzymes. Among these enzymes, LDHA has a great importance because it maintains the proliferation, survivability, and invasiveness of tumor cells (Huang et al., 2016; Zhao et al., 2013; Zhou et al., 2019). In cancer patient, increased LDHA levels have been related to poor prognosis and resistance to treatment in different types of tumor (Fiume et al., 2014; Zhao et al., 2013), including melanoma (Gao and Ma, 2017). Thus, the inhibition LDHA is considered a feasible strategy for the development of new therapies (Zhou et al., 2019). Here, we evidenced a decrease in LDHA gene expression after treatment with 22-HTG in melanoma cell line. However, once the enzyme is present in the system a minor reduction in LDHA activity was observed, our results suggest that this effect must have occurred due to cytotoxicity of 22-HTG and no by direct interaction between substance and enzyme activity.

Knockdown of LDHA may reduce invasion of hepatocellular carcinoma cells through multiple mechanisms, such as loss of MMP-2 expression (Sheng et al., 2012). Moreover, in the process of cell migration and signaling, molecules as vimentin, MMP-2 and MMP-9 play an important role (Cui et al., 2019). 22-HTG reduced activity of both MMPs, but not the expression of vimentin. In cancer, increase in MMP activity leads to the degradation of the extracellular matrix, which, in addition to other factors, favors the process of migration and invasion by tumor cells (Rajoria et al., 2011) which is observed in the most lethal forms of melanoma (De Luca et al., 2017). Our results suggests that 22-HTG invasion inhibition may occur due to decreased MMP-2 and MMP-9 activity. Furthermore, MMPs are associated with proliferation of different types of cancer and the inhibition of the activity of these enzymes decreases the growth of tumor cells and metastasis (Yelken et al., 2017). Positive correlation of the data are in accordance with other studies that have shown that celastrol inhibits MMP-9 expression, thus inhibiting the cell invasion (Sethi et al., 2007; Mi et al., 2014; Zhu et al., 2010), as observed in our study. To our knowledge, this is the first time that 22-HTG inhibits cell proliferation and migration by melanoma cells, bringing perspectives for the use of this natural product for the therapy of melanoma.

5. Conclusion

22β-hydroxytingenone has anti-proliferative and anti-invasive effects against human melanoma cells. Reduction of LDHA gene expression and MMPs activity (MMP-2 and MMP-9) may inhibit proliferation, migration, and invasiveness of melanoma cells. This study provides further information on the biological activities of quinonemethide triterpene, focusing on 22-HTG, which was identified as a promising

substance with anticancer activity, whose mechanisms need to be further investigated.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2020.104879.

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6. CAPÍTULO III

22*β*-hidroxitingenona induz apoptose e suprime a invasão de células de melanoma humano pela inibição da atividade de MMP-9 e sinalização de MAPK[3]

Elenn Suzany Pereira Aranha, Adrhyann Jullyanne de Sousa Portilho, Leilane Bentes de Sousa, Emerson Lucena da Silva, Felipe Pantoja Mesquita, Felipe Moura Araújo da Silva, Waldireny C. Rocha, Emerson Silva Lima, Ana Paula Negreiros Nunes Alves, Hector Henrique Ferreira Koolen, Raquel Carvalho Montenegro, Marne Carvalho de Vasconcellos

Neste capítulo é mostrado a indução de morte celular em células de melanoma humano (SK-MEL-28) provocada após a exposição à 22*β*-hidroxitingenona. Este capítulo aborda ainda o potencial antimetastático de 22*β*-hidroxitingenona usando um modelo 3D in vitro de pele humana reconstruída. A indução de apoptose após a exposição à 22*β*-hidroxitingenona foi confirmada e os efeitos moleculares investigados mostram redução na expressão de genes da via MAPK, importante via de estudo no melanoma. Por fim, mostramos que 22*β*-hidroxitingenona diminuiu a invasão de células de melanoma na derme usando o modelo 3D, demonstrando o potencial antimetástatico da substância. Em conjunto, esses dados ajudam a explicar o efeito citotóxico de 22*β*-hidroxitingenona em células de melanoma, elucidando o processo de morte celular e criam boas expectativas para a continuação dos estudos usando essa substância.

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GRAPHICAL ABSTRACT

Title: 22*β*-hydroxytingenone induces apoptosis and suppresses invasiveness of melanoma cells by inhibiting MMP-9 activity and MAPK signaling

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Graphical Abstract

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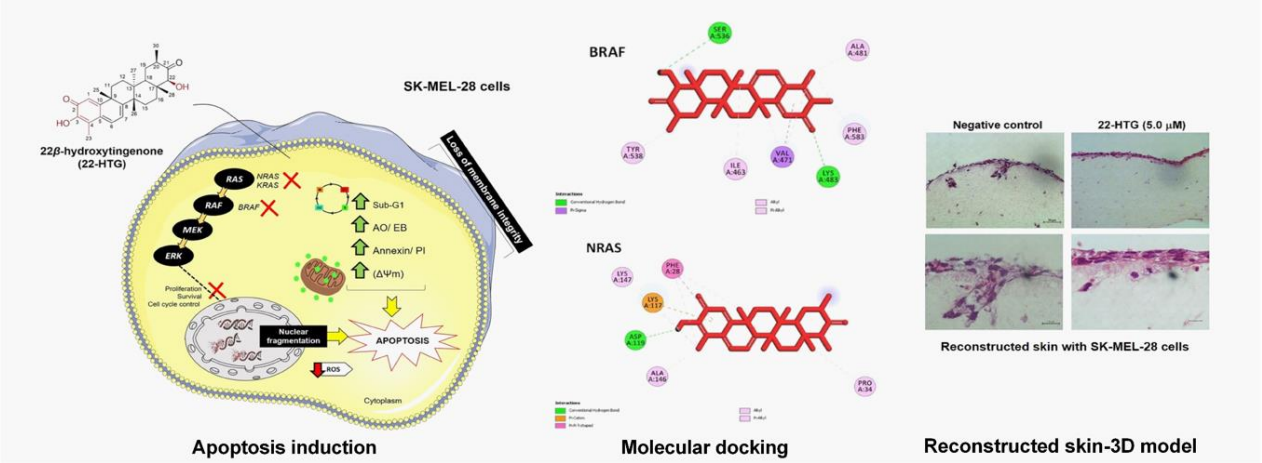

Title: 22*β*-hydroxytingenone induces apoptosis and suppresses invasiveness of melanoma cells by inhibiting MMP-9 activity and MAPK signaling

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Abstract

Ethnopharmacological relevance: 22*β*-hydroxytingenone (22-HTG) is a quinonemethide triterpene isolated of *Salacia impressifolia* (Miers) A. C. Smith belong to the family Celastraceae, which has been used used for medicinal purposes in traditional medicine. However, the anticancer effects of 22-HTG and the underlying molecular mechanisms in melanoma cells have not been elucidated.

Aim of the study: The present study investigated apoptosis induction and antimetastatic potencial of 22-HTG in SK-MEL-28 human melanoma cells.

Materials and Methods: First, the in vitro cytotoxic activity of 22-HTG in cultured cancer cells was evaluated. Then, cell viability was determined by the trypan blue assay in melanoma cells (SK-MEL-28), following cell cycle, annexin V-FITC/propidium iodide assays (Annexin/PI), mitochondrial membrane potential, production of reactive oxygen species (ROS) by flow cytometry, and fluorescence microscopy with acridine orange/ ethidium bromide assay (AO/BE) staining. RT-qPCR was performed to evaluate the expression of BRAF, NRAS, and NRAS genes and docking mocelular was executed. The anti-invasiveness potential of 22-HTG was evaluated in a three-dimensional (3D) model of reconstructed human skin.

Results: 22-HTG reduced viability of SK-MEL-28 cells and caused morphological changes, as cell shrinkage, chromatin condensation, and nuclear fragmentation. Furthermore, 22-HTG caused apoptosis demonstrated by increased stained with AO/BE and Annexin/PI. Apoptosis may have been caused by mitochondrial instability without the involvement of ROS production. Expression of *BRAF*, *NRAS*, and *KRAS*, important biomarkers in melanoma development, was reduced by 22-HTG treatment and was strong binding affinity with BRAF and NRAS in molecular docking. In reconstructed skin model, 22-HTG was able to decrease capacity of melanoma cells to invade into the dermis.

Conclusions: Our data indicate that 22-HTG has anti-tumorigenic properties in melanoma cells by induction of cell cycle arrest, apoptosis and inhibition of invasiveness potential in 3D model, and provide new insights for future work on investigating the utilization of 22-HTG in malignant melanoma treatment.

Keywords: SK-MEL-28, Apoptosis, Invasion, Quinonemethide triterpenes.

Abbreviations: 22*β*-hydroxytingenone (22-HTG); Mitogen-Activated Protein Kinase (MAPK); dimethyl sulfoxide (DMSO); human melanoma cells (SK-MEL-28), human doxorubicin (doxo) resistant uterine sarcoma cells (MES-SA/DX), human prostate cancer cell line (DU 145), normal cell derived from human pulmonary fibroblast (MRC-5); Dulbecco's Modified Eagle's Medium (DMEM); 50% inhibitory concentration (IC_{50}); propidium iodide (PI); acridine orange (AO); ethidium bromide (EB); 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA); quantitative real-time PCR (qRT-PCR); actin beta (ACTB); three-dimensional (3D); reactive oxygen species (ROS).

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1. Introduction

The liana, *Salacia impressifolia* (Miers) A. C. Smith is distributed from Central to South America, especially at the Brazilian Amazon rainforest (Lombardi, 2010). In the North of Brazil, *S. impressifolia* is popularly known as “miraruíra” or "cipó-miraruíra", which is also the popular name for Amazonian *Connarus* spp. and both are used (stem decoction) to treat inflammations and diabetes (Lorenzi and Matos, 2002). In the Peruvian Amazon, *S. impressifolia* it is known among the folk as “panu” and is applied to different treatments, such as against dengue, renal affections, rheumatism and cancer (Brako and Zarucchi, 1993; Clavo et al., 2003).

The activity cytotoxic of the extract and fraction of *S. impressifolia* were reportated against different cell lines (Rodrigues et al., 2019). The authors demonstrated anticancer potential in vitro and in vivo and pointed out the presence of quinonemethide triterpenoids, especially compounds tingenone and 22*β*-hydroxytingenone (22-HTG). In especial, results obtained against the SK-MEL-28 cell line (human melanoma cells) (Aranha et al., 2020) with 22-HTG encouraged more studies to evaluate its mechanism of action. Among types of skin cancer, melanoma is the most aggressive and lethal form of tumor (Leonardi et al., 2018) and most associated with genetic changes, which lead to uncontrolled cell proliferation and the appearance of an invasive cell phenotype (Savoia et al., 2019).

Mutations in oncogenes *BRAF* and *NRAS* are fundamentally reported in patients with melanoma and the dysregulation in these genes can lead to alterations in some signaling pathways cell, as Mitogen-Activated Protein Kinase (MAPK), also known as RAS/RAF/MEK/ERK. The loss of control of signaling processes in the MAPK pathway can lead to uncontrolled cell proliferation, invasion, metastasis, survival, angiogenesis, and apoptosis inhibition, which are steps involved in melanoma development (Moreira et al., 2020).

Prognosis of patients with early-stage melanoma has been improved in recent years with the advances in the development of targeted therapies using specific inhibitors and immunotherapies (Berning et al., 2019). Despite the important progress of these therapies and the advancement that has been made in the clinical of melanoma there is still a significant risk in the development of drug resistance or overstimulation of the immune system, which in the long-term can lead to a poor prognosis (Berning et al., 2019; Kozar et al., 2019). For this reason, the identification of new alternative therapies capable to affect molecular events enhancing the life quality of patients with melanoma is still needed (Menezes et al., 2018).

Therefore, considering that natural products are a promising source of active principles, which may even be used in combination in existing therapy to enhance the pharmacological effect and reduce toxicity (Oprean et al., 2018), the present work describes biological activity of 22-HTG, associated with induction of apoptosis, reduction of invasiveness potential, and investigation the molecular mechanism associated of these effects.

2. Materials and methods

2.1. Extraction and isolation of 22β-hydroxytingenone

Trunk and twigs of *S. impressifolia* (Miers) A. C. Smith (family Celastraceae) were collected at Adolpho Ducke Reserve (coordinates 2°56′58.2'' S, 59°56′36.3'' W) located in highway AM-010, km 26, municipality of Manaus, Amazonas state, Brazil. A voucher specimen (#4699) was deposited in the herbarium of the National Institute for Amazonian Research, Brazil. The acess was registered (number: A715C2F) in SisGen -Brazilian National System of Genetic Resource Management and Associated Traditional Knowledge- and complied with their guidelines. The plant extraction, isolation workflow, structural confirmation, and purity of the *22β-hydroxytingenone* (22-HTG) were previously described (Aranha et al., 2020; da Silva et al., 2016). In the in vitro experiments, 22-HTG (Figure 1) was dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium. The final concentrations of solvent in all experiments did not exceed 0.2% (v/v). This concentration does not influence cell proliferation as shown previously (Boncler et al., 2014).

2.2. Cell line and culture conditions

Human melanoma cells (SK-MEL-28), human doxorubicin (doxo) resistant uterine sarcoma cells (MES-SA/DX), human prostate cancer cell line (DU 145), and the normal cell derived from human pulmonary fibroblast (MRC-5) were utilized in this study. Cells were grown in 75 cm^2 culture flasks, containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco™ #12100046), supplemented with 10% bovine fetal serum (FBS, Gibco™ #12657029) and 1.0% penicillin-streptomycin (Pen Strep, Gibco™ #15070063), and maintained at 37°C in a humidified atmosphere of 5%. Cells were passaged after reaching 90% confluence, detached with 0.05% Trypsin-EDTA (Gibco™ #25300062), and continuously cultured from the passage no. 20 until passage no. 50, for all experiments.

2.3. Cell viability assay

The 50% inhibitory concentration (IC_{50}) was determined by Alamar blue assay (resazurin 7-Hydroxy- 3H-phenoxazin- 3-one 10-oxide) (Ansar Ahmed et al., 1994). For the assay, SK-MEL-28, MES-SA/DX, DU 145, and MRC-5 were plated (0.5×10^4/ well) in a 96-well plate and allowed to attach for 24 h. 22-HTG (20 – 0.625 μM) was then added to each well with a volume of 100 μL/well and after 72 h of 22-HTG exposure, 10 μL of 0.02% Alamar blue solution in DMEM were added to each well. Fluorescence was measured by using 465 nm excitation and 540 nm emission in a microplate reader (DTX 800 Beckman Coulter Multimode Detector). This assay was used to determine the cell line and concentrations used in subsequent tests.

Cell viability was assessed by trypan blue exclusion assay. SK-MEL-28 cells (0.3 x 10^5 cells/well) were plated in 12-well-plate and after 24 h the medium was replaced with 22-HTG (2.5 and 5.0 μM) by 24 h of treatment. After, cells were harvested to estimate cellular viability by differentiating living cells from dead ones in a hemocytometer chamber using trypan blue solution 0.4% (Strober, 2001).

2.4. Analysis of cell morphology

SK-MEL-28 cells (0.5 x 10^5 cells/well) were plated in 24-well-plate and after 24 h the medium was replaced with 22-HTG (2.5 and 5.0 μM) by 24 h of treatment. After treatment, cells were resuspended in the culture medium, and slides were made by cytocentrifugation (450 g/ 5 minutes). Cells were fixed and stained with a LaborClin® rapid panoptic dye kit. The cells were analyzed and morphological changes in the cytoplasm and nucleus were photographed using light microscopy (Eclipse Ni-U-Nikon).

2.5. Cell cycle analysis

The potential of the 22-HTG to interfere with the cell cycle of SK-MEL-28 human melanoma cells was evaluated by flow cytometry using propidium iodide (PI) as a cell marker (Vartholomatos et al., 2015). SK-MEL-28 cells (0.5 x 10^5 cells/well) were plated in 24-well-plate and after 24 h the medium was replaced with 22-HTG (2.5 and 5.0 μM) for 24 h of treatment. Thus, cells were harvested, centrifuged at 247 g for 5 min at 4°C, and the cell pellet was fixed using cold ethanol 70% overnight. Then, cells were centrifuged at the same conditions as the previous step, supernatant removed, and the cells were stained with 100 μL PI solution (5 μg/mL) for 30 minutes at 4ºC. The fluorescent emission was measured in a flow cytometer (FACSCanto II, BD Biosciences, OR, USA).

2.6. Acridine orange and ethidium bromide cell staining

Acridine orange (AO) and Ethidium bromide (EB) staining were used to distinguish viable from apoptotic, and necrotic cells (Ralph et al., 2016). SK-MEL-28 cells (0.3 x 10^5 cells/well) were plated in 24-well-plate and after 24 h the medium was replaced with 22-HTG (2.5 and 5.0 μM) for 24 h of treatment. After the time of exposure to the substance, cells were harvested, centrifuged at 247 g for 5 min at 4°C, resuspended in 30 μL of culture medium and stained with 2 μL of an aqueous solution AO/EB (100 μg/mL; AO/EB). The cell suspension was immediately examined using fluorescence microscopy (Eclipse Ni-U-Nikon). Images obtained were then analyzed and cells percentage was quantified as viable (uniform bright green nuclei with an organized structure), apoptotic (orange to red nuclei with condensed to fragmented chromatin and green cytoplasm) and necrotic cells (uniformly orange to red nuclei with an organized structure and red cytoplasm).

2.7. Apoptosis assay

Apoptosis was evaluated by double-labeled flow cytometry analysis with Annexin V-FITC and PI, which allows the detection of apoptotic and necrotic cells due to modifications on the plasmatic membrane (Guerra et al., 2017). SK-MEL-28 cells (0.3 x 10^5 cells/well) were plated in 24-well-plate and after 24 h the medium was replaced with 22-HTG (2.5 and 5.0 μM) for 24 h of treatment. Subsequently, the cells were harvested and centrifuged at 247 g for 5 min at 4°C. The cell pellet was stained with 5 μL of Annexin V-FITC and 5 μL of PI for 15 min at room temperature (25°C) in the dark, as recommended by FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™). Finally, the fluorescence was evaluated by flow cytometry (FACSCanto II, BD Biosciences, OR, USA).

2.8. Evaluation of mitochondrial membrane potential

The evaluation of the mitochondrial membrane potential was performed to investigate the 22-HTG effect on mitochondrial metabolism by fluorescent labeling with rhodamine 123 (Amaral-Machado et al., 2019). SK-MEL-28 cells (0.3 x 10^5 cells/well) were plated in 12-well-plate and after 24 h the medium was replaced with 22-HTG (2.5 and 5.0 μM) for 24 h of treatment. Next, cells were harvested, centrifuged at 247 g for 5 min at 4°C, and cell pellets were resuspended in rhodamine123 solution (5 μg/mL) for 30 minutes at 37°C and 5% CO_2 atmosphere in dark. Then, the mitochondrial membrane potential was evaluated by flow cytometry (FACSCanto II, BD Biosciences, OR, USA).

2.9. Evaluation of intracellular reactive oxygen species (ROS) levels

2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent dye was used to determine the intracellular ROS levels of the SK-MEL-28 cell line (Ribeiro et al., 2018). SK-MEL-28 cells (0.3 x 10^5 cells/well) were plated in 12-well-plate and after 24 h the medium was replaced with 22-HTG (2.5 and 5.0 μM) for 3 h of treatment. Subsequently, the cells were incubated with DCFH-DA solution (10 μM) for 30 minutes at 37°C and 5% CO_2 atmosphere. After, the cells were washed twice with PBS, harvested, and centrifuged at 247 g for 5 min at 4°C. Cell pellets were resuspended with 400 μL of PBS and the intracellular ROS levels were determined by flow cytometry (FACSCanto II, BD Biosciences, OR, USA).

2.10. Total mRNA isolation and expression analysis by qRT-PCR

The effects of 22-HTG on the mRNA expression BRAF, NRAS, and KRAS were analyzed. SK-MEL-28 cells (0.4 x 10^5 cells/well) were plated in 24-well-plate and after 24 h the medium was replaced with 22-HTG (2.5 μM) for 24 h of treatment. Next, cells were trypsinized, collected, and total mRNA was extracted by TRIzol® Reagent (Life Technologies, USA). RNA integrity was verified by electrophoresis and concentration, the quality of the RNA was determined using NanoDrop (Thermo Scientific). RNA concentration was normalized to 20 ng/μL and the reverse-transcription was performed using the High-Capacity cDNA kit, according to the manufacturer's protocol (Life Technologies, USA).

Quantitative real-time PCR (qRT-PCR) was performed by the Fast SyberGreen kit (Applied Biosystems, USA). Expression levels of *BRAF*, *NRAS*, and *KRAS* were determined in triplicate using stable expressed Actin Beta (ACTB) gene as the endogenous control. Primer efficiency was determined for all genes. PCR primers sequence of each gene was designed using OligoPerfect Designer (Invitrogen) and are described in Table 1.

All requirements proposed in Minimum Information for Publication of Quantitative Real-Time PCR Experiments - MIQE Guidelines were followed (Bustin et al., 2009). The expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), considering negative control samples as a calibrator of the experiments.

2.11. Molecular docking

The docking studies were carried out according to a previously reported approach (de Lima et al., 2019). First, the three-dimensional (3D) structure of 22*β*-hydroxytingenone was downloaded from PubChem (PubChem CID: 73147) (https://pubchem.ncbi.nlm.nih.gov/). Then, the structure was treated to the geometry optimization by the semi-empirical method

PM7 (Stewart, 2013) using the MOPAC2016 software (Stewart, 2016). Finally, the ligand was prepared via Autodock tools (Morris et al., 2009) and saved as PDBQT files. On the other hand, the 3D crystal structures of the B-Raf Kinase V600E oncogenic mutant (BRAF) (PDB ID: 3OG7) and human NRAS GTPase (NRAS) (PDB ID: 3CON) were retrieved from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (http://www.rcsb.org) as PDB files. These receptors were prepared via Autodock tools as previously described (de Lima et al., 2019) and saved as PDBQT files. Finally, a docking analysis was carried out using Autodock Vina (Trott and Olson, 2010) with Discovery Studio (AccelrysInc, 2016) being used to analyze the binding conformations.

2.12. Artificial skin reconstruction

The Human Skin Equivalent was reconstructed according to (Boelsma et al., 2000; Maria and Wada, 1997; Pedrosa et al., 2017) with modifications. MRC5 cells (25 x 10^4 cells/skin) were gently mixed with 700 µL of Collagen Type I (2.3 mg/mL, A10483-01 Gibco™), 108 µL of reconstruction buffer 10X (NaOH 0.05 M, $NaHCO_3$ 2.2%, HEPES 200 mM), and 108 µL of Ham's F12 Nutrient Mixture 10X. After gently mixing gel solution with MRC5, this solution (800 µL/insert) was quickly transferred to a Corning transwell® culture 12 mm diameter inserts with 0.4 µm (12-well plate), and incubated at 37°C with 5% CO_2 atmosphere for 30 min to allow jellification. After, Collagen type IV (6µg/mL – C7521 SIGMA) coating was performed on the dermal matrix with fibroblasts embedded in collagen, DMEM/F12 medium supplemented with growth factors was added outside the insert and incubated at 37°C for 1 hour for pH adjustment. HaCat (50 x 10^4 cells/skin) and SK-MEL-28 (25 x 10^4 cells/skin) cells were mixed and seeded a dermal matrix to full thickness skin. After 24 hours of submersion, skins were raised to the air-liquid interface by removing media from insert and cultured by 10 days. During experiments, the medium was exchanged for each 48 h of incubation until 10 days.

2.13. Histological analysis

Skins samples were fixed in 10% buffered formaldehyde at 4ºC, dehydrated with alcohol and embedded in paraffin. For morphological analyses, the samples were stained with hematoxylin and eosin.

2.14. Determination of MMP activity by gelatin zymography

Activity MMP-2 and MMP-9 into conditioned organotypic media was determined by gelatin zymography. In the end experiments, the media were collected and centrifugated at 425 x *g* for 10 min at 4°C to remove cell debris. Total protein contents were normalized by protein determination method of Bradford. The cell-conditioned medium was subjected to substrate-gel electrophoresis. A similar amount of protein containing the conditioned media was applied on 10% polyacrylamide gels containing 1.0 mg/mL gelatin. After electrophoresis, polyacrylamide gels were washed with 2.5% Triton X-100 at room temperature to remove sodium dodecyl sulfate. Gels were then incubated overnight at 37˚C in a buffer containing 5.0 mM $CaCl_2$, 50 mM Tris–HCl pH 8.5 and 5.0 μM $ZnCl_2$ to digest gelatin by MMP. The gels were stained with 1% Brilliant Blue R in 45% methanol and 10% glacial acetic acid. After 30 min, the gels were destained in the same solution without the Coomassie blue dye. Proteolytic activity was detected as clear zones against the background stain of undigested substrate and then intensities of the bands were estimated using ImageJ Software®.

2.15. Statistical analysis

All data were presented as the mean ± standard deviation (SD) from three independent experiments performed in triplicate. Statistic difference ($p<0.05$) was obtained compared with negative control (DMSO 0.2%) by analysis of variance (ANOVA) followed by Tukey's or Bonferroni's posttest using GraphPad Prism 6.0 for Windows (Institute Software for Science, San Diego, CA).

3. Results

3.1. 22-HTG induces reduction on viability cell in SK-MEL-28 cells

Figure 1-(B) presents the cell survival curves obtained after exposure of 22-HTG using a small panel of cancer cell lines derived from the prostate (DU 145), uterus (multidrug-resistant) (MES-SA/DX), melanoma (SK-MEL-28), and the normal one established from lung (MRC-5). The concentrations of the compound that reduced cell growth by 50% (IC_{50} values) are shown in Table 2. Cytotoxicity of 22-HTG was also evaluated in noncancerous cells (MRC-5), showing low selectively. Doxorubicin, an effective chemotherapeutic anticancer drugs, exhibited higher cytotoxicity to noncancer cells than 22-HTG.

The lowest IC_{50} value for 22-HTG in cancer cells was in SK-MEL-28 (3.2 μM). This *in vitro* melanoma model presents a high resistance to toxicity due to mutations such as

TP53R273H (Ralph et al., 2016), becoming a relevant clinical study model for the research of drugs in therapy for this type of cancer (Rossi et al., 2018), therefore was used for the subsequent experiments in this study.

According to IC_{50} value in SK-MEL-28 cells, the concentrations 2.5 and 5.0 µM were defined to evaluate the effect of 22-HTG on cell viability of SK-MEL-28 cells by trypan blue staining. Treatment for 24 h with 2.5 and 5.0 µM of 22-HTG caused a significant decrease ($p<0.05$) in the number of viable cells (Figure 1-C).

3.2. Cell cycle analysis

Cell cycle arrest analysis was performed to evaluate whether the treatment with 22-HTG causes changes in the cell cycle progression. The cellular distribution in different phases of the cell cycle was illustrated in relation to the intracellular DNA content. As shown in Figure 1-(D), after 24 h of treatment with 22-HTG, both concentrations decreased the number of cells in the G0/G1 and induced a significant accumulation of cells in the sub-G1 phase ($p < 0.05$), which is considered an important effect for control tumor growth. Treatment with doxorubicin (5.0 µM) increased the percentage of cells in the G2/M phase and reduced cells in G0/G1 and S phase.

3.3. 22-HTG induces apoptosis in SK-MEL-28 cells

Initially, signs of apoptosis were investigated by analysis of cell morphology. Treatment with 22-HTG caused changes in the morphology of SK-MEL-28 cells, as compared to negative control group cells. The main morphological changes found in cells SK-MEL-28 after treatment with 22-HTG were condensation and nuclear fragmentation. It is also possible to observe a reduction in the cell and nucleus volume, intracellular vacuole, irregularities, and instability of the plasma membrane (Figure 2-A). The positive control doxorubicin (5.0 µM) reduced the number of cells, induced chromatin condensation, and nuclear fragmentation and blebbing of the plasmatic membrane. The morphologic changes described suggest cell death by apoptosis and reflect the cytotoxic potential of 22-HTG in SK-MEL-28 cell line.

Apoptosis was evaluated by staining with AO/EB. Acridine orange is a vital dye that will stain both living and dead cells, whereas EB will stain only those cells that have lost their membrane integrity. Living cells stain in green and can thus be distinguished from apoptotic cells (Kasibhatla, 2006). After treatment with 22-HTG was detected a significant decrease ($p<0.05$) in the viability of SK-MEL-28 cells and the number of cells with green and red-

stained (apoptotic) increased ($p<0.05$) in both concentrations tested (Figure 2-B). Cells stained in red only were observed in the doxorubicin treatment. Thus, these results indicate that cell death after treatment with 22-HTG occurred through apoptosis.

Apoptotic cells were also confirmed with annexin V-FITC and PI by flow cytometry. Phosphatidylserine is a phospholipid presented in the cell membrane, normally localized on the internal surface. During the apoptosis process, this lipid is externalized and available to detection by Annexin V-FITC conjugate, which is considered a marker of apoptosis cell death (Tischlerova et al., 2017). The use of PI in this assay allows the evaluation of the necrotic cells since this cellular process is characterized by the loss of membrane integrity (Amaral-Machado et al., 2019). 22-HTG induced a significant increased ($p<0.05$) in apoptotic cell populations in early apoptosis (AnnexinV-positive/PI-negative) and late apoptosis (Annexin V-positive/PI-positive) compared with the negative control (Figure 2-C). Doxorubicin (5.0 µM) also induced apoptosis ($p<0.05$).

End-stage apoptosis is characterized by loss of the plasma membrane integrity which is also possible to observe in the necrotic phenotype (Galluzzi et al., 2018). Treatment with 22-HTG not induced an increase in necrotic cells in AO/EB and Annexin/PI assay. Therefore, taken together, these results suggest that 22-HTG is able to induce apoptosis in SK-MEL-28 cells.

3.4. Evaluation of mitochondrial membrane potential

Mitochondria is an important cellular organelle and changes in the mitochondrial membrane potential and permeability are indicators of mitochondrial dysfunction, resulting in apoptosis induction (Amaral-Machado et al., 2019; Tischlerova et al., 2017).

Integrity or disruption of the mitochondrial membrane potential can be detected using rhodamine 123, a green-fluorescent dye stored in active mitochondria. A decrease of mitochondrial membrane potential is indicated by a reduction in fluorescence intensity (Rodrigues et al., 2018). Treatment with 22-HTG caused significantly ($p<0.05$) mitochondrial membrane potential loss in the SK-MEL-28 cells, showing that 22-HTG induced mitochondrial damage (Figure 3). Positive control doxorubicin (5.0 µM) also caused significant mitochondrial damage.

3.5. Evaluation of intracellular ROS levels

DCFH-DA is a specific ROS indicator (Chen et al., 2015) and was used to investigate whether 22-HTG causes increased intracellular ROS levels, which would be involved with its

cytotoxic effect. Figure 4 shown a reduction in the number of positive DCF-DA cells after treatment with 22-HTG, revealing that exposure of 22-HTG (2.5 and 5.0 μM) decreased significantly ($p<0.05$) the production of ROS in SK-MEL-28 cells.

3.6. mRNA isolation and expression analysis by RT-qPCR

It was investigated whether 22-HTG affect *BRAF*, *NRAS*, and *KRAS* gene expression, important biomarkers in melanoma due to involvement in MAPK signaling pathway, described in approximately 90% of cases of melanoma (Strickland et al., 2015). The transcripts levels of all genes evaluated were significantly reduced by treatment with 22HTG (2.5 μM) after 24h, compared with negative control ($p < 0.05$), as shown in Figure 5-(A).

3.7. Molecular docking

In order to investigate the main interactions and the binding affinity between 22HTG and the BRAF, and NRAS proteins, a preliminary docking study was conducted. Initially, docking analysis revealed scoring function values for 22HTG (-10.4 kcal/mol) close to that obtained for the previously described inhibitor PLX4032 (redocking binding free energy = -11.6 kcal/mol, RMSD < 2 Å), which suggests the establishment of favorable interactions for the 22HTG-BRAF complex. Also, for NRAS, the binding free energies for 22HTG (-9.7 kcal/mol) and the previously described inhibitor guanosine-5'-diphosphate (redocking binding free energy = − 10.8 kcal/mol, RMSD < 2) were close (Figure 5-B).

In regards to the interactions, 22HTG presented specific intramolecular interactions with the binding site of BRAF, especially of the quinone moiety with Lys483 via an hydrogen bond, and via π-sigma and π-alkyl interactions with Val471. Other interaction were observed at the 22*β*-hydroxy group with Ser536 through an hydrogen bond. Against NRAS, near all interactions occurried at the quinone ring system. Most significant ones were hydrogens bond with Asp129, π-cation with Lys117 and π-π T-shaped interaction with Phe28.

3.8. Invasion decreases after 22-HTG treatment in 3D models

We used a reconstructed skin model with SK-MEL-28 cells, a model more similar to the in vivo 3D tumor environment, to test the effects of 22-HTG. As shown Figure 6 (A-*arrow*), untreated skin reconstructs were marked by melanoma invasion and/or migration, showed a delimited tumor area, with melanoma cells in dermis-epidermis junction toward the dermis, characterizing the invasion process. After 22-HTG treatment (5.0 μM), this reconstructed skin with SK-MEL-28 cells presented a decrease of invasion points (Figure 6 (A)- *arrow*). Furthermore, using reconstructed skin model, 22-HTG (5.0 μM) decreased

significantly MMP-9 activity (latent and active form), however MMP-2 did not occur reduction (Figure 6-B).

The reduction of MMM-9 activity is agreement with the findings of the 2D experiments (Aranha et al., 2020). We believe that reduction of MMP-9 activity after exposure to 22-HTG may be involved in the reduction of invasion observed in the histological analysis of reconstructed skin model with SK-MEL-28 cells.

4. Discussion

Natural products represent an source for drug discovery, since that compounds isolated from medicinal plants with cytotoxic effect are important to identify new drug with anticancer potential (Ahmed and Halaweish, 2014; Rodrigues et al., 2019). Recents studies have identified the cytotoxic potential of *S. impressifoia* and this effect was associated with quinonemethide triterpenes (Rodrigues et al., 2019), which are considered potent anticancer agents, inhibiting proliferation, angiogenesis, metastasis, and causing apoptosis or autophagy in tumor cells (Salvador et al., 2017), becoming clinically interesting due to the anticancer potential (Sachan et al., 2018). Studies of our research group have reported the role of 22-HTG against melanoma cells (Aranha et al., 2020), but the mechanism leading to the potent cytotoxic effect needs further elucidation. In the current study we identify potential anti-melanoma of 22-HTG, by induction of apoptosis, reduced invasiveness of SK-MEL-28 cells in 3D model, and interfered with the MAPK signaling.

Cell proliferation may be regulated by control of cell cycle and apoptosis (Bi et al., 2018). The loss of normal cell cycle control is an important feature of cancer which may induce uncontrolled proliferation (Menezes et al., 2018). Likewise, the sub-G1 phase of cell cycle is a typical marker of DNA fragmentation observed in the late event of the apoptosis pathway (Beberok et al., 2019). In our study, we found that, in addiction to the cytotoxic effect, 22-HTG induced morphological changes and sub-G1 peak in cell cycle analysis. Thus, these results indicate that the antiproliferation effects were mediated by apoptosis.

Apoptosis is an important mechanism in anticancer drug research for the discovery and development of new therapeutic agents (An et al., 2019). Previous work has shown that 22-HTG induced apoptosis in cancer stem cells of breast cancer (Cevatemre et al., 2016). In the current study, we also demonstrated that 22-HTG induced apoptosis in SK-MEL-28 cells. Several studies using quinonemethide triterpene, as Celastrol, shown induction of apoptosis on human myeloma (Tozawa et al., 2011), breast (Mi et al., 2014), and pancreatic cancer cells (Zhao et al., 2014). Pristimerin, other quinonemethide triterpene, show induction of

apoptosis in the breast (Lee et al., 2013), prostate (Liu et al., 2014), and pancreatic cancer cells (Deeb et al., 2014). These results corroborate with the present study and reinforce the idea that 22-HTG induce apoptotic-like cell death in SK-MEL-28 cells.

After verifying the events of apoptosis caused by the treatment with 22-HTG, it was investigated if this process occurs by disruption of mitochondrial membrane potential, as a hallmark of apoptosis (Beberok et al., 2017). In cancer cells, mitochondrial damage may be a promising strategy in the regulation of apoptosis (Sharma et al., 2017). Cevatemre et al. (2016) (Cevatemre et al., 2016) showed that 22-HTG induced a decrease in the mitochondrial membrane potential in cancer stem cells of breast cancer.These findings suggest that mitochondrial dysfunction is likely with induction of apoptosis by intrinsic pathway after treatment with 22-HTG.

Several studies in the literature have shown that the cytotoxic effect of anticancer substances is caused by the intracellular increase of ROS, resulting in oxidative damage and consequently induction of apoptosis (Cho et al., 2018; Lee et al., 2019; Wang et al., 2018; Zhang et al., 2015). However, in this study we show that apoptosis induced by 22-HTG was not directly associated with ROS production in melanoma cells. A similar effect has been demonstrated using pristimerin (Wu et al., 2005), where the authors demonstrated that pristimerin did not enhance the generation of ROS. Among other findings, Wu et al. (Wu et al., 2005) showed in a cell-free system, that pristimerin was able to induce cytochrome *c* release from isolated mitochondrial fractions, indicated the induction of mitochondrial membrane permeabilization by a direct action on mitochondria.

In melanoma there is high heterogeneity tumoral, leading phenotypic plasticity to support melanoma progression and resistance to drug exposure to treatment (Arozarena and Wellbrock, 2019). In current study 22-HTG induced apoptosis and reduced invasion of population of melanomas with genetic profile of SK-MEL-28 cells, with significant inhibition MAPK signalling. SK-MEL-28 cells have genetic alterations, as B-Raf^{V600E}, TP53^{R273H} and PTEN, which notoriously confer them a immense resistance to the most targeted anticancer therapies (Daveri et al., 2015; Ralph et al., 2016).

MAPK pathway is involved in the regulation of important cellular functions such as cell cycle control, proliferation, survival, migration, and programmed cell death (Abd El Maksoud et al., 2019; Shao et al., 2018) and play an important role in the advancement and progression of melanoma (Cicenas et al., 2017). Inhibition of the MAPK pathway is demonstrated as an inducer of apoptosis, causing inhibition of tumor growth (Ahmed and Halaweish, 2014). BRAF and NRAS are proteins involved with signaling MAPK pathway

(Dumaz et al., 2019), which coupled to other mutations are involved with resistance mechanism, one of the major obstacles in the melanoma treatment (de Sousa et al., 2019). In this study, we have demonstrated that 22-HTG was able to inhibit genic expression and has stronger binding affinity *in silico* of BRAF (V600E) and NRAS.

In current study was also demonstrated that 22-HTG decreased invasion of melanoma cells into the dermis and reduced the action of MMP-9 using 3D human skin reconstruct model, which is an ideal model to dissect each step of melanoma development and progression (Li et al., 2015). Previous study have shown that 22-HTG reduced migration, invasion, and MMPs activity in 2D models (Aranha et al., 2020), which is in accordance with what was demonstrated in this study and reflects the antimetastatic potential of 22-HTG.

5. Conclusion

22*β*-hydroxytingenone has anti-tumorigenic properties in melanoma cells including the inhibition of proliferation, apoptosis induction, and inhibition of invasiveness potential. The MAPK pathway might also be involved, with inhibition *BRAF* and *NRAS*. Our results showed that 22*β*-hydroxytingenone has anticancer potential and all this information could be used to support new investigations intended to study a natural product with anti-melanoma action.

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Table 1. Sequence of oligonucleotides used for qRT-PCR.

Gene	Sequence (5'- 3')	NCBI reference sequence
BRAF	F- CATCCACAGAGACCTCAAGAGT	NM_001354609.2
	R- ATGACTTCTGGTGCCATCC	
NRAS	F- TCCAGCTAATCCAGAACCAC	NM_002524.5
	R- TTCGCCTGTCCTCATGTATT	
KRAS	F- TTGTGGTAGTTGGAGCTGGT	
	R- ACTCCTCTTGACCTGGTGTG	NM_001369787.1
ACTB[a]	F- CTGGAACGGTGAAGGTGACA	
	R- AAGGGACTTCCTGTAACAACGCA	NM_001101.5

[a]Actin Beta (ACTB) gene was used as the endogenous control.

Table 2- IC_{50} values on cancer cell line (DU 145, SK-MEL-28, and MES-SA/DX) and one non-tumor cell line type (MRC-5) after 72 h of exposure to 22-HTG using Alamar blue assay.

	IC_{50} µM (confidence intervals)[a]	
Cell lines	**22-HTG**	**Doxorrubicin[b]**
DU 145	6.94 (6.67- 7.38)	0.39 (0.32 – 0.48)
SK-MEL-28	3.2 (3.05- 3.37)	0.22 (0.05 – 0.88)
MES-SA/DX	8.02 (7.6- 8.79)	0.42 (0.20 – 0.87)
MRC5	2.61 (2.29-2.95)	0.14 (0.10 – 0.17)

[a]Data are presented as half-maximal inhibitory concentration (IC_{50}) value and 95% confidence intervals (CI95%) from three independent experiments performed in triplicate.

[b]Doxorubicin was used as the positive control.

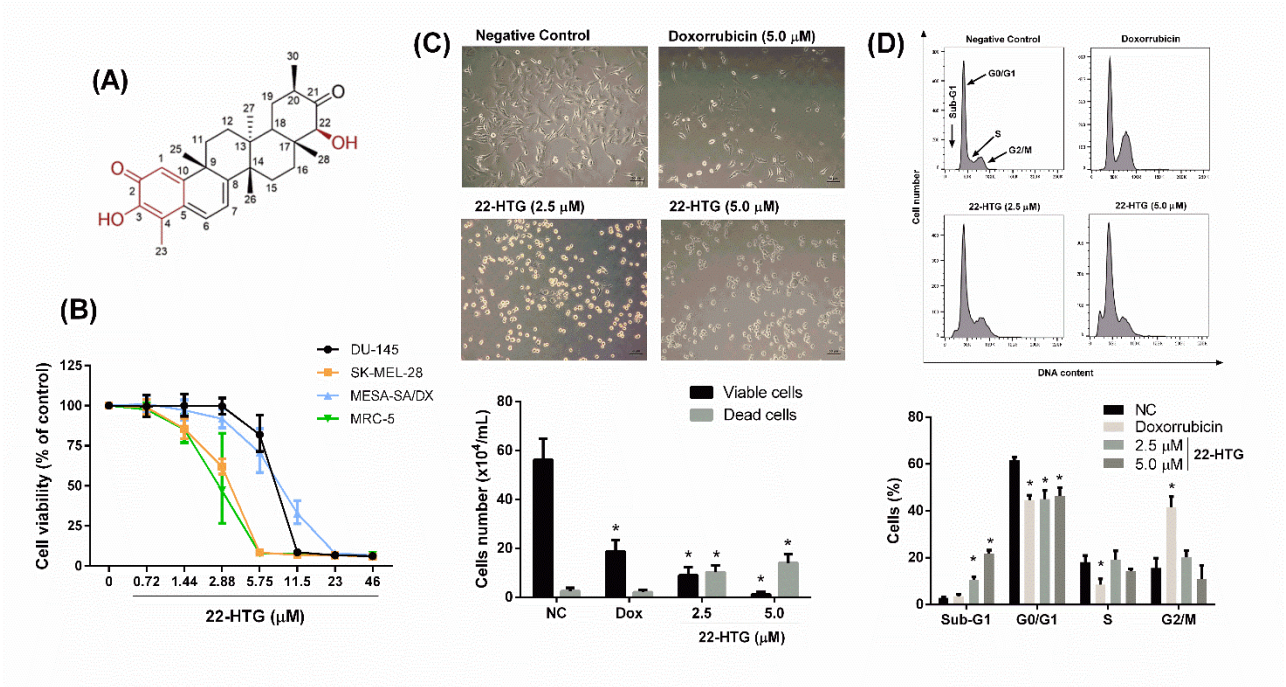

Figure 1. Inhibition of cell proliferation by treatment with 22*β*-hydroxytingenone (22-HTG). (A) Chemical structure of 22-HTG. (B) Cell survival curves obtained by Alamar blue assay after 72 h of incubation. Cancer cells: human prostate cancer cell line (DU 145); human malignant melanoma (SK-MEL-28); human doxorubicin-resistant uterine sarcoma cells (MES-SA/DX). Noncancerous cells: MRC- 5 (human lung fibroblast). (C) Effect of 22-HTG on cell viability of SK-MEL-28 cells after 24 h of exposure. (D) Cell cycle distribution in SK-MEL-28 cells after 24 h treatment with 22-HTG. The images and values represent results from three independent experiments, mean ± SD. *p<0.05 was considered significant when compared to the negative control (NC). Dox- Doxorrubicin (5.0 μM) was used as the positive control.

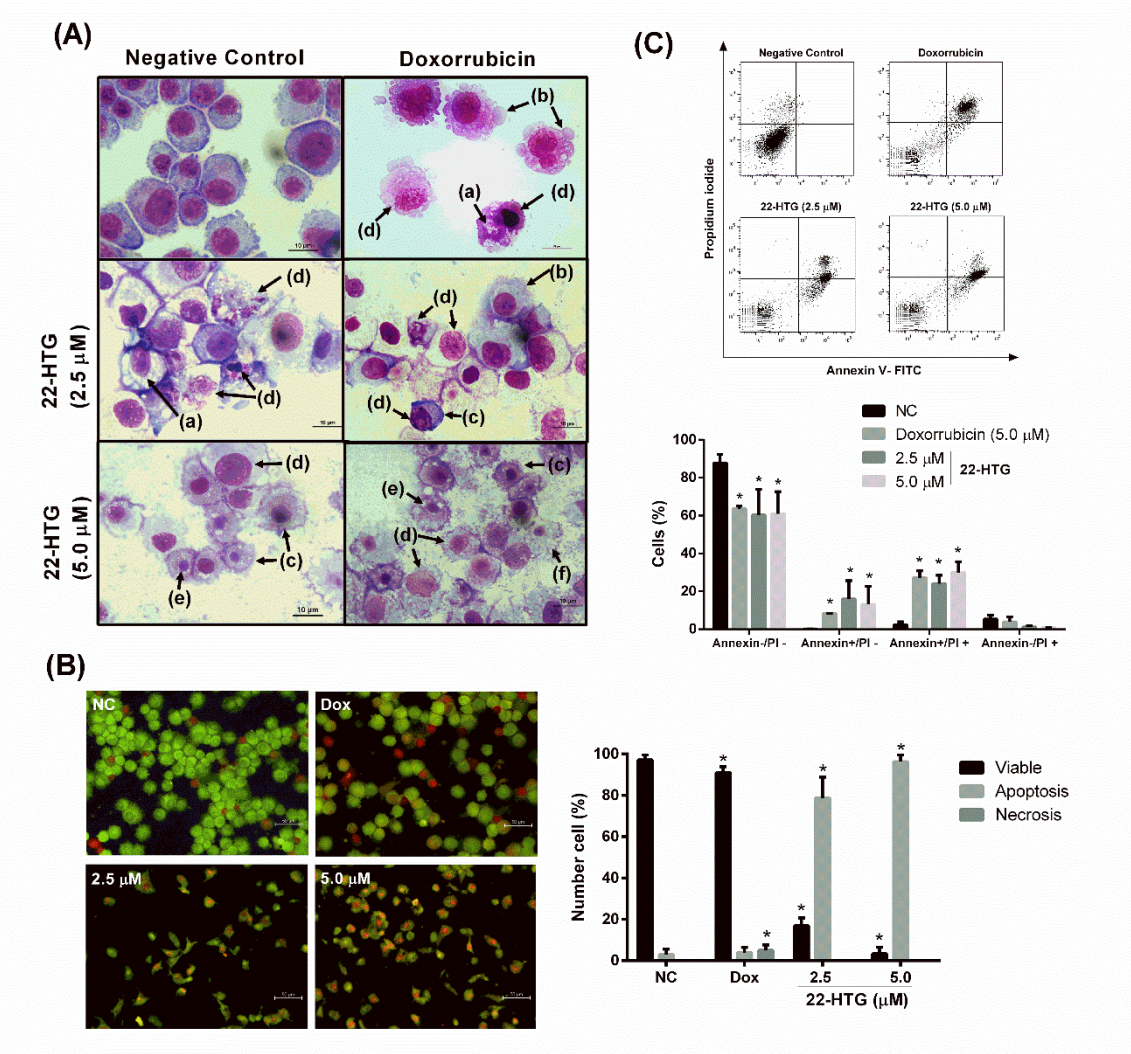

Figure 2. Induction of apoptosis by 22*β*-hydroxytingenone (22-HTG). (A) Morphological changes in SK-MEL-28 cells after exposure to 22-HTG (2.5 and 5.0 µM) for 24 h by the rapid panoptic dye. The images demonstrate (a) intracellular vacuole, (b) plasma membrane irregularities, (c) cell retraction, (d) condensation and nuclear fragmentation, (e) cell volume reduction, and (f) instability of the plasma membrane, at a magnification of 1000x. (B) Detection of apoptosis using Aridine Orange/ Ethidium bromide staining in SK-MEL-28 cells. After 24 h of treatment with 22-HTG, cells were analyzed by fluorescence microscopic as green, green and red arrows, and red as representative viable, apoptotic and necrotic cells respectively. (C) 22-HTG increased apoptotic cells in human melanoma cells. Percentage of apoptotic cells after 24 h treatment with 22-HTG corresponding to viable and non-apoptotic (Annexin-/ PI-), early apoptotic (Annexin+/ PI-), late apoptotic (Annexin+/ PI+) and necrosis cells (Annexin-/ PI+). Results are expressed as mean ± SD of three independent experiments. *$p<0.05$ was considered significant when compared to the negative control (NC). PI- Propidium iodide. Doxorrubicin (5.0 µM) was used as the positive control.

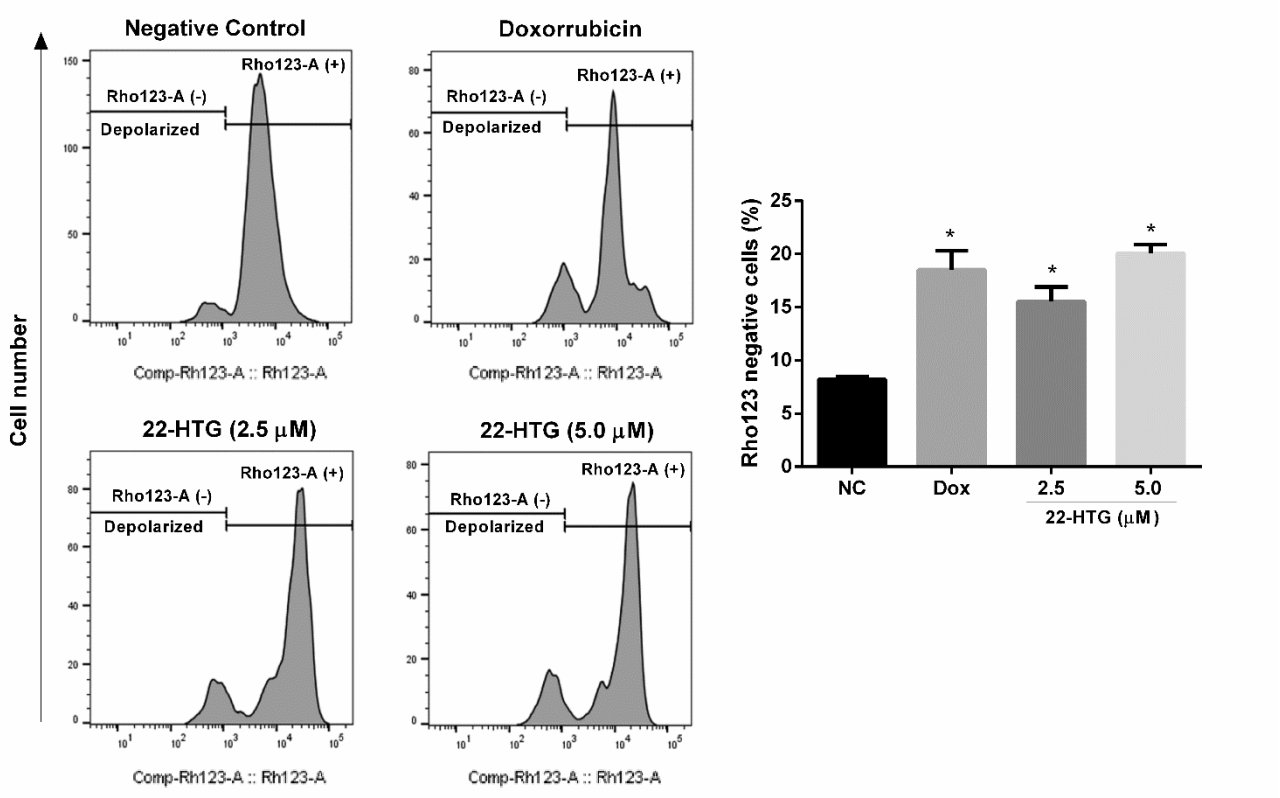

Figure 3. Mitochondrial membrane potential of SK-MEL-28 cells using rhodamine123 after 24 h of treatment with 22-HTG. Results are expressed as mean ± SD of three independent experiments. *p<0.05 was considered significant when compared to the negative control (NC). Rho123- Rhodamine 123. Dox- Doxorrubicin (5.0 μM) was used as the positive control.

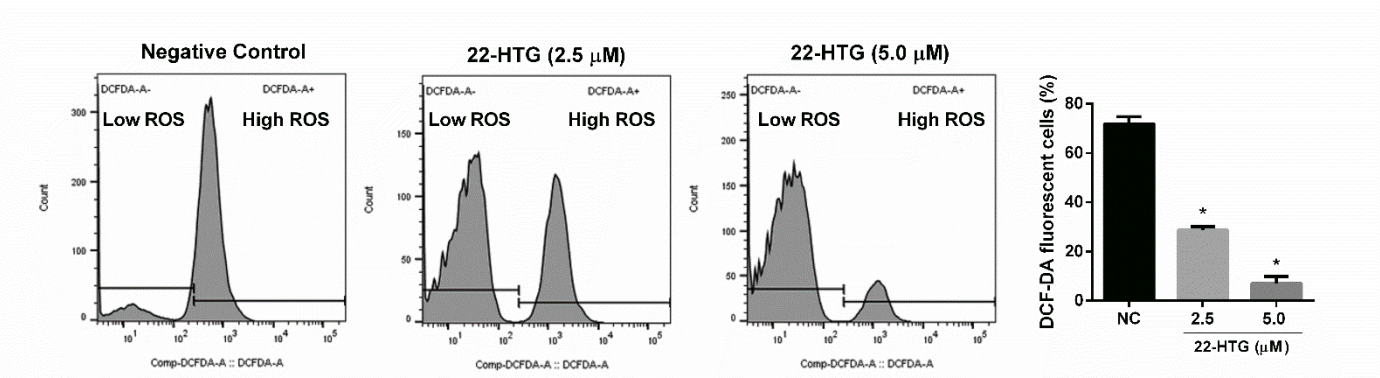

Figure 4. Detection of intracellular Reactive Oxygen Species (ROS) level in SK-MEL-28 cells after 3 h of treatment with 22-HTG. ROS level was observed using DCFH-DA fluorescence staining procedure through flow cytometry. Results are expressed as mean ± SD of three independent experiments. *$p<0.05$ was considered significant when compared to the negative control (NC).

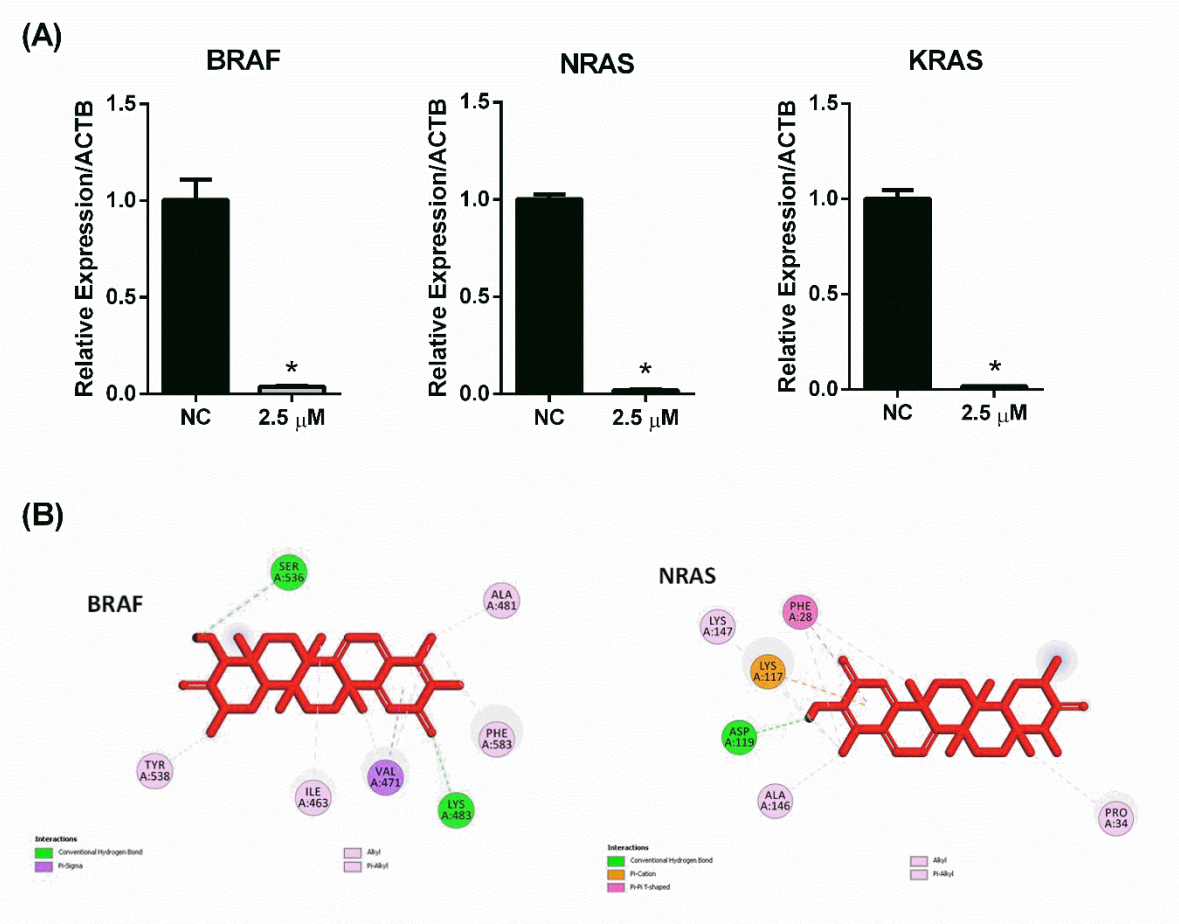

Figure 5. Genic expression in vitro in SK-MEL-28 cells and molecular docking. **(A)** Expression relative of *BRAF*, *NRAS*, and *KRAS* genes in SK-MEL-28 cell line after treatment with 22-HTG (2.5 μM) at 24 h. Relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ method and the actin beta (ACTB) gene was used as the internal control. **(B)** General interactions for 22-HTG against BRAF and NRAS. Results are expressed as the mean ± SD of three independent experiments. *$p<0.05$ was considered significant when compared to the negative control (NC).

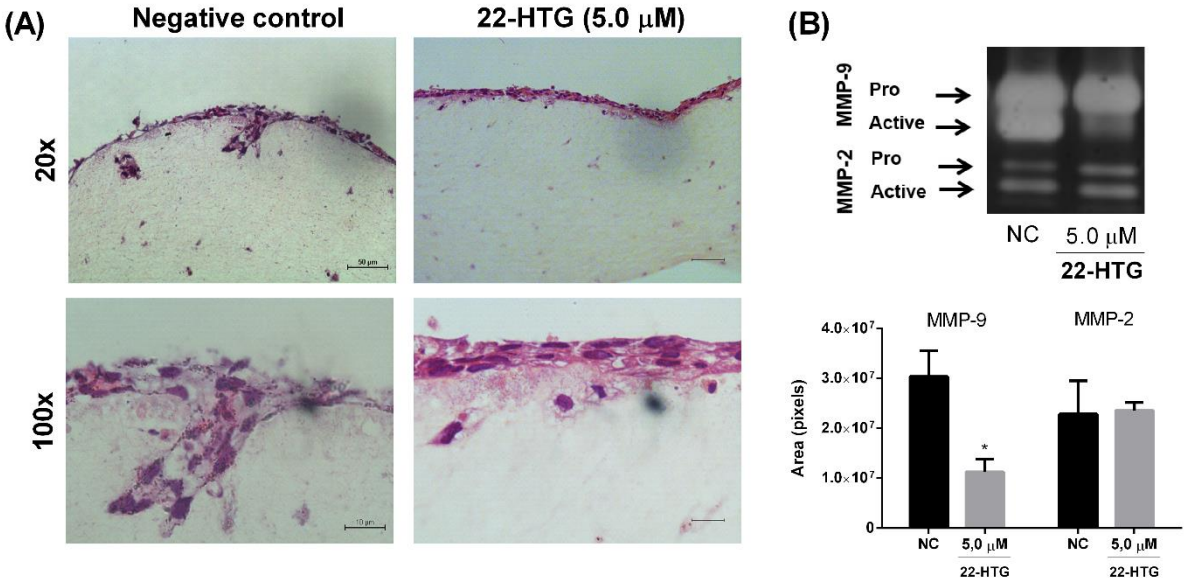

Figure 6. Decreased invasive potential of SK-MEL-28 cells induced by 22*β*-hidroxytingenone (22-HTG) using 3D model. (A) Reconstructed skin with SK-MEL-28 melanoma cells untreated and treated with 5.0 μM of 22-HTG for 72 h. The images shown hematoxylin/eosin staining (20x and 100x magnification). (B) Proteolytic action of MMP-2 and MMP-9 using reconstructed skin model with SK-MEL-28 melanoma cells after treatment with 22-HTG (5.0 μM). The activity of the enzymes appeared through the light bands, indicating degradation of the substrate. Results are expressed as the mean ± SD of three independent experiments. *$p<0.05$ was considered significant when compared to the negative control (NC).

7. DADOS SUPLEMENTARES

Outras informações relevantes obtidas usando 22*β*-hidroxitingenona (22-HTG):

7. Predição *in silico*

7.1 – Propriedades farmacocinéticas e toxicológicas - admetSAR

O perfil farmacocinético e toxicológico de 22-HTG foi obtido através software gratuito admetSAR (http://lmmd.ecust.edu.cn/admetsar2/).

O estudo de novas substâncias ativas com potencial efeito biológico e candidatos a novos fármacos deve envolver a investigação de características importantes, como: absorção oral, distribuição corporal, metabolismo, excreção, baixa toxicidade, além de sua atividade farmacológica. Nesse sentido, as ferramentas de triagem virtual se tornam importantes no desenvolvimento de medicamentos, pois permitem a previsão desses parâmetros com ajuda de softwares especializados (NASTASA et al., 2019).

A análise da predição das propriedades ADMET para 22-HTG é mostrada na tabela 1. De acordo com a predição, 22-HTG possui parâmetros positivos para: absorção intestinal, permeabilidade a Caco-2, biodisponibilidade oral. É possível que 22-HTG possua boa absorção, o que é importante para administração oral. Através do valor de logS (-3,602) é possível predizer uma boa solubilidade em água, uma vez que valores de logS entre -1 e -5 são encontrados para a maioria dos fármacos e caracteriza a polaridade necessária para a solubilidade aquosa e o caráter lipofílico adequado para a passagem através da membrana (JORGENSEN; DUFFY, 2002). Outros parâmetros preditos mostram que não existe promiscuidade inibitória de enzimas do citrocromo P450, uma vez que 22-HTG não inibe e não é substrato de CYP3A4, CYP26A, CYP2D6, CYP2C9, CYP2C19 e CYP1A2. A porcentagem de ligação a proteínas plasmáticas é de 95,4%, o que significa que apenas 4,6% da fração livre pode exercer o efeito farmacológico, no entanto a porção ligada pode atuar como um reservatório ou depósito a partir do qual a droga pode ser liberada lentamente (BELHASSAN et al., 2019).

Em relação as informações toxicológicas, os resultados mostram hepatotoxicidade negativa, assim como para corrosão e irritação dos olhos. A toxicidade oral aguda é considerada moderada, de acordo com resolução da diretoria colegiada (RDC) nº 294, de 29 de julho de 2019- anexo IV/ seção 1, a qual dispõe sobre os critérios para avaliação e classificação toxicológica (BRASIL, 2019). De acordo com o previsto, 22-HTG pode não induzir carcinogênese ou a formação de micronúcleos, além de apresentar toxicidade negativa no teste de Ames, o qual é um ensaio amplamente utilizado para avaliação de agentes

mutagênicos (KAUFFMANN et al., 2020). Entretanto, de acordo com a predição existe risco de inibição dos canais de potássio *either-a-go-go* (HERG- *Human ether-a-go-go related gene channel*), envolvidos no controle da atividade elétrica do coração. É importante ressaltar que todos os parâmetros mencionados são previstos.

A análise em conjunto dos dados gerados pelo admetSAR permite concluir que 22-HTG apresenta boas propriedades farmacocinéticas, mas algumas limitações devem ser consideradas para garantir o efeito contra o melanoma, mas também o uso racional e seguro.

Tabela 1. Perfil de predição ADMETsar para estudo de propriedades farmacocinéticas e toxicológicas *in silico* usando a substância 22*β*-hidroxitingenona (22-HTG).

Parâmetros analisados	**22-HTG**
Absorção intestinal humana	+
Caco-2	+
Barreira hematoencefálica	-
Biodisponibilidade oral	+
Ligação a proteínas plasmáticas (%)	95,4
Glicoproteína-P (inibição)	-
Glicoproteína-P (substrato)	-
CYP3A4 (substrato)	+
CYP26A (substrato)	-
CYP2D6 (substrato)	-
CYP3A4 (inibição)	-
CYP2C9 (inibição)	-
CYP2C19 (inibição)	-
CYP2D6 (inibição)	-
CYP1A2 (inibição)	-
CYP- promiscuidade de inibição	-
Canais de potássio *either-a-go-go* (inibição)	+
Irritação e corrosão dos olhos	-
Carcinogenicidade	-
Mutagênese (Ames)	-
Micronúcleo	-
Hepatotoxicidade	-
Toxicidade oral aguda	III
Solubilidade em água (logS)	-3,602

7.2 – Análise dos alvos moleculares de 22-HTG

Os possíveis alvos moleculares de 22-HTG foram avaliados usando a ferramenta computacional SwissTargetPrediction (http://www.swisstargetprediction.ch/).

Cada composto natural pode ter vários alvos moleculares para exercer seus efeitos farmacológicos. Técnicas computacionais podem ser empregadas para prever os alvos de um determinado fitoquímico (LIU et al., 2017a). Para ajudar a explorar os efeitos de 22-HTG contra células de melanoma, investigamos os seus potenciais alvos moleculares usando a ferramenta *SwissTargetPrediction*. Foram identificados treze alvos moleculares de acordo com a estrutura química de 22-HTG. Desse total, para seis dos alvos preditos foram encontrados dados na literatura que mencionam o envolvimento com a progressão do melanoma (Tabela 2). Dentre esses alvos identificados para o melanoma, destaca-se o gene PTPN11 (*Tyrosine-protein phosphatase non-receptor type 11*), o qual codifica a proteína tirosina fosfatase SHP2, a qual participa da ativação da via RAS/ RAF/ MAPK (HILL et al., 2019).

O gene PTPN11 é frequentemente ativado em amostras de melanoma humano e também em linhagens celulares, sendo considerado um gene necessário para a ativação completa da sinalização da via MAPK (HILL et al., 2019). Assim, a inibição de PTPN11 bloqueia a sinalização nessa via e impede eventos celulares como: sobrevivência, proliferação, migração, invasão e indução de apoptose (PALMIERI et al., 2015; PRAHALLAD et al., 2015). Sendo assim, a informação que PTPN11 é um alvo molecular de 22-HTG na predição *in silico*, pode ser relacionada a inibição da expressão gênica de BRAF, NRAS e KRAS demonstrada no Capítulo II deste estudo, o que em conjunto pode ajudar a explicar os efeitos na redução da proliferação, migração, invasão celular e indução de apoptose causados pela exposição à 22-HTG contra células de melanoma.

Tabela 2. Potenciais alvos moleculares da substância 22β-hidroxitingenona.

Gene	Nome do alvo	Probabilidade	Melanoma	Referência
AKR1B10	Aldo-ceto redutase família 1 (B10)	98%	-	-
AKR1B1	Aldose redutase	98%	-	-
HSF1	Fator de choque térmico 1	58%	Sim	Nakamura, et al. 2014; Cho and Grim, 2015; Toma-Jonik, et al. 2015
PTPN11	Tirosina fosfatase não-receptor tipo 11	58%	Sim	Hill, et al. 2019; Oliveira; Dalmaz; Zeidán-Chuliá, 2018; Prahallad, et al. 2015
TLR9	Receptor do tipo Toll 9	58%	-	-
PTPN6	Tirosina fosfatase- não receptor do tipo 6	58%	-	-
AR	Receptor andrógeno	28%	Sim	Wang, et al. 2016; Schmidt, et al. 2016; Schmidt, et al. 2019
ESR1	Receptor de estrogênio alfa	25%	Sim	Mori et al., 2006
ESR2	Receptor de estrogênio beta	25%	Sim	Marzagalli et al., 2016; Giorgi, et la. 2014;
CYP17A1	Citocromo P450 17α-hidroxilase-C17,20-liase	23%	-	-
SHBG	Globulina ligadora de hormônios sexuais	20%	-	-
NR3C1	Receptor de glicocorticóides	14%	Sim	Huang, et al., 2017; Lai et al., 2013
PGR	Receptor de progesterona	14%	-	-

8. CONCLUSÕES GERAIS

- Foi demonstrado que 22*β*-hidroxitingenona possui efeito antitumoral usando células de melanoma humano, reduzindo a proliferação, migração, invasão celular e atividade de metaloproteinases.
- A exposição a 22*β*-hidroxitingenona provocou a indução de morte celular por apoptose, causando dano mitocondrial e interferência da via de sinalização celular MAPK através da redução da expressão de genes BRAF, NRAS e KRAS.
- Usando um modelo 3D de pele artificial, no qual é possível simular um ambiente mais complexo de cultura células, o tratamento com 22*β*-hidroxitingenona reduziu a invasão de células de melanoma na derme, evidenciando o potencial antimetástático da substância.
- O presente trabalho é o primeiro a descrever a atividade da substância 22*β*-hidroxitingenona contra células de melanoma humano.

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ANEXO

Journal of Ethnopharmacology

22β-hydroxytingenone induces apoptosis and suppresses invasiveness of melanoma cells by inhibiting MMP-9 activity and MAPK signaling

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	SK-MEL-28, Apoptosis, Invasion, Quinonemethide triterpenes
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Abstract:	Ethnopharmacological relevance: 22β-hydroxytingenone (22-HTG) is a quinonemethide triterpene isolated of Salacia impressifolia (Miers) A. C. Smith belong to the family Celastraceae, which has been used used for medicinal purposes in traditional medicine. However, the anticancer effects of 22-HTG and the underlying molecular mechanisms in melanoma cells have not been elucidated. Aim of the study: The present study investigated apoptosis induction and antimetastatic potencial of 22-HTG in SK-MEL-28 human melanoma cells. Materials and Methods: First, the in vitro cytotoxic activity of 22-HTG in cultured cancer cells was evaluated. Then, cell viability was determined by the trypan blue assay in melanoma cells (SK-MEL-28), following cell cycle, annexin V-FITC/propidium iodide assays (Annexin/PI), mitochondrial membrane potential, production of reactive oxygen species (ROS) by flow cytometry, and fluorescence microscopy with acridine orange/ethidium bromide assay (AO/BE) staining. RT-qPCR was performed to evaluate the expression of BRAF, NRAS, and NRAS genes and docking mocelular was executed. The anti-invasiveness potential of 22-HTG was evaluated in a three-dimensional (3D) model of reconstructed human skin. Results: 22-HTG reduced viability of SK-MEL-28 cells and caused morphological changes, as cell shrinkage, chromatin condensation, and nuclear fragmentation. Furthermore, 22-HTG caused apoptosis demonstrated by increased stained with AO/BE and Annexin/PI. Apoptosis may have been caused by mitochondrial instability without the involvement of ROS production. Expression of BRAF, NRAS, and KRAS, important biomarkers in melanoma development, was reduced by 22-HTG treatment and was strong binding affinity with BRAF and NRAS in molecular docking. In reconstructed skin model, 22-HTG was able to decrease capacity of melanoma cells to invade into the dermis. Conclusions: Our data indicate that 22-HTG has anti-tumorigenic properties in melanoma cells by induction of cell cycle arrest, apoptosis and inhibition of invasiveness potential in 3D model, and provide new insights for future work on