TATIANE RENATA FAGUNDES

AVALIAÇÃO *IN VITRO* DA ATIVIDADE ANTITUMORAL DO ÁCIDO GRANDIFLORÊNICO E TIOIDANTOÍNAS SOB AS LINHAGENS CELULARES MCF-7, HuH7.5 E A549

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Tese apresentada ao programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de doutor em Patologia Experimental.

Orientador: Prof. Dr. Wander R. Pavanelli
Co-orientadora: Profª Dra. Carolina Panis

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Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota.

Madre Teresa de Calcutá

FAGUNDES, TATIANE R**. Avaliação *in vitro* da atividade antitumoral do ácido grandiflorênico e tioidantoínas sob as linhagens celulares MCF-7, HuH7.5 e A549.** 2018. 78 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

O câncer é a segunda principal causa de morte no mundo, e estima-se qye seja responsável por 9,6 milhões de mortes em 2018. Dentre os fatores limitantes do tratamento dos tumores malignos com medicamentos é a resistência e a baixa eficácia das terapias disponíveis, além dos efeitos colaterais que se estendem também a todo tipo celular em divisão. As tioidantoínas são compostos sintéticos que apresentam efeitos biológicos, tais como: antiparasitários, antivirais, antitumorais, antioxidantes, anti-inflamatória. O ácido grandiflorênico é um diterpeno, extraído da parte aérea da planta *Sphagneticola trilobata,* e já descrito por suas ações anti-inflamatórias, antileishmanicida, antibacteriana e antitumoral. Considerando novas abordagens para o tratamento do câncer, pesquisamos o efeito desses compostos nas linhagens celulares tumorais de mama (MCF-7), de pulmão (A549) e de fígado (HuH7.5), bem como os prováveis mecanismos de morte envolvidos.

As tioidantoínas foram testadas em diferentes concentrações (25, 50 e 100 µM), através de ensaios de citotoxicidade pelo MTT, vermelho neutro e azul de tripan, sob as celulas MCF-7 e hemácias ovinas. A avaliação do volume celular e análise do ciclo celular foi realizada por citometria de fluxo, e análises de fluorescência para determinação do mecanismo de morte induzido pelas tioidantoínas. Três experimentos independentes foram realizados, cada um em triplicata. O tratamento com as tioidantoínas promoveu a morte das células tumorais de mama MCF-7, porém não causou hemólise em hemácias ovinas. O efeito citotóxico resultou no aumento da produção de ROS, e parada do ciclo celular na fase G_1/G_0, com redução do volume celular, perda de integridade de membrana, despolarização mitocondrial e aumento da fluorescência para anexina, indicando morte celular por apoptose, e aumento da formação de vacúolos autofágicos.

O GFA *in vitro* foi avaliado quanto à citotoxicidade através do teste de avaliação mitocondrial do MTT, vermelho neutro e azul de tripan, nas linhagens tumorais MCF-7 (câncer de mama), A549 (câncer de pulmão) e HuH7.5 (câncer de fígado). A avaliação do volume celular foi realizada por citometria de fluxo, e análises de fluorescência para determinação do mecanismo de morte, através da exposição de fosfatidilserina, integridade de membrana, e formação de vacúolos autofágicos. Foi avaliada ainda a possível indução da formação de espécies reativas de oxigênio por fluorescência. O tratamento com GFA inibiu significativamente a proliferação celular nas três linhagens estudadas, que foi acompanhada de diminuição do volume celular, principalmente na concentração de 400 nM. A avaliação dos mecanismos de morte mostrou que os tratamentos aumentaram: a produção de ROS, a exposição de folfatidilserina, a despolarização de membrana mitocondrial, e formação de vacúolos autofágicos.

Observamos que os compostos estudados se mostraram citotóxicos para as linhagens tumorais, alcançando uma indução de apoptose e autofagia nessas células, em proporções maiores que nas células normais testadas, demonstrando

que os dois compostos, devem ser melhores estudados como candidatos à terapia antineoplásica.

Palavras-chave: Ácido grandiflorênico. Tioidantoínas. Câncer de mama. Câncer de fígado. Câncer de pulmão.

FAGUNDES, TATIANE R**. In vitro evaluation of the antitumor activity of grandiflorenic acid and thiohydantoins under the MCF-7, HuH7.5 and A549 cell lines.** 2018. 75 p. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2018.

ABSTRACT

Cancer is one of the leading causes of death worldwide, and the limiting factors for the treatment of malignant tumors is the resistance and low efficacy of the available therapies, as well as the side effects that also extend to every type of cell division. Thioidantoins are synthetic compounds that have biological effects, such as: antiparasitic, antiviral, antitumor, antioxidant, anti-inflammatory and anticarcinogenic. The grandiflorenic acid is a diterpene, extracted from the aerial part of the plant *Sphagneticola trilobata*, and already described by its anti-inflammatory, antileishmanicida, antibacterial and antitumoral actions. Considering new approaches to cancer treatment, we investigated the effect of these compounds on the tumor cell lines of breast (MCF-7), lung (A549) and liver (HuH7.5), as well as the probable mechanisms of death involved.

Thioidantoins were tested at different concentrations (25, 50 and 100 μM) by MTT, neutral red and trypan blue cytotoxicity assays under MCF-7 cells and ovine red blood cells. Evaluation of cell volume and cell cycle analysis was performed by flow cytometry, and fluorescence analysis to determine the mechanism of death induced by thioidantoins. Three independent experiments were performed, each in triplicate. Treatment with thioidantoins promoted the death of MCF-7 breast tumor cells, but did not cause hemolysis in ovine red blood cells. The cytotoxic effect resulted in increased ROS production and cell cycle arrest in the G1 / G0 phase, with reduction of cell volume, loss of membrane integrity, mitochondrial depolarization and increased fluorescence to annexin, indicating cell death by apoptosis, and increased formation of autophagic vacuoles.

GFA in vitro was evaluated for cytotoxicity through the MTT mitochondrial evaluation, neutral red and tripan blue, in MCF-7 (breast cancer), A549 (lung cancer) and HuH7.5 liver). Cell volume evaluation was performed by flow cytometry, and fluorescence analysis for determination of the mechanism of death, through phosphatidylserine exposure, membrane integrity, and autophagic vacuoles formation. The possible induction of the formation of reactive oxygen species by fluorescence was also evaluated. Treatment with GFA significantly inhibited cell proliferation in the three lines studied, which was accompanied by a decrease in cell volume, mainly at the concentration of 400 nM. The evaluation of the mechanisms of death showed that the treatments increased: ROS production, folphatidilserin exposure, mitochondrial membrane depolarization, and autophagic vacuoles formation.

We observed that the compounds studied were cytotoxic to the tumor cell lines, achieving an induction of apoptosis and autophagy in these cells, in proportions higher than the normal cells tested, demonstrating that the two compounds should be better studied as candidates for antineoplastic therapy.

Key words: Grandiflorenic acid. Thioidantoins. Breast cancer. Liver cancer. Lung cancer.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AC	Ácido caurenóico
AG	Ácido grandiflorênico
BRCA1	do inglês Breast cancer tipo 1
BRCA2	do inglês Breast cancer tipo 2
cDNA	Ácido desoxirribonucleico complementar
CHC	Carcinoma hepatocelular
c-Kit	Receptor tirosina quinase
COX2	Ciclo-oxigenase 2
ADN	Ácido desoxirribonucleico
EGFR	Receptor de crescimento epidermal
EROS	Espécies reativas de oxigênio
HER2	Receptor de crescimento epidermal humano do tipo 2
LPS	Lipopolissacarídeo
NSCLC	Carcinoma de pulmão de células não pequenas
PDGF	Fator de crescimento derivado de plaquetas
PGE2	Prostaglandina E2
RAF	Proteína Serina/treonina quinase
RE	Receptor de estrógeno
RP	Receptor de progesterona
SCLC	Câncer de pulmão de c
VEGF	Fator de crescimento vascular epidermal

SUMÁRIO

1 INTRODUÇÃO

1.1 Aspectos gerais do câncer

O câncer é uma doença genética cuja evolução conduz a mutações no DNA, resultando no crescimento anormal de células, e em alguns casos tem o poder de invadir tecidos adjacentes e à distância, além de sofrerem transformações até se tornarem malignas, apresentando uma desestabilização inespecífica de múltiplas vias moleculares, associados à sua iniciação e progressão (PLANKAR; JERMAN; KRAŠOVEC, 2011).

Atualmente são conhecidos mais de cem tipos e subtipos de câncer, diferenciados pela etiologia, processo de evolução e forma de tratamento e, por isso, o câncer não pode mais ser considerado como uma patologia única (BRENTANI; COELHO; KOWALSKI, 2003).

A célula cancerígena caracteriza-se pela perda de função em consequência da ausência de diferenciação, proliferação descontrolada, invasividade dos tecidos adjacentes e metástase. A origem dessa célula é consequência de alterações genéticas que podem ser produzidas por diversos mecanismos como a inativação de genes supressores de tumor, ativação de oncogenes, inativação de genes responsáveis pela apoptose e mutações produzidas por agentes químicos, físicos e biológicos (BRENTANI; COELHO; KOWALSKI, 2003; BRUNTON; LAZO; PARKER, 1998)

Em 2018, A Organização Mundial de Saúde (OMS) prevê que 9,6 milhões de pessoas morrerão por câncer em todo o planeta, sendo que uma em cada seis mortes são relacionadas à doença. As formas mais comuns desta doença são os tumores de pulmão (2,09 milhões de casos), mama (2,09 milhões de casos), colorretal (1,8 milhões de casos) e fígado (788.000 de casos) (WHO- About the International Agency for Research on Cancer, 2017).

A existência de vários tipos de tumores com diferentes caracteristicas, variações genéticas e epigenéticas e desfechos clínicos, dificultam a compreensão dessa doença, os mecanismos de ação dos quimioterápicos e a criação de novas terapias (VARGO-GOGOLA; ROSEN, 2007). A resistência ao tratamento do câncer constitui um obstáculo importante para a melhora da sobrevida dos pacientes acometidos por essa doença (LORD; ASHWORTH, 2013).

Atualmente o tratamento com medicamentos tem baixa eficácia em virtude do mecanismo de ação que os fármacos supressores possuem, inibindo o crescimento ou matando as células malignas. A maior parte desses quimioterápicos são genotóxicos, produzindo dano ao DNA e mutações, que podem induzir a formação outras células neoplásicas. Os princípios ativos mais procurados são aqueles que induzem as células a apoptose, levando apenas células malignas à morte (SUBHASHINI; MAHIPAL; REDDANNA, 2005).

1.1.1 Câncer de mama

O câncer de mama é a neoplasia mais frequente entre as mulheres, considerado o segundo tipo mais frequente de neoplasia no mundo; no Brasil corresponde a 22% dos novos casos a cada ano, com estimativa de 59.700 novos casos para o ano de 2018 (MINISTÉRIO DA SAÚDE- BRASIL, 2018). Quando diagnosticado precocemente as chances de cura são elevadas (ESTEVA; HORTOBAGYI, 2004).

O carcinoma invasivo de mama é definido como um grupo de tumores epiteliais malignos caracterizados por invadir o tecido adjacente e ter alta tendência de metástases à distância (TAVASSOÉLI, F.A., DEVILEE, 2003). Caracterizam-se como neoplasias heterogêneas, com vários subtipos patológicos e diferentes aspectos histológicos, além de apresentações clínicas, e diferentes respostas ao tratamento (VAN ’T VEER et al., 2002).

Desde 2001 uma nova classificação para os carcinomas mamários tem sido adotada. Esta nova classificação leva em consideração aspectos moleculares da doença e vem mostrando um importante avanço a partir da identificação dos perfis de expressão gênica. Propostos por Perou e colaboradores (PEROU et al., 2000a), esta classificação é baseada em estudos com cDNA *microarrays* com mais de 8.000 genes humanos (PEROU et al., 2000a; SORLIE et al., 2003), resultando na classificação molecular dos subtipos tumorais que podem ser associados com parâmetros clínicos relevantes, como o tempo de sobrevida e o tempo livre de doença (BERTUCCI; BIRNBAUM; GONCALVES, 2006; NIELSEN et al., 2004; SORLIE et al., 2003; VIEIRA et al., 2008). Os subtipos moleculares utilizados na clínica são**:** luminal A, luminal B**,** *HER-2*, triplo negativo, mama normal e baixa claudina (EROLES et al., 2012).

O subtipo luminal A é o mais frequente, e constitui tumores malignos que revelam alta expressão de receptores de estrogênio (RE) e progesterona (RP), e baixos índices de genes relacionados à proliferação celular (BERTUCCI; BIRNBAUM; GONCALVES, 2006; SASCO, 2003).

O segundo subtipo é o luminal B; que está associado a pior prognóstico, sendo particularmente relacionado à recidiva tumoral, por apresentar possíveis similaridades com os tumores RE negativos, e pode ou não apresentar a amplificação do gene que codifica o *HER-2* (receptor do fator de crescimento epidermal humano 2) (SORLIE et al., 2003; SØRLIE et al., 2001). Também apresentam maiores taxas de proliferação celular, o que favorece o uso de quimioterápicos e hormonioterapia como tratamentos complementares ao tratamento cirúrgico (SORLIE et al., 2003). No grupo dos tumores luminais, a hormonioterapia, com os medicamentos Tamoxifeno, Anastrozol, Letrozol, constitui uma forma eficaz de terapêutica associada ou não ao tratamento cirúrgico (SØRLIE et al., 2001).

Os subtipos *HER-2* e triplo negativo, são relevantes pela sua frequência e evolução clínica, e possuem a característica em comum da não expressão de RE e RP. O primeiro se caracteriza por amplificação do oncogene *HER-2**,*** que está associado à maior agressividade clínica, com elevada taxa de proliferação celular, mas responde ao tratamento com medicamentos que bloqueiam a atividade do *HER-2*, como, por exemplo, o anticorpo monoclonal trastuzumab (BERTUCCI; BIRNBAUM; GONCALVES, 2006; PEROU et al., 2000a; SHAK, 1999).

O subtipo triplo negativo é representado por tumores com altos altos índices de proliferação celular, principalmente a via de sinalização *ki67* (CAREY et al., 2010), e não apresentam expressão imunohistoquímica de receptores de estrógeno, progesterona e HER-2 (SORLIE et al., 2003), e é um dos mais intrigantes subtipos tumorais, pois tem associação com pior prognóstico (DUNKLER; MICHIELS; SCHEMPER, 2007; TURNER; REIS-FILHO, 2006), e não possui alvo terapêutico definido, como os outros subtipos (BERTUCCI; BIRNBAUM; GONCALVES, 2006).

O subtipo de mama normal são tumores responsáveis por cerca de 5% a 10% de todos os carcinomas de mama. Eles são mal caracterizados e foram agrupados na classificação dos subtipos intrínsecos com fibroadenomas e amostras normais de mama (PEROU et al., 2000b), e expressam genes característicos do tecido adiposo, apresentando um prognóstico intermediário entre o luminal e o triplo negativo, sem a expressão de ER, PR e *HER-2*, e geralmente não respondem à quimioterapia

neoadjuvante (EROLES et al., 2012). Ainda restam dúvidas sobre sua real existência e alguns pesquisadores acreditam que podem ser um artefato técnico de alta contaminação com tecido normal durante os microarrays (WEIGELT et al., 2010).

O subtipo claudina- baixa é caracterizado por uma baixa expressão de genes envolvidos em junções e adesão intercelular, incluindo claudinas, cingulina, ocludina e E-caderina (HERSCHKOWITZ et al., 2007). Apresentam baixa expressão de *HER-2* e e possui características do subtipo luminal, e superexpressam ainda um conjunto de 40 genes relacionados à resposta imune, indicando uma alta infiltração de células do sistema imunológico de tumores (PRAT et al., 2010). Esses tumores apresentamum prognóstico ruim, embora apresentem uma baixa expressão de genes relacionados à proliferação celular, e superexpressam um subconjunto de genes intimamente ligados à diferenciação mesenquimal e transição epitelial-mesenquimal, que estão associados com a aquisição de fenótipo de células-tronco de câncer (EROLES et al., 2012).

A predisposição genética é responsável por cerca de 10% dos casos de câncer de mama. Os genes *breast cancer* 1 (BRCA1) e *breast cancer* 2 (BRCA2) estão relacionados com o desenvolvimento de doença em mulheres jovens (KOCH, HILTON AUGUST; PEIXOTO, JOAO EMILIO; NEVES, 2000). Para os demais casos, onde não há predisposição genética, muitos fatores como o envelhecimento da população, localização geográfica, etnia e sobrepeso estão relacionados com o aumento de risco (KOCH, HILTON AUGUST; PEIXOTO, JOAO EMILIO; NEVES, 2000; MINISTÉRIO DA SAÚDE- BRASIL, 2018). Além disso, doenças benignas de mama, menarca precoce, menopausa tardia, nuliparidade, uso de contraceptivos orais (principalmente se houver a combinação de progesterona e estrogênio) e primeiro parto em idade avançada também são considerados fatores de risco (KOCH, HILTON AUGUST; PEIXOTO, JOAO EMILIO; NEVES, 2000).

De um modo geral pouco se conhece sobre possíveis métodos de prevenção da doença, de forma que o rastreamento, seja pelo auto-exame da mama e acompanhamento médico, constitui a melhor forma de identificar precocemente a doença, melhorando o prognóstico, facilitando o tratamento e aumentando a sobrevida das pacientes.

A conduta habitual no tratamento consiste de cirurgia, seguida por radioterapia. O tratamento sistêmico é determinado de acordo com o risco de recorrência (idade da paciente, comprometimento linfonodal, tamanho tumoral, grau de diferenciação),

assim como marcadores moleculares que irão ditar a terapia mais apropriada (MINISTÉRIO DA SAÚDE- BRASIL, 2018). O fenótipo da célula tumoral auxilia na escolha da terapia. Em vários países, a quimioterapia padrão adotada para o tratamento do câncer de mama em estágios iniciais é a combinação de antraciclinas, como a doxorrubicina, com taxanos de forma simultânea ou sequencial (WARDLEY, 2006).

O termo terapia alvo tem sido empregado para descrever novos fármacos visam marcadores moleculares específicos, como moduladores de RE (tamoxifeno), inibição da produção sistêmica do hormônio (anastrozol) (REIS-FILHO; WESTBURY; PIERGA, 2006). A progesterona também participa da patogenia do câncer de mama, muitos tipos de carcinomas além de RE também expressam RP, e por isso também tem sido desenvolvidos moduladores seletivos para RP (REIS-FILHO; WESTBURY; PIERGA, 2006). O traztuzumab usado no tratamento de tumores *HER-2*, melhora a sobrevida de mulheres acometidas por esse tumor, porém muitos efeitos colaterais são relatados, e uma das complicações mais significativas é seu efeito no coração e está associada a disfunção cardíaca em 2% a 7% dos casos (TRIULZI et al., 2018).

A carência de terapias direcionadas ao tratamento do câncer de mama triplo negativo é realidade devido à dificuldade de alvos específicos da via, e esses pacientes são tratados com quimioterapia convencional (CETIN; TOPCUL, 2014)

De um modo geral, embora existam muitas opções terapêuticas para o câncer de mama, as pacientes necessitam de opções menos tóxicas, que reduzam os riscos de progressão da doença, e diminuam o aparecimento de células tumorais resistentes aos medicamentos utilizados.

1.1.2 Câncer de pulmão

O câncer de pulmão é a causa mais comum de mortalidade entre todos os tumores malignos, além de possuir grande incidência entre homens e mulheres (TRAVIS, 2011). No Brasil são estimados para o ano de 2018, 30.000 novos casos, sendo 18.740 em homens e 12.530 em mulheres (MINISTÉRIO DA SAÚDE-BRASIL, 2018).

A literatura classifica os tumores pulmonares em dois subtipos mais importantes, o carcinoma de pulmão de células não pequenas (NSCLC), que representa qualquer tipo de câncer de pulmão epitelial, acometendo 85% dos

indivíduos, e o carcinoma de pulmão de pequenas células (SCLC), que abrange todos os 13 a 15% restantes (CARTER et al., 2014; KURIBAYASHI; FUNAGUCHI; NAKANO, 2016; TRAVIS, 2011).

A relação causal entre o hábito de fumar e câncer de pulmão foi estabelecida por estudos epidemiológicos, realizados nas décadas de 50 e 60. O risco de câncer de pulmão na população de fumantes é dezessete vezes maior em homens e onze vezes maior em mulheres, quando comparados com não fumantes (DELA CRUZ; TANOUE; MATTHAY, 2011; MOLINA et al., 2008; SUN; SCHILLER; GAZDAR, 2007; XU et al., 2012). Além disso, outros fatores estão associados com a evolução desta doença, dentre eles, os fatores genéticos e ambientais, como exposição à compostos químicos como o asbesto, arsênico, cádmio, hidrocarbonetos, gás mostarda, entre outros, ainda mais quando associados ao hábito de fumar (DELA CRUZ; TANOUE; MATTHAY, 2011; SUN; SCHILLER; GAZDAR, 2007).

O tratamento para pacientes com NSCLC é individualizado, podendo ser curativo ou paliativo, devido à baixa eficácia terapêutica (DELA CRUZ; TANOUE; MATTHAY, 2011). Já os pacientes com SCLC na maioria das vezes têm a doença disseminada no momento do diagnóstico, pois esse tipo possui uma alta malignidade e agressividade, e fraca diferenciação. Com a falta de tratamento, a sobrevida é de cerca de 6 a 12 semanas para aqueles com doença em estágio extenso e cerca de 3 a 6 meses para aqueles com doença em estágio limitado (SUN; SCHILLER; GAZDAR, 2007).

Os fármacos usados tradicionalmente no tratamento do câncer de pulmão são baseados principalmente em uma combinação dos medicamentos gemcitabina, carboplatina, cisplatina, irinotecano, e etoposídeo (CACCIOLA et al., 2015; WANG et al., 2016; XIAO et al., 2014). Embora o Gefitinib (Iressa), inibidor de EGFR (receptor do fator de crescimento epidérmico), um importante alvo molecular para o câncer de pulmão, apresentar um aumento na taxa de sobrevivência dos doentes com NSCLC avançado, sua eficácia é limitada, além de efeitos adversos como pneumonite intersticial (CACCIOLA et al., 2015; LIU; WANG, 2015; TANG et al., 2011; XIAO et al., 2014).

1.1.3 Câncer de fígado

O carcinoma hepatocelular (CHC) é uma neoplasia com prevalência crescente, e a terceira principal causa de morte relacionada ao câncer no mundo

(BOSCH et al., 2004; JEMAL et al., 2011). É um dos tipos de tumores mais difíceis de tratar, e as atuais estratégias terapêuticas destinadas a induzir a apoptose não são eficazes o suficiente para eliminar completamente o tumor (BERGAMASCHI et al., 2003).

Ao contrário de outros tumores sólidos, a maioria dos CHC evoluem de doenças hepáticas crônicas, especialmente associada ao fígado, como cirrose resultante da hepatite viral ou do consumo de etanol (BAFFY; BRUNT; CALDWELL, 2012; EL–SERAG; RUDOLPH, 2007). Mesmo se o diagnóstico for realizado precocemente, e realizados tratamentos com quimioterapia adjuvante, cirurgias e até mesmo transplante de fígado, a cura é difícil por conta da alta taxa de recorrência ou metástase dentro de 5 anos após o tratamento nesses pacientes (LLOVET et al., 2008).

Quando o CHC é detectado no estágio inicial, o transplante hepático é a terapia mais eficaz, pois permite o tratamento simultâneo do câncer e da doença associada; no entanto com a falta de doadores disponíveis e às diminutas compatibilidades entre dador e doente, esta terapia torna-se útil em apenas 5% dos casos (LLOVET et al., 2008).

Em relação aos fármacos, o primeiro agente testado no tratamento do CHC foi o antimetabólito fluorouracil, porém os melhores resultados encontrados foram obtidos com o medicamento doxorubicina, o qual apresentou taxas de resposta entre 10-30%, e o sorafenibe, um inibidor de angiogênese e tirosinoquinases, que atua bloqueando a sinalização de PDGF (fator de crescimento derivado de plaquetas), VEGF (fator de crescimento do endotélio vascular), c-kit e RAF tanto nas células tumorais quanto nas células endoteliais circundantes, podendo levar a efeitos colaterais como problemas no fluxo sanguíneo do coração e perfurações no estômago e intestinos (LIU et al., 2006). Algumas combinações de fármacos também têm sido estudadas, como os esquemas de doxorrubicina associado a sorafenibe, bevacizumabe e erlotinibe, dentre outras, apresentando baixos níveis de resposta (SANTOS et al., 2018).

1.2 Resistência a quimioterápicos

Os primeiros estudos significativos a respeito dos quimioterápicos antineoplasicos surgiram na década de 40, na tentativa de usar a mostarda nitrogenada como terapia antitumoral. Os farmacologistas Alfred Gilman e Louis

Goodman, da Universidade Yale descobriram seus efeitos fez contra o linfoma murino, e em 1943, o primeiro uso de um composto de mostarda para tratar o câncer humano foi registrado; um paciente que sofria de linfoma não-Hodgkin e apresentava obstrução grave das vias aéreas (ELBAGOURY; KOTB, 2018; GOODMAN et al., 1946).

Em meados dos anos 50, a descrição da estrutura helicoidal do DNA proposta por Watson e Crick (H C CRICK, 1953), foi marcante para a biologia molecular, criando oportunidades de gerar hiposteses racionais sobre a citocinética e o modo de ação de drogas, a taxa de reparo dde tecidos normais e tumorais após danos induzidos pelos quimioterápicos (LESHER; BAUMAN, 1969). O entusiasmo de tratar o câncer levou a descoberta de várias familias de drogas antitumorais, como antibióticos (actinomicina D, antraciclinas), antimitóticos (alcalóides da vinca), análogos de nucleosídeos e nucleobases (citarabina, 6-mercaptopurina, 6-tioguanina e fluoropirimidinas), cisplatina, etoposídeo ou procarbazina (BONADONNA et al., 1969; FALKSON et al., 1965; HEIDELBERGER et al., 1957; JOHNSON et al., 1963; MATHE et al., 1962; PINKEL, 1962).

Todo esse avanço da quimioterapia aliada aos avanços da biologia molecular, impulsionou a descoberta de novos farmacos para algumas neoplasias, mas com pouco ou nenhum resultado em outras. Existem casos em que as limitações da quimioterapia tornam a doença de dificil tratamento, e a maioria dos tumores é resistente à terapia, ou torna-se resistente após uma resposta inicial, problema atribuido à presença de mecanismos celulares mediadores de resistância a drogas, que resultam de alterações genéticas e bioquimicas que permitem as células tumorias sobreviverem durante o tratamento quimioterápico (LEONARD; FOJO; BATES, 2003; LONGLEY; JOHNSTON, 2005).

Para que a terapia antioneoplásica seja bem sucedida, o agente quimioterápico deve atingir as células tumorias em quantidades ideiais para exercer sua atividade, e, precisamente, uma das principais causas da resistência a drogas é a incapacidade de atingir essas concentrações intracelulares ótimas de medicamento, já que o microambiente tumoral também está envolvido no crescimento e sobrevivência do tumor (GALMARINI; GALMARINI; GALMARINI, 2012).

Uma população de células neoplásicas malignas pode tornar-se resistente a partir da seleção de uma célula geneticamente resistente através do tratamento

com o agente citotóxico ou através da seleção de uma subpopulação de células com características fenotípicas relacionadas à resistência, sendo que nesse caso, a expansão dessas variáveis aumenta as chances de que células geneticamente mutadas (resistentes) sejam geradas (PRADOS et al., 2012).

O transporte celular pode ser mediado por uma família de transportadores de membrana que podem colaborar na absorção do agente quimioterápico, por transferência passiva, caso que ocorre por exemplo com a doxorrubicina e vinblastina, ou facilitar a difusão, ativando o transporte como os análogos de nucleosídeos (JULIANO; LING, 1976; MANSOORI et al., 2017). A redução da absorção dos medicamentos pode ocorrer através da diminuição de ligação das drogas aos receptores, ou redução do número de transportadores específicos que as drogas usam para entrar nas células, resultante de mutações nesses transportadores que inibem e reduzem a absorção das drogas (CROOP et al., 1989; MANSOORI et al., 2017).

A resistência a quimioterápicos pode ser diminuida tambem por inibição da morte celular. A nível celular esse evento é mediado pela necrose, apoptose e autofagia, processos que diferem entre si por caracteristicas biológicas, porém todos eles facilitam a morte celular. A apoptose pode ocorrer através de vias intrinsecas e extrinsecas; em sua via extrinseca, os ligantes e receptores de morte celular, como FAS, TNF-R, caspases-3, -6, -7 e -8 estão envolvidos, resultandona proteolise da cascata reguladora, ativação de caspases que culminará na morte por apoptose, e a via intrinseca mitocondrial, regulada pelas proteinas pró- apoptóticas Bcl-2, Bax e Bak que também culminam nos eventos apoptóticos(LUQMANI, 2005). Nas duas vias, a desregulação de proteinas pró e antipoptóticas em células tumorais estao associadas ao aumento da resistência à quimioterapia (HIGGINS, 1992). Além disso, a resistência as drogas pode ocorrer pelas mutações no gene p53, responsável por induzir apoptose em situações de estresse celular e danos no DNA, prejudicando a conexão entre danos no DNA (causada por agentes quimioterápicos) e a ativação da apoptose (DE VREE et al., 1998).

O efeito dos agentes quimioterápicos depende tambem da conformação de seus alvos nas celulas tumorias. Modificações como mutações e mudanças nos niveis de expressão deles podem levar a mecanismos de resistência aos quimioterápicos (JONES et al., 2009). As enzimas topoisomerases são responsaveis pela abertura da molécula de DNA durante a replicação, o fármaco antitumoral

doxorrubicina, usada principalmente no tratamento de tumores de mama e pulmão, tem por função a inibição das topoisomerases, e por consequência inibição da multiplicação celular (SIMON; KINGSTON, 2013). Porém celulas tumorais com mutações nessas enzimas alteram o objetivo da droga mencionada (HOUSMAN et al., 2014).

Os agentes quimioterápicos a base de platina, como a cisplatina, danificam direta ou indiretamente o DNA das células tumorais, levando essas células à apoptose (BORST et al., 2000). Algumas células podem se tornar resistentes a esse mecanismo que levaria a morte celular, através da ativação do sistema de reparo de excisão de nucleotídeos e mecanismos de reparo de recombinação homólogos nas células tumorais, aumentando a taxa de reparo do DNA (BEACH; PALMITER, 1981; DE PAGTER; KLOOSTERMAN, 2015).

1.3 Alternativas terapêuticas

1.3.1 Ácido grandiflorênico

Sphagneticola trilobata Pruski (*Wedelia paludosa* D.C., *Acmella brasiliensis)* (Asteraceae) (Figura 1), é uma planta herbácea com caule castanho-avermelhado e folhas opostas, encontrada em várias regiões do Brasil, principalmente nos estados de Pernambuco, Bahia, Minas Gerais, São Paulo e Santa Catarina, e conhecida popularmente como “pseudo-arnica”, “margaridão” ou “pingo-de-ouro” (BRESCIANI; CECHINEL-FILHO; YUNES, 2000). Estudos fotoquímicos apontam que essa planta possui alta concentração de compostos terpênicos, fenólicos e esteroidais, e cada parte da planta possui uma constituição química diferente, com similaridade entre os compostos encontrados (BACCARIN et al., 2009; FILHO et al., 2004).

Figura 1. *Wedelia paludosa* D.C. (disponível em: http://avancine.com.br/produto/vedelia/)

A planta possui caráter ornamental e também é usada na medicina popular, atuando no tratamento de doenças respiratórias, infecciosas, com ação analgésica, expectorante e anticonvulsivo, além de apresentar propriedades biológicas como antinociceptivo (BLOCK et al., 1998; MIZOKAMI et al., 2012), tripanocida (BATISTA; CHIARI; DE OLIVEIRA, 1999).

Entre seus constituintes encontram os compostos diterpenos pertencente a classe dos terpenos, constituídos por 20 átomos de carbono, com predominância de dois compostos, o ácido caurenóico (AC) (ent-kaur-16-en-19-oic acid) e o ácido grandiflorênico (AG) (Figura 2) (ent-kaur-9(11),16-dien-19- oic acid), retirados das partes aéreas da planta (BACCARIN et al., 2009; BATISTA et al., 2009). Esses compostos são semelhantes, diferindo apenas em uma dupla ligação de hidrogênio na sua estrutura química, que alteram significativamente as propriedades biológicas desses compostos, tais como atividade antiplasmodial contra *P. falciparum* e citotoxicidade em células neoplásicas (BATISTA et al., 2009, 2013), capacidade leishmanicida do ácido caurenóico contra *L. amazonensis* em cultura de macrófagos (MIRANDA et al., 2015),

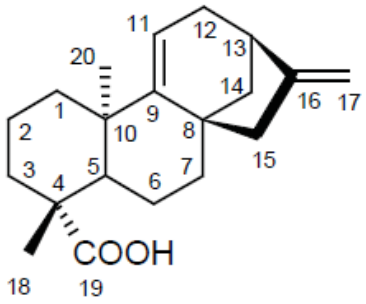

Figura 2. Estrutura química do ácido grandiflorênico (*ent*-kaur-9(11),16-dien-19-oic acido).

Alguns estudos demonstraram ainda que o diterpenóide AG exerce atividades biológicas como a sinalização de apoptose na linhagem celular de macrófagos RAW 264.7 (HUESO-FALCÓN et al., 2010), no entanto, pouco se sabe sobre os mecanismos de ação exercidos pelo diterpenóide AG sobre linhagens tumorais.

1.3.2 Tioidantoínas

Os fármacos de origem sintética representam significativa parcela do mercado farmacêutico, constatando-se que 62% deles são estruturas heterocíclicas, dentre os quais 95% apresentam-se nitrogenados, demonstrando a importância da química dos heterocíclicos, nas quais muitas vezes pode ocorrer a presença de mais de um heteroátomo no mesmo sistema (MENEGATTI; FRAGA; BARREIRO, 2001).

As tioidantoínas são compostos heterocíclicos biologicamente ativos com anéis de cinco membros, contendo dois heteroátomos. Muitos trabalhos de pesquisa têm sido descritos sobre a química e propriedades das tioidantoínas (2-tioxo-imidazolidina-4-ona) e seus derivados há mais de 120 anos, principalmente porque o anel tioidantoínico é um importante grupo farmacóforo presente em compostos ativos biológicos atribuídos a reatividade química e afinidade com biomacromoléculas (HADY, 2012; SUDHA VENGURLEKAR; RAJESH SHARMA; PIYUSH TRIVEDI, 2012). As tioídantoínas, descobertas por Klason em 1890, são análogos de enxofre da hidantoína com um grupo carbonila substituído pelo grupo tioxo, que possuem propriedades químicas similares aos compostos carbonílicos correspondentes (EDWARD; NIELSEN, 1957).

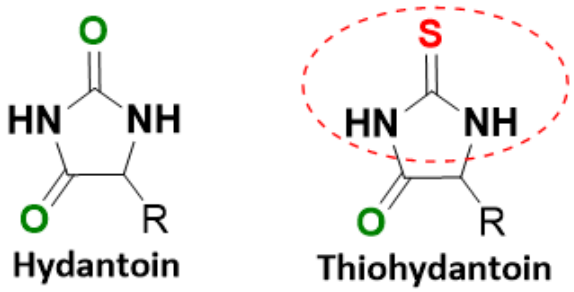

Figura 3: Estrutura geral das hidantoínas e tioidantoínas.

As tioidantoínas são notavelmente conhecidas devido às suas amplas aplicações, como na estrutura química de vários agentes farmacêuticos usados como antiparasitários contra promastigotas de *L. donovani* (PORWAL et al., 2009), contra *M. tuberculosis* da cepa H_{37}Rv (POYRAZ et al., 2017) e agente causador da malária *P. falciparum* da cepa W2 (CQ-R) (RAJ et al., 2014), além de apresentarem atividade antitumoral contra células tumorais de fígado HePG-2 (ABUBSHAIT, 2017).

1.4 Justificativa

Os aspectos relacionados à biologia do câncer determinam a dificuldade no seu tratamento. Embora existam muitas terapias direcionadas a cada tipo de tumor, estas ainda apresentam baixa eficácia e alta toxicidade às células normais, causando efetios adversos que podem ser letais. No processo de desenvolvimento de novos fármacos, observa-se baixa percentual de sucesso apesar de muitas moléculas apresentaram significativa atividade antitumoral em ensaios laboratoriais, tornando-se necessária, a busca de novos fármacos para o tratamento do câncer.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar *in vitro* a atividade biológica do ácido grandiflorênico e tioidantoínas em linhagens celulares tumorais de mama (MCF-7), fígado (Huh7.5) e pulmão (A549), bem como elucidar os mecanismos de morte envolvidos.

2.2 Objetivos específicos

Avaliar a atividade antiproliferativa ou citotóxica dos compostos, sobre as linhagens tumorais;

Determinar a concentração citotóxica dos compostos sobre as linhagens tumorais;

Determinar a quantidade de EROS induzidos pelos tratamentos no sobrenadante das culturas;

Elucidar os mecanismos de morte dos compostos nas linhagens celulares.

3 ARTIGOS PARA PUBLICAÇÃO

O presente trabalho originou 2 artigos científicos que serão incluídos nessa tese. Ambos foram realizados na Universidade Estadual de Londrina, no Laboratório de Imunoparasitologia de Doenças Negligenciadas e Câncer, em colaboração com os laboratórios de Biologia de Tumores, da Universidade Estadual do Oeste do Paraná, Laboratório de Biotransformação e Fotoquímica, da Universidade Estadual de Londrina e Laboratório de propriedades e síntese de substâncias orgânicas, da Universidade Estadual de Londrina.

O primeiro trabalho foi submetido para publicação na revista “Cancer Cell International”, e intitula-se “Mechanisms of action of thioidantoins under MCF-7 tumor cells, involves cell cycle arrest and ROS production”. O artigo está formatado de acordo com as normas da revista

O segundo trabalho intitula-se “Grandiflorenic acid induces changes associated with apoptosis and autophagy in tumor cells by increasing the production of reactive oxidative species”, e será submetido à revista Phytomedicine.

3.1 Effects of thioidantoins in MCF-7 tumor cell, involving cell cycle arrest and ROS production

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Abstract

Introduction: Breast cancer is a more frequent neoplasm in women worldwide. If diagnosed early, as chances of cure are high, but at an advanced stage, its treatment is difficult. Drugs are effective in fighting the crisis, side effects and tumor resistance. Literature reports demonstrate that thioidantoins, biologically active heterocyclic compounds, have several biological activities, such as: antiparasitic, antiviral, antitumor, antioxidant, anti-inflammatory and anticarcinogenic. This vision, the study of the use of thioidantoins the new age of antitumor against MCF-7 anti-cancer cells.

Methods: MTT, neutral red and tripan blue tests were used to evaluate the possible cytotoxicity of the compounds under MCF-7 cells and ovine red blood cells. Evaluation of cell volume and cell cycle analysis was performed by flow cytometry, and fluorescence analysis to determine the mechanism of death induced by thioidantoins. Three independent experiments were performed, each in triplicate.

Results: Treatment with 25, 50 and 100 μM thiohydantoins resulted in the death of MCF-7 breast tumor cells, but did not cause haemolysis in ovine red blood cells. The cytotoxic effect resulted in increased ROS production and cell cycle arrest in the G1 / G0 phase, with reduction of cell volume, loss of membrane integrity, mitochondrial depolarization and increased fluorescence to annexin, indicating cell death by apoptosis, and increased formation of autophagic vacuoles.

Conclussions: Our data indicate that thioidantoins are cytotoxic to breast tumor cells, and this effect is linked to increased ROS production, possibly causing direct DNA damage. This phenomenon alters tumorigenic pathways that lead to cell cycle arrest in G1 / G0, an important point of error checking in the DNA that culminates in the activation of apoptosis. In addition, the results indicate increased autophagy, which is linked to decreased lysosomal viability, and considered as a mechanism of tumor suppression cell death.

Key words: thioidantoins, breast cancer, ROS, MCF-7, apoptosis.

1. Introduction

The World Health Organization (WHO) predicts that 2.09 million women will be affected by breast cancer in the year 2018 worldwide, being a more frequent neoplasm among women (1). Invasive breast carcinoma is defined as a group of malignant epithelial tumors

characterized by invading adjacent tissue and having a high tendency for distant metastases (2).

Many factors such as population aging, geographic location, ethnicity, and overweight are related to increased risk, in addition, benign breast diseases, late menopause, nulliparity, use of oral contraceptives (especially if there is a combination of progesterone and estrogen) and first birth in old age are also considered risk factors for breast cancer (3,4). The genetic predisposition is responsible for about 10% of breast cancer cases, and are related to the development of disease in young women (4).

The existence of several types of tumors with genetic and epigenetic variations and clinical outcomes make it difficult to understand this disease, the mechanisms of action of chemotherapeutic agents and the creation of new therapies (5). Although different therapeutic strategies are available for the treatment of breast cancer in its different subtypes, a major problem currently encountered is the growing appearance of drug resistant tumor cells and the low efficacy of existing treatments.

Reports in the literature have described the chemical and biological properties of thioidantoins (2-thioxoimidazolidine-4-one), biologically active heterocyclic compounds with pharmacophoric groups with affinity to biomacromolecules (6–8).

The thioidantoins, discovered by Klason in 1890, are sulfur analogs of hydantoin with a carbonyl group substituted by the thioxo group, which have similar chemical properties to the corresponding carbonyl compounds (9–11). Thiohydantoins are notably known because of their presence in the chemical structure of various pharmaceutical agents antituberculosis (10), antimalarials (12) and antitumorals (9).

In view of the above, we proposed to investigate the biological effect of thioidantoins under MCF-7 breast cancer cells.

2. Méthods

Cell culture and treatments

MCF-7 cells, kindly provided by Professor Dr. Vanessa Jacob (IFRJ, Rio de Janeiro), were cultured in 25 cm² culture flasks with Dulbecco's modified eagle's medium (Gibco Invitrogen, New York, USA)) supplemented with 10% fetal bovine serum (SBF-Gibco, Invitrogen, New York, USA), 10 U / ml penicillin and 10 μg / ml streptomycin (Invitrogen-Gibco). Cells were kept in an oven (Sanyo, Japan) at 37 ° C, 5% CO2.

The assays were performed by plating MCF-7 cells at defined concentrations through a screening with 22 thioidandoins, kindly provided by Professor Fernando Cesar de Macedo Junior, Laboratory of Properties and Synthesis of Organic Substances (Universidade Estadual de Londrina), and among the tested (paper in preparation), the tioidantoínas 7 and 13 were those that presented the most significant effects on the cell line MCF-7. The concentrations used were 25, 50 and 100 μM, determined from previous tests, for 24 hours. Doxorubicin (Pfizer®) was used as a positive control at a dose of 25 μM, dose considered IC_{50} in the cells studied (13).

Mitochondrial viability and determination of CC_{50}

The determination of the inhibitory concentration to 50% of the cells (IC50) was obtained by logarithmic regression of the results of the MTT ((3- (4,5-mymethylthiazol-2-yl) - 2,5-dyphenyltetrazolium bromide) assay described by Mosmann (1983) (14), which estimates cell viability through mitochondrial cell activity. MCF-7 cells ($2x10^5$ cells / well) were treated with the 25, 50 and 100 μM thioidantoins in 24-well plates per 24 hours (37ºC, 5% CO_2). After that time, MTT (5 mg / mL) was added for 3h. Cells under the same conditions without treatment were used as controls; 0.025% DMSO was the vehicle, and doxorubicin (25 μM) was used as the positive control. The plates were read using a spectrophotometer (Thermo Scientific, Multiskan GO) at 550 nm. The results were expressed as percentage of viability in relation to the control.

Inviable cell count

MCF-7 cells ($2x10^5$ cells / well) in 24-well plates were treated with the compounds as described previously, then the cells were washed with trypsinized PBS (saline phosphate), resuspended in trypan blue (0.05%) and counted the non-viable cells using Neubauer's chamber. The cell count of the four outer quadrants of the chamber allowed for the identification of cell number increase or decrease by evaluating the total number of viable and non-viable cells (incorporated with the dye).

Lysosomal Viability

MCF-7 cells (2x105 cells / well) in 24-well plates were treated with thioidantoins as described above; supernatants were removed from the lines and added 200 μL of Neutral Red solution (Sigma-Aldrich-St. Louis, MO, USA) (40 μg / mL) were incubated in an oven for 2 h. The supernatant was then removed and diluent solution was added and the

spectrophotometer read at 540 nm (Thermo Scientific, Multiskan GO) according to Babich and Boreunfreund (15).

Hemolytic assay

The toxicity of the compound was tested in the hemolytic assay using sheep erythrocytes (Ethics Committee on Animal Experimentation of the niversidade Estadual de Londrina: 82862016.60). Sheep blood was collected with heparin, and prepared in a suspension of 2% erythrocytes, treated with 25, 50 and 100 μM thioidantoins, incubated under study for 3 hours, 37 ° C, 5% CO_2. The absorbance was measured at absorbance at 550 nm.

Determination of cell volume

MCF-7 cells (5x10 5 cells / well) were treated with 100 μM thioidantoins, washed with PBS and collected for cell volume analysis (FSC-H) according to Bortoleti et al. (16). Analyzes were performed using the BD Accuri ™ C5 Plus flow cytometer (BD Biosciences, San Jose, CA). Histograms were generated and FSC-H represented the cell volume. A total of 10,000 events were analyzed. Untreated cells were used as a negative control.

Generation of reactive oxygen species

To evaluate the generation of reactive oxygen species (ROS), MCF-7 cells ($1x10^4$ cells / well) were treated with 50 and 100 μM thiohydantoins for 24 hours, and the assay was performed according to Bortoleti *et al* (16); H_2O_2 was used as a positive control.

Determination of cell membrane integrity

MCF-7 cells (2x105 cells / well) were collected, washed with PBS and incubated directly with 0.50μg / mL propionic iodide (PI) (PI-Sigma, St. Louis, MO) , USA) for 15 minutes. Cell analysis was performed immediately using a microplate fluorescence reader (Victor X3, PerkinElmer, Finland), with excitation wavelength of 480 nm and emission of 580 nm. Digitonin (40 μM) (Sigma, St. Louis, MO, USA) was used as a positive control. Fluorescence values were normalized to the total number of cells from each treatment.

Determination of phosphatidylserine exposure

Phosphatidylserine exposure was detected using Annexin-V FITC (Sigma- Aldrich, St. Louis, MO, USA). After treatment with GFA as described above, MCF-7, A549 and HuH7.5 cells (2x105 cells / well) were washed and resuspended in 100 ul of binding buffer

(140mM NaCl, 5mM CaCl2 e 10 mM Hepes-Na, pH 7,4), followed by the addition of 5 µl of Annexin-V FITC for 15 minutes. After incubation, 200µL of binding buffer was added, and the data were obtained using a microplate fluorescence reader (Victor X3, PerkinElmer, Finland) at the excitation (480nm) and emission wavelength (520nm). 10 µM Camptothecin (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control. Fluorescence values were normalized to the total number of cells from each treatment.

Determination of the formation of autophagic vacuoles

For quantification of autophagic vacuoles, GFA-treated MCF-7, A549 and HuH 7.5 cells (2x105 cells / well) as described above were washed with PBS and incubated with monodanzilcadaverin (50µM) (Sigma- Aldrich, St. Louis, MO, USA). The data were obtained using a microplate fluorescence reader (Victor X3, PerkinElmer, Finland), with excitation wavelength (380 nm) and emission (525 nm). Fluorescence values were normalized to the total number of cells from each treatment.

Flow cytometry for cell cycle determination

MCF-7 cells were plated ($5x10^5$ cells/well), treated with 100 µM thiohydantoins, for 24 hours. After the treatments the cells were washed with PBS, centrifuged and fixed in 70% ice-cold ethyl alcohol for 1 hour at 4ºC, washed with PBS, incubated with 0.05% ribonuclease A (RNAse A-Sigma-Aldrich, St. Louis, MO, USA) and 50 µg/ml propidium iodide (Sigma, St. Louis, MO, USA) for 15 minutes. Cells were analyzed on the BD Accuri ™ Plus flow cytometer, and 10,000 events were analyzed for population sub-G_1, G_0/G_1, S, G_2/M.

Statistical analysis

Data were expressed as mean ± standard error of the mean. Three independent experiments were performed, each in triplicate. The data were analyzed using the statistical software GraphPad Prism (GraphPad Software, Inc., USA, 500.288). Significant differences between groups were determined using One-way ANOVA, followed by the Tukey test for multiple comparisons. The differences were considered statistically significant when $p \leq 0.05$.

3. Results

Thioidantoins exert cytotoxic effect under MCF-7 cells

Our first results were observed through the Tripan Blue assay; treatment with thioidantoins 7 and 13, at doses 25, 50 and 100 μM for 24 hours, promoted death of MCF-7 cells (Fig. 1A).

In the MTT and neutral red assays, we observed a significant reduction in mitochondrial ($p < 0.0001$) and lysosomal ($p < 0.0001$) viability respectively (Fig. 1B e 1C), the inhibitory concentration of both thiohydantoins were calculated after the treatments by the mitochondrial viability test (MTT), the IC_{50} of the thioidantoin 7 treatment was 88.50 ± 0.2, and the thioidantoin 13 was 43.40 ± 0.01.

It is important to report that treatment with thioidantoins 7 and 13 did not show hemolytic activity at the concentrations tested (25, 50 and 100 μM) (Fig. 2).

Thioidantoins reduce MCF-7 cell volume

When analyzing the cell volume of MCF-7 cells, we found that treatment with thioidantoins 7 and 13 at the dose of 100 μM (Fig. 3A, B), promoted a reduction in the volume of MCF-7 cells after 24 hours. This result is most evident when we evaluate volume reduction by fluorescence intensity (Fig. 3C).

Thiohydantoins promote increased ROS production in MCF-7 cells

In an attempt to find the mechanism involved in the cytotoxicity verified in the above tests, we evaluated ROS production. It was observed that treatment with both thiohydantoins at concentrations of 50 and 100 μM induced the production of ROS ($p < 0,0001$) (Fig. 4A).

Thioidantoins promote cell death pattern associated with apoptosis, autophagy, and membrane integrity alteration

When MCF-7 cells were treated with thioidhydantoins 7 and 13 at doses 25, 50 and 100 μM, we found a significant increase in the fluorescence intensity of Annexin V, suggesting cell death by apoptosis ($p < 0,0001$) (Fig. 5A). When the fluoride intensity of monodanzilcadaverin was analyzed, the same phenomenon was observed, also being able to infer cell death by autophagy ($p < 0.0001$) (Fig. 5B). Figure 5C shows an increase in fluorescence intensity for PI indicating change in plasma membrane integrity ($p < 0.0001$).

Tioidantoínas promovem parada no ciclo celular em G_0/G_1

Treatment with thioidantoins 7 and 13, at the dose of 100 μM, was able to induce "stop" in the cell cycle of MCF-7 cells, in the G_0/G_1 phase (Fig 6A, B e C). The observed

values were approximately 50.5% of the cells in the G_0/G_1 phase (Fig. 6D) for treatment with thioidantoin 7, and 55.5% (Fig. 6D) with the thioidanotine 13 also in the G_0 / G_1 phase, of the 10,000 events analyzed.

4. Discussion

Cancer is a complex disease and its occurrence and development are closely related to the abnormal system of intracellular signal transduction (17). Malignant breast tumors are characterized as heterogeneous neoplasms, with several pathological subtypes, with a marked tendency of distant metastasis, different clinical presentations, which causes a great variation in the responses to the treatment (18,19).

The choice of drugs used for breast tumors are chosen according to the tumor cell phenotype. The standard chemotherapy used for the treatment of early-stage breast cancer is the combination of anthracyclines, such as doxorubicin, with taxanes either simultaneously or sequentially (20). New drugs have been employed targeting specific molecular markers such as ER modulators (tamoxifen) (21); traztuzumab used in the treatment of HER-2 tumors improves the survival of women with this tumor, but many side effects are reported, and one of the most significant complications is its effect on the heart and is associated with cardiac dysfunction in 2% to 7 % of cases (22). Thus, there is a lack of therapies directed to the treatment of breast tumors, and despite the therapeutic options, patients need less toxic options, that reduce the risk of disease progression, and decrease the appearance of drug resistant tumor cells used.

Thus, in recent years, many researchers have been incessantly pursuing the discovery of novel compounds that exhibit desirable characteristics of an effective drug.

Thioidantoins are a group of heterocyclic compounds with thioidantoin ring and with important affinity characteristic with biomacromolecules (23–25). In the present study, we demonstrated that thioidantoins 7 and 13 induced toxicity in MCF-7 tumor cells, without altering the viability of erythrocytes. Zuo et al. (26), synthesized and tested various indoline thiohydantoin derivative compounds against LNCaP cells, and found that compound 48c showed antiproliferative effect with IC_{50} between 15.3 and 8.7 μM.

Wu Fu et al. (9), reported that some thioidantoins (compounds 16 and 18) were able to inhibit the proliferation of tumor stem cells taken from patients with glioma and cultured. These same compounds also showed cytotoxicity against HeLa (IC_{50}: 1.17 mM), MCF-7 (IC_{50}: 11.4 mM), and decreased numbers of HCT116 and HEK293 cells without toxicity (IC_{50}> 50 mM) (27,28).

Currently, there is no literature report demonstrating the mechanisms by which thioidantoins cause cytotoxicity in tumor cells. Among the main mechanisms of death, apoptosis plays a central role when analyzing the effectiveness of new drugs with antitumor activity.

Apoptosis is a process of cell death that requires energy for self-destruction; and has features such as changes in morphological appearance, including cellular retraction, discrete mitochondrial membrane depolarization, nuclear fragmentation, phosphatidylserine translocation to the cell surface, loss of plasma membrane integrity, and condensation and chromatin degradation (29).

In addition, apoptotic cell death is accompanied by a change in plasma membrane structure by surface exposure of phosphatidylserine, which may be detected by its affinity for annexin V, membrane phospholipid binding protein (30). It was exactly this phenomenon that we observed in our results for both thioidantoins tested.

Concerning the increase in fluorescence for PI, we observed that our compounds decrease membrane integrity. This characteristic usually refers to the necrosis process, but in recent years it is believed that this morphological alteration is linked to a late process in apoptosis, the process called secondary necrosis, which refers to the progressive loss of cell membrane plasma integrity apoptotic (31). It is believed to occur in vivo, especially in tumor cells from patients undergoing selected chemotherapy and some types of radiotherapy, when apoptotic cells are not efficiently cleansed by phagocytic cells, as well as observed in lysosomal disorders, or when cells are infected by apoptosis-inducing pathogens such as vesicular stomatitis virus or encephalomyocarditis virus (32–35). This alteration can be stimulated even by the formation of reactive oxygen species (36,37), fact that we observed in our treatments.

Our results also point to a death pattern associated with autophagy due to increased fluorescence for monodanzilcadaverine, a dye that binds to autophagic vacuoles (38). In cancer this process has a double role, and can present as a mechanism of cellular survival, and also act as a tumor suppressor, preventing the accumulation of damaged proteins and organelles (39). Regarding the ability of autophagy to limit tumors, authors point out that this process may limit necrosis and chronic inflammation, which are associated with the release of pro-inflammatory HMGB1. Thus, even when we observed significant levels of marking for necrosis, when we assessed the intensity of labeling for monodanzilcadaverina, that is, death due to autophagy, the levels were higher, therefore we believe that autophagy may be acting as an attenuator of chronic inflammation (40), as well as suppressing tumor growth.

Additionally, our results still show a decrease in lysosomal viability, a phenomenon that may have been initiated by the rupture of this organelle from the exogenous stimulus used.

The release of lysosomal enzymes into the cell cytoplasm may initiate a cascade of intracellular degradation events (41). These enzymes can attack the mitochondria directly and induce the release of cytochrome C; still directly or indirectly increase the formation of ROS, a fact also observed in our results, thus activating pro-apoptotic proteins.

Concerning DNA damage, under normal conditions the cycle proceeds without interruptions, however, if growing cells receive some kind of exogenous stimulus that leads to damage, they generally have the ability to temporarily pause in the phase G1, S or G2 for possible repair and reactivation of the cell cycle, and when the damage is severe, the cells may alternatively undergo apoptosis or enter an irreversible G0 state (42). The G1-phase checkpoint depends on increased expression and activation of the p53 gene.

In this context, we observed that treatment with thioidantoins 7 and 13 promoted an increase in cellular events in G1. This result associated with the increase in ROS production allows us to infer that there was indeed a "stop" in the G1 cycle, possibly caused by oxidative stress (42–45).

Indeed, in some solid and hematological malignancies, oxidative stress may induce apoptosis, and this can be explored in selective cancer therapy strategies (46). Ionizing radiation and chemotherapeutic agents induce directly or indirectly the generation of ROS and NO followed by accumulation of oxidized proteins, DNA damage and cell cycle arrest. This ROS generation increases apoptosis through increased proapoptotic Bap protein expression, caspase-3 activation, and internal mitochondrial membrane rupture (47).

5. Conclusions

Our data suggest that thioidantoins 7 and 13 exert a cytotoxic effect on breast tumor cells (MCF-7 strain), because they cause direct damage to the DNA, due to the increase in ROS production, consequently altering tumorigenic pathways that lead to the stop of the cell cycle in G1/ G0, an important checkpoint that can result in apoptosis.

In addition, it is possible to infer that both thioidantoins promote autophagy, which is linked to a decrease in lysosomal viability. Therefore, we conclude that both compounds have antitumor activity, but further studies should be performed to better define its efficacy as a drug.

Abbreviations

CaCl2	Calcium chloride
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
FSC-A	Forward Scatter- Area
H_2O_2	hydrogen peroxide
IC_{50}	inhibitory concentration
MTT	3-(4,5-dimethylthi azol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
PBS	phosphate buffered saline
PI	Propidium iodide
RNAse-A	Ribonuclease A
ROS	Reactive Oxygen Species
WHO	World Health Organization

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Consent for publication

All authors agree to submit this manuscript and declare there are no competitors of interest related to this work.

Availability of data and materials

One-way ANOVA was used for comparisons between groups, and multiple comparisons were performed with Tukey's post-test.

Interest conflicts

All authors declare no conflict of interest.

Authors' contributions

Tatiane Renata Fagundes (Guarantor of the integrity of the whole study, study concepts, study design, bibliographic research, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript revision), Vírgínia Márcia Concato (experimental studies, data acquisition, data analysis, statistical analysis), Bruna Taciane da

Silva Bortoleti (data analysis, statistical analysis), Fernanda Tomiotto-Pellissier (data analysis, statistical analysis), Elaine Siqueira (experimental studies), Priscila Góes Camargo (bibliographic search, manuscript preparation), Amanda Cristina Machado Carloto (pesquisa bibliográfica), Taylon Felipe Silva (manuscript preparation), Carolina Panis (study design, handwriting review), Fernando Cesar Macedo Junior (handwriting review), Ivete Conchon-Costa (handwriting review), Wander Rogério Pavanelli (handwriting review). All authors read and approved the final manuscript.

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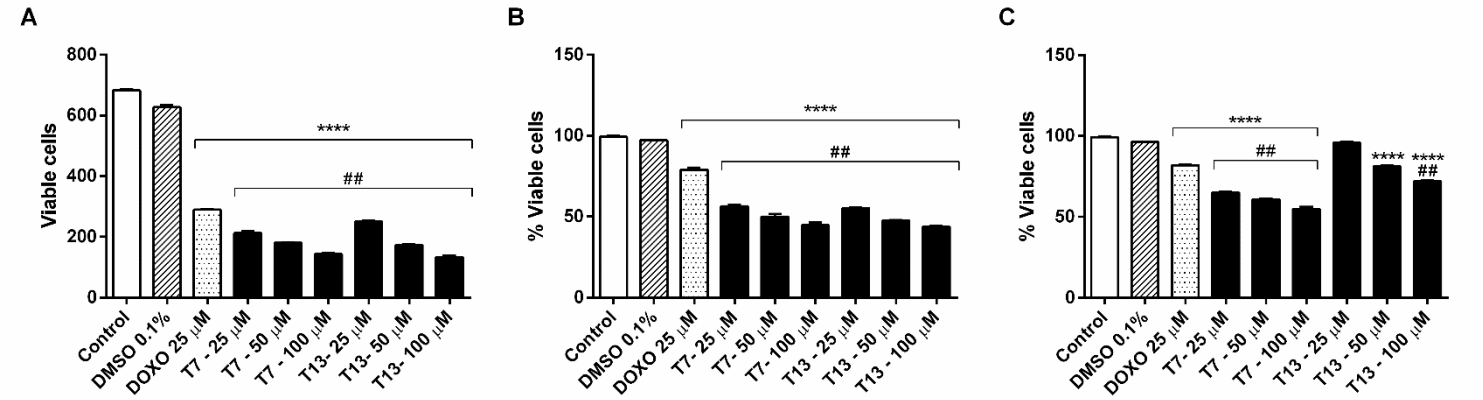
List of figures

Figure 1. Viability of the MCF-7 tumor line treated with the T7 and T13 thioidantoins. Cells were treated with thioidantoins at concentrations of 25, 50 and 100 μM for 24 h and analyzed



by tripan blue (A), MTT viability assays (B) and neutral red (C). Control (DMEM medium), DMSO 0.01% (vehicle) and Doxorubicin (DOXO) 25 μM (positive control). The values represent the mean ± SEM of three different experiments performed in duplicate. **** Significant difference in relation to control ($p \leq 0.0001$). ## Significant difference in relation to the positive control ($p \leq 0.0001$).

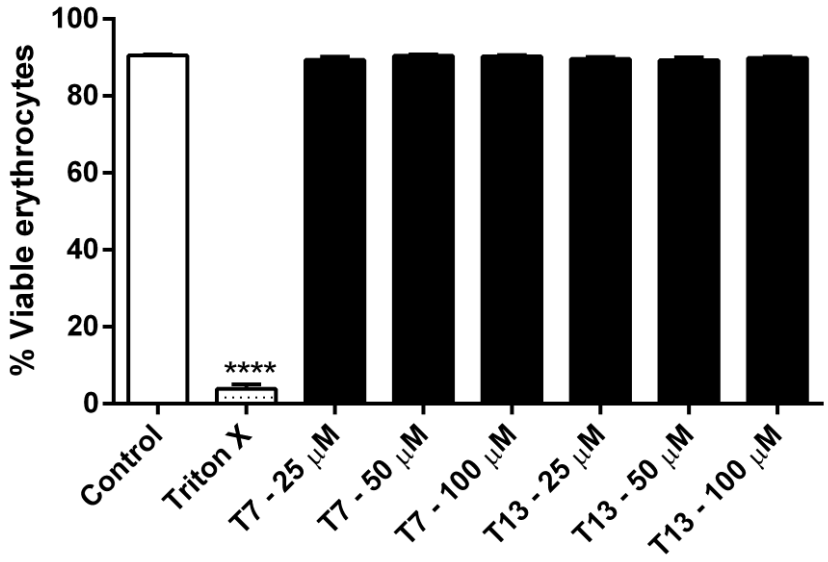


Figure 2. Cytotoxicity of thioidantoins in ovine erythrocytes. The erythrocytes were treated with T7 and T13 at concentrations of 25, 50 and 100 μM for 3h to determine the percentage of viable erythrocytes. Control (erythrocytes + PBS), 0.1% DMSO (vehicle) and Triton X (positive control) for hemolysis. Values represent the mean ± SEM of three independent experiments performed in triplicate. **** Significant difference in relation to the negative control ($p \leq 0.0001$).

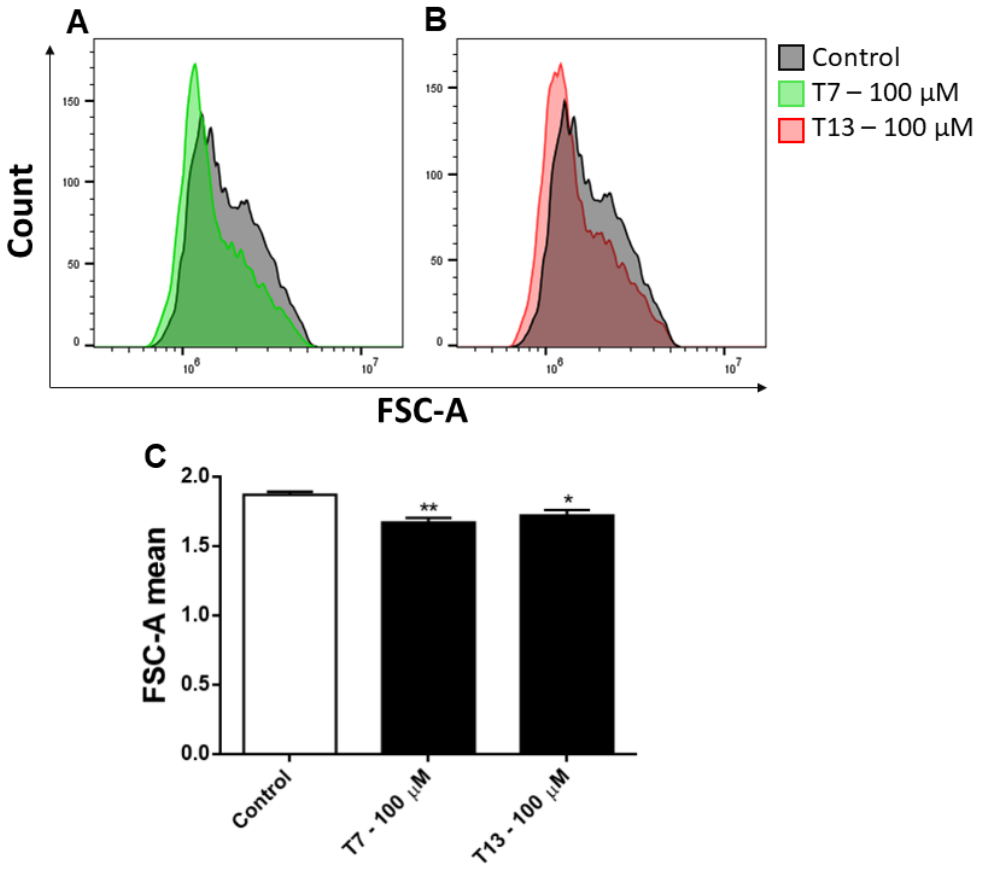


Figure 3. Cell volume of MCF-7 tumor line after treatment with thioidantoins. The cells were treated with T7 and T13 at concentration of 100 µM and the cell volume was analyzed by flow cytometry. The FSC-A parameter was considered a function of cell size. Typical histograms of at least three independent experiments are shown (A, B). Mean ± SEM of the fluorescence intensity of FSC-A are shown (C). * Significant difference in relation to control ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$).

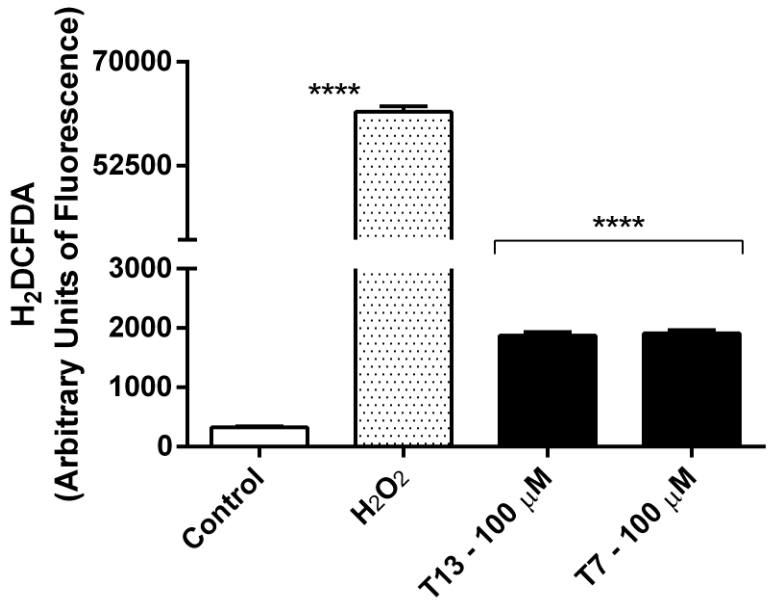


Figure 4. Production of reactive oxygen species produced by the thioidantoin-treated MCF-7 tumor cell. Cells were treated with T7 and T13 at concentrations of 50 and 100 μM for 24 h and analyzed by the H2DCFDA probe for analysis of reactive oxygen species levels. H2O2 was used as a positive control. The values represent the mean ± SEM of three different experiments performed in duplicate. **** Significant difference in relation to control ($p \leq 0.0001$).

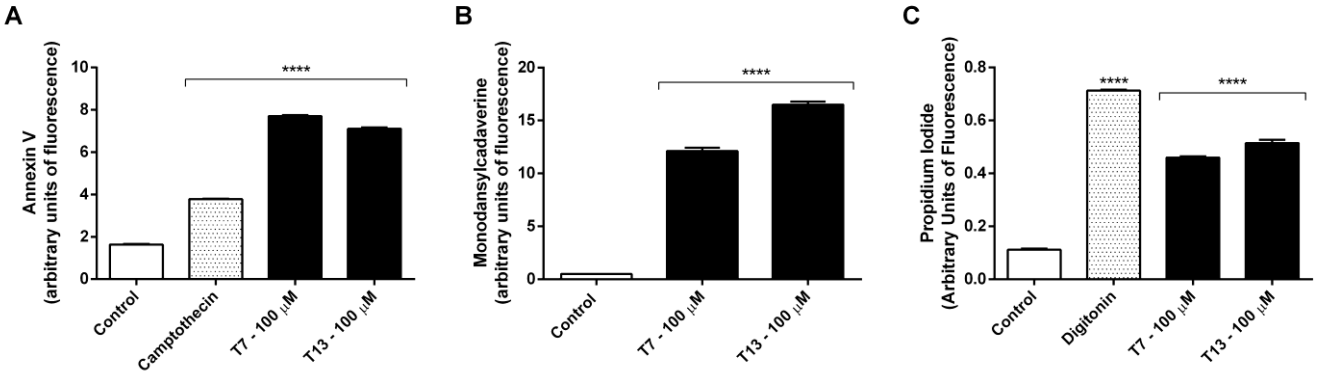


Figure 5. Thioidantoin-induced death mechanisms in the MCF-7 tumor line. Cells were treated for 24 h with T7 and T13 at concentrations of 25, 50, 100 μM and the exposure of phosphatidylserine by annexin V (A) labeling, autophagic vacuole formation by monodansilcadaverine (B) labeling and integrity analysis of the plasma membrane by propidium iodide (C). The data represent the mean ± SEM of three independent experiments performed in duplicate. **** Significant difference in relation to control ($p \leq 0.0001$). Camptothecin and digitonin were used as positive controls.

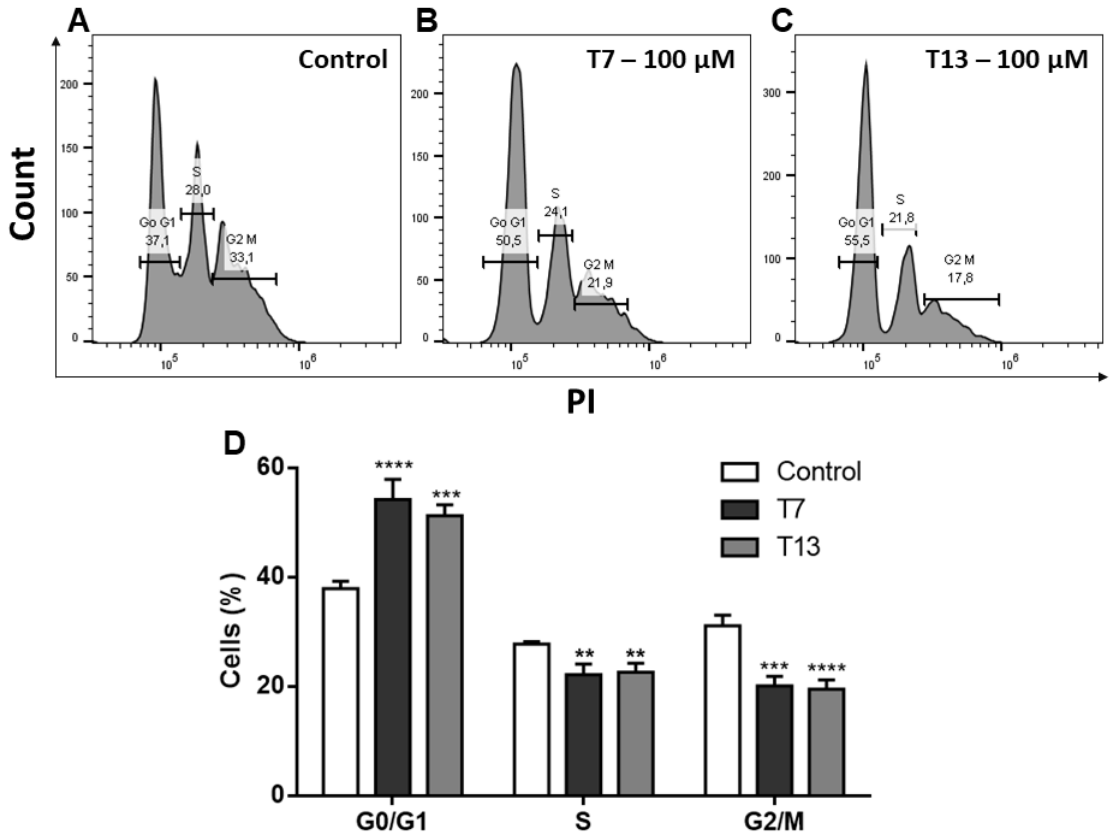


Figure 6. Cell cycle of MCF-7 tumor line after treatment with thioidantoins. Cells were treated with T7 and T13 at concentration of 100 µM and the DNA content assessed by propidium iodide labeling and flow cytometric analysis. Histograms typical of at least three independent experiments are shown (A, B and C). Mean ± SEM of fluorescence intensity are given (D). ** Significant difference in relation to control ($p \leq 0.01$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$).

3.2 Grandiflorenic acid induces changes associated with apoptosis and autophagia in tumor cells.

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ABSTRACT

Background: Cancer is a molecularly heterogeneous disease, caused by several factors, including environmental and genetic. It is estimated that by the year 2018 there will be 9.6 million deaths from cancer worldwide, mainly from breast, lung and liver tumors. The complexity of the disease leads to low response rates of the treatments used, being necessary the discovery of new drugs to control the disease. *Sphagneticola trilobata* is a plant that has diterpenes as the main constituent, including grandiflorenic acid (GFA), which has been reported for its anti-inflammatory, antileishmanicidal, antibacterial and antitumoral actions.

Objective: the aim of our study was to evaluate whether GFA has antitumor activity under breast cancer cell lines (MCF-7), liver (HuH7.5) and lung (A549).

Methods: GFA in vitro was evaluated for cytotoxicity through the MTT mitochondrial evaluation, neutral red and tripan blue, in MCF-7 (breast cancer), A549 (lung cancer) and HuH7.5 liver). Cell volume evaluation was performed by flow cytometry, and fluorescence analysis for determination of the mechanism of death, through phosphatidylserine exposure, membrane integrity, and autophagic vacuoles formation. The possible induction of the formation of reactive oxygen species by fluorescence was also evaluated.

Results: GFA treatment at concentrations of 12.5, 25, 50, 100, 200, and 400 nM significantly inhibited cell proliferation in the three lines studied, which was accompanied by a decrease in cell volume, mainly at the concentration of 400 nM. The evaluation of the mechanisms of death showed that the treatments increased ROS production, folphatidylserine exposure, mitochondrial membrane depolarization, and decreased plasma membrane integrity, indicating mechanisms related to apoptosis, in addition to the formation of autophagic vacuoles.

Conclusions: GFA presented cytotoxic activity under tumor cell lines MCF-7, HuH7.5 and A549, promoted aspects related to apoptotic morphology and autophagy, probably due to increased ROS production. Further experiments will be needed to elucidate the signaling pathways involved in the characteristics related to cell death caused by the compound.

Key words: cancer, breast, liver, lung, grandiflorenic acid, apoptosis.

INTRODUCTION

Cancer is a molecularly heterogeneous, genetic disease whose evolution leads to numerous changes in DNA, characterized by abnormal cell growth and in some cases has the

power to invade adjacent and distant tissues (Louzada et al., 2012). Currently, more than 100 types of cancer are known, differentiated by etiology, evolution process and form of treatment and, therefore, cancer can no longer be considered as a single pathology (Brentani et al., 2003). In 2018, the World Health Organization (WHO) estimated 9.6 million cancer deaths, with lung cancer (1.7 million), liver (782,000) and breast (627,000) being among the deaths ("WHO | About the International Agency for Research on Cancer," 2017).

Lung cancer is the most common type of tumor in the world, representing a major public health problem (Plummer et al., 2016). Smoking is the major etiological risk factor for this type of cancer, contributing to a 10-fold increase in risk in long-term smokers compared to nonsmokers. Other risk factors that evidence the environment as an inducer of this type of cancer include exposure to radiation, asbestos, heavy metals (arsenic, chromium, nickel), polycyclic aromatic hydrocarbons and chloromethyl ethers (Pukkala et al., 2009).

Liver cancer is an aggressive cancer that often occurs as a result of chronic liver disease and cirrhosis (Liu et al., 2015), being the fifth most common tumor in men and the seventh most common cancer in women, and the third leading cause of cancer-related death in the world (Ferlay et al., 2015; Jemal et al., 2011). Despite advances in treatment, liver cancer remains one of the most difficult cancers to treat. In patients with early diagnosis, surgery, and liver transplantation provide curative potential, however, recurrence remains a major problem after curative treatment, reaching an incidence of more than 70% at 5 years (Llovet et al., 2008). In addition, traditional systemic chemotherapy has low efficacy and few survival benefits (Verslype et al., 2009).

Breast cancer is the one with the highest incidence and mortality among women, with different types and causes, with the main factors associated with age, family history, use of contraceptives, alcohol consumption, smoking, previous breast disease, exposure to radiation and obesity ("WHO | About the International Agency for Research on Cancer," 2017). Although the treatment and diagnosis of breast cancer has made significant progress in the last two decades, the treatment consists of surgery, followed by radiotherapy and systemic therapy, a combination of chemotherapy and hormonal modulators (Reis-Filho et al., 2006; Wardley, 2006). Although different therapeutic strategies are available for the treatment of breast cancer, a major problem currently encountered is the growing appearance of drug resistant tumor cells used (Tripathy, 2005).

Grandiflorenic acid (GFA) is a diterpene compound belonging to the class of terpenes derived from the herbaceous plant *Sphagneticola trilobata* (L.) Pruski (*Wedelia paludosa* DC.) (Asteraceae) (Bresciani et al., 2000). It is described in the literature that

diterpene compounds have pharmacological properties as antiprotozoal against trypomastigotes of *T. cruzi*, the causal agent of Chagas disease (Batista et al., 1999), leishimanicidal effect against promastigote forms of *L. amazonensis* (Bortoleti et al., 2018), anti-inflammatory activity, inhibiting nitric oxide and inducing the release of prostaglandins (PGE2), cyclooxygenases-2 (COX-2), in RAW264.7 macrophages induced by LPS (lipopolysaccharides) (Choi et al., 2011). Some studies have shown that GFA diterpenoid exerts biological activities such as the signaling of apoptosis in the macrophage cell line RAW 264.7 (Hueso-Falcón et al., 2010). However, little is known about the biological activities of GFA diterpenoid in MCF-7, HuH7.5 and A549 tumor lines.

Even if current medications are considered effective, the search for therapeutic alternatives of natural origin, which eventually have antitumor activity without causing several side effects, will always be a reason for investigation by some researchers.

In this way, the aime of the study was to evaluate the effect of GFA on tumor cell lines MCF-7 (breast cancer), A549 (lung carcinoma) and HuH7.5 (hepatocellular carcinoma), seeking to elucidate the possible mechanisms of death.

MATERIALS AND METHODS

Cell Culture

MCF-7, HuH7.5 and A549 cells, kindly provided by Professor Dr. Juliano Bordignon (Fiocruz, Curitiba), were cultured in 25 cm^2 culture flasks with DMEM culture medium (Dulbeco's modified eagle's medium) (Gibco Invitrogen, New York, USA) supplemented with 10% fetal bovine serum (SBF- Gibco, Invitrogen, New York, USA), 10 U / mL penicillin and 10 μg / mL streptomycin (Invitrogen-Gibco). Cells were kept in an oven (Sanyo, Japan) at 37 ° C, 5% CO2. For all tests the positive control used was doxorubicin at the dose of 25 μM, dose considered IC_{50} in the cells studied (Fornari et al., 1994).

Grandiflorenic acid (GFA)

The plant material of *S. trilobata* was collected in the Medicinal Plants Garden of the Center of Agricultural Sciences of the Universidade Estadual de Londrina (UEL) (23˚32'64”N-51˚20'63”W). The isolation and structural chemical elucidation of GFA was performed according to Bortoleti et al. (Bortoleti et al., 2018), as well as the concentrations used. A stock solution of GFA diluted in 1% DMSO (dimethylsulfoxide) (Gibco) was prepared, without exceeding 0.015% in all experiments.

Mitochondrial viability and determination of CC_{50}

Determination of the 50% inhibitory concentration of the cells (IC 50) was obtained by logarithmic regression of MTT assay results ((3- (4,5-mymethylthiazol-2-yl) -2,5-dyphenyltetrazolium bromide) described by Mosmann (1983) which estimates cell viability through mitochondrial cell activity. MCF-7, A549 and HuH 7.5 cells ($2x10^5$ cells / well) were treated with GFA at concentrations of 25-400 nM in 24-well plates per 24 h (37 °C, 5% CO_2). After that time, MTT (5 mg / mL) was added for 3h. Cells under the same conditions without treatment were used as controls; 0.015% DMSO was the vehicle, and the positive control was 25 μM doxorubicin. The plates were read using a spectrophotometer (Thermo Scientific, Multiskan GO) at 550 nm. The results were expressed as percentage of viability in relation to the control.

Inviable cell count

MCF-7, A549 and HuH 7.5 cells ($2x10^5$ cells / well) were treated with GFA as described previously, then the cells were washed with PBS (saline biphosphate) and trypsinized, resuspended in tripan blue (0.05%) and counted infeasible cells using Neubauer's chamber.. The cell count of the four outer quadrants of the chamber allowed for the identification of cell number increase or decrease by evaluating the total number of viable and non-viable cells (incorporated with the dye).

Lysosomal Viability

MCF-7, A549 and HuH 7.5 cells ($2x10^5$ cells / well) were treated with GFA as described previously, line supernatants were removed and added 200 μL of Neutral Red solution (Sigma-Aldrich-St. Louis, MO, USA) (40 μg / mL), then incubated in an oven for 2 h. The supernatant was then removed, diluent solution added and spectrophotometer read at 540 nm (Thermo Scientific, Multiskan GO) (Babich and Borenfreund, 1991).

Hemolytic assay

The toxicity of the compound was tested in the hemolytic assay using sheep erythrocytes (Ethics Committee on Animal Experimentation of the Universidade Estadual de Londrina: 82862016.60). Sheep blood was collected with heparin, and prepared in a suspension of 2% erythrocytes, treated with 12.5, 25, 50, 100, 200 and 400 nM GFA, incubated in an oven for 3 hours, 37 ° C in 5% CO2. The absorbance was measured at absorbance at 550 nm.

Determination of cell volume

MCF-7, A549 and HuH 7.5 cells ($5x10^5$ cells / well) were treated with 100, 200 and 400 nM GFA per 24 h (37 °C, 5% CO_2), washed with PBS and collected for cell volume analysis (FSC-H) (Bortoleti et al., 2018). Analyzes were performed using the BD Accuri ™ C5 Plus flow cytometer (BD Biosciences, San Jose, CA). Histograms were generated and FSC-H represented the cell volume. A total of 10,000 events were analyzed. Untreated cells were used as a negative control.

Alteration of mitochondrial membrane potential

To investigate the internal potential of the mitochondrial membrane, staining with tetramethylrhodamine-ester (TMRE) (Sigma- Aldrich- St. Louis, MO, USA). Cells ($1x10^4$ cells / well) were treated with 200 and 400 nM GFA per 24 hours, and incubated with 25 nM TMRE for 30 min. Fluorescence was measured on the fluorescence microplate reader (Victor X3, PerkinElmer, Finland) with the excitation wavelength (480 nm) and emission (580 nm). To compare the different treatments, the fluorescence values obtained were normalized with the respective number of cells. CCCP was used as a positive control.

Generation of reactive oxygen species

To evaluate the generation of reactive oxygen species (ROS), MCF-7, A549 and HuH 7.5 cells ($1x10^4$ cells / well) were treated with 200 and 400 nM GFA for 24h (37 ° C, 5% CO2) and the assay was performed according to Bortoleti et al (Bortoleti et al., 2018). H_2O_2 was used as a positive control.

Determination of cell membrane integrity

MCF-7, A549 and HuH 7.5 cells ($2x10^5$ cells / well) were treated with 25-400 nM GFA for 24h (37ºC, 5% CO 2), washed with PBS and incubated directly with propionic iodide at 0, 50 μg / mL (PI- Sigma, St. Louis, MO, USA), for 15 minutes. Cell analysis was performed using a microplate fluorescence reader (Victor X3, PerkinElmer, Finland) with excitation wavelength (480 nm) and emission (580 nm). Digitonin (40 μM) (Sigma, St. Louis, MO, USA) was used as a positive control. Fluorescence values were normalized to the total number of cells from each treatment.

Determination of phosphatidylserine exposure

Phosphatidylserine exposure was detected using Annexin-V FITC (Sigma-Aldrich, St. Louis, MO, USA). After treatment with GFA as described above, MCF-7, A549 and HuH7.5 cells ($2x10^5$ cells / well) were washed and resuspended in 100 ul of binding buffer (140mM NaCl, 5mM $CaCl_2$ e 10 mM Hepes-Na, pH 7,4), followed by the addition of 5 µl of Annexin-V FITC for 15 minutes. After incubation, 200µL of binding buffer was added, and the data were obtained using a microplate fluorescence reader (Victor X3, PerkinElmer, Finland) at the excitation (480nm) and emission wavelength (520nm). 10 µM Camptothecin (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control. Fluorescence values were normalized to the total number of cells from each treatment.

Determination of the formation of autophagic vacuoles

For quantification of autophagic vacuoles, GFA-treated MCF-7, A549 and HuH 7.5 cells (2x105 cells / well) as described above were washed with PBS and incubated with monodanzilcadaverin (50µM) (Sigma- Aldrich, St. Louis, MO, USA). The data were obtained using a microplate fluorescence reader (Victor X3, PerkinElmer, Finland), with excitation wavelength (380 nm) and emission (525 nm). Fluorescence values were normalized to the total number of cells from each treatment.

Statistical analysis

Data were expressed as mean ± standard error of the mean. Three independent experiments were performed, each in triplicate. The data were analyzed using the statistical software GraphPad Prism (GraphPad Software, Inc., USA, 500.288). Significant differences between groups were determined using One-way ANOVA, followed by the Tukey test for multiple comparisons. The differences were considered statistically significant when $p \leq 0.05$.

RESULTS

GFA exerts cytotoxic effect under the MCF-7, A549 and HuH7.5 cell lines 7

When evaluating the effect of GFA on the MCF-7, A549 and HuH7.5 lines, it was observed that the 24-hour treatment with doses 12.5, 25, 50, 100, 200 and 400 nM showed a significant reduction in viability of the three tumor cell lines ($p<0,00001$) (Fig. 1A, B and C), in relation to the control. By MTT assay was possible to observe a decrease in mitochondrial viability ($p< 0,0001$) (Fig. 1D, E and F), , when the neutral red test was used ($p< 0,0001$) (Fig. 1G, H and I), we also observed a reduction in lysosomal viability in the three strains studied,

when compared to the control, as well as the chemotherapy drug doxorubicin at the dose of 25 μM for the MCF-7 and HuH7.5 lines.

In all three trials, we observed a decrease in cell viability in a dose-dependent manner. Thus, after these assays the IC_{50} was determined; for MCF-7 cells the inhibitory concentration was 198.4 nM ± 0.01, in the A549 cells was 181 nM ± 0.01, and in the HuH7.5 line, 123.3 ± 0.01.

In turn, treatment with GFA at doses 12.5, 25, 50, 100, 200 did not show hemolytic activity, only the concentration of 400 nM was cytotoxic to red blood cells (Fig. 2).

GFA drastically reduces the cell volume of MCF-7, A549 and HuH7.5 cells

Cell volume analysis by flow cytometry revealed after 24 hours of treatment at the doses of 100, 200 and 400 nM an intense decrease in cell volume in the three cell lines tested, MCF-7 (Fig. 3A and D), A549 (Fig. 3B and E), HuH7.5 (Fig. 3C and F).

GFA produces mitochondrial depolarization and increased ROS production

After observing cytotoxic effects of GFA in the studied strains, we sought to find the mechanism by which the compound acts on these tumor cells.

We found that treatment with 200 and 400nM GFA causes mitochondrial membrane depolarization (Fig. 4A, B and C), mainly in MCF-7 and A549 lines; and HuH7.5 cells in a less significant manner. These same doses of GFA were able to promote increased ROS production (Fig. 4D, E and F).

GFA alters phosphatidylserine exposure, formation of autophagic vacuoles, and alteration of membrane integrity

Upon investigating the type of cell death, we found that concentrations between 25 and 400 nM increased the labeling for Annexin V, indicating phosphatidylserine exposure (Fig. 5A, B and C), as well as monodanzilcadaverine (Fig. 5D, E and F) and consequently formation of autophagic vacuoles in the three strains tested.

Treatment with GFA caused changes in plasma membrane integrity, as shown in Figure 5G, H and I.

DISCUSSÃO

Cancer is a disease characterized by intense cellular heterogeneity, leading to difficulty in treatment (Louzada et al., 2012). In recent years many researchers have

investigated the biological effect of products or compounds from herbs and plants under tumor cells, looking for natural compounds that exert antitumor activity without causing adverse reactions, or at least the decrease of these events.

In this sense, our study sought to elucidate the mechanisms of action of GFA, a diterpene derived from the Wedelia paludosa plant, under the cell lines MCF-7 (breast adenocarcinoma), A549 (lung cancer) and HuH7.5 (liver cancer). We found that GFA (25, 50, 100, 200 and 400 nM) was able to reduce the viability and number of cells tested. In investigating the role of caurenic acid (CA), also considered a diterpene, AC was able to inhibit the cellular viability of RAW 264.7, Hela, HepG2 and HT-29 leukemic macrophages, by inducing apoptosis via the mitochondrial pathway (Hueso-Falcón et al., 2010).

Apoptosis is a physiological process that seeks to eliminate a specific group of cells, in order to preserve the body's homeostasis. Its activation can occur through the extrinsic pathway (via TNF death receptors) (Okada and Mak, 2004), or by the intrinsic (mitochondrial) pathway, activated by intracellular or extracellular stress such as growth factor deprivation, DNA damage, hypoxia or activation of oncogenes (Finkel, 2001).

In fact, numerous studies on apoptosis point to mitochondria as the main target of this type of death, which occurs through increased mitochondrial permeabilization and consequent release of pro-apoptotic molecules present in it (Kim et al., 2006). It was this phenomenon, increased mitochondrial permeabilization, that we observed in the treatments with the GFA, suggesting that apoptosis is a possible mechanism of death induced by this diterpene.

There are other morphological and biochemical characteristics that confirm cell death by apoptosis, among them: loss of cell volume, condensation and nuclear fragmentation, formation of apoptotic bodies, phosphatidylserine externalization, mitochondrial changes and activation of caspases (Bortner and Cidlowski, 2002). Among the aforementioned, cell volume loss was also another characteristic observed in the three tumor lines treated with 200 and 400 nM GFA. This characteristic of cell volume loss or cell shrinkage, also called apoptotic volume decrease (DVA) has been a ubiquitous aspect of apoptosis, which ironically has been ignored, particularly from the standpoint of regulation. This process occurs passively, to facilitate the breaking of the cell into smaller apoptotic bodies, aiding in its eventual phagocytosis by neighboring cells or macrophages (Bortner and Cidlowski, 2002).

Apoptotic cell death is also accompanied by a change in plasma membrane structure, termed phosphatidylserine surface exposure, which can be detected by its affinity for annexin V (van Engeland et al., 1998), which is also observed in our results.

Recent research has drawn attention to the plasma membrane. The formation of pores in the plasma membrane increases its permeability, and consequent rupture in a process called secondary necrosis in cells that initiated the process of apoptosis (Rogers et al., 2017). Our data show an increase in fluorescence for PI, indicating a change, or loss of plasma membrane integrity, leading to a late apoptosis (Berghe et al., 2010).

Studies also show that apoptotic cell death caused by oxidative stress stimuli undergoes a transition to secondary necrosis, by mechanisms that inactivate caspases, by oxidation of their thiol group, or by a reduction in ATP levels, due to a decrease in function mitochondrial (Chandra et al., 2000; McConkey, 1998).

In addition to apoptosis, autophagy is also characterized by a "clean" cell death pattern, as it is an important process in cell metabolism because it maintains the balance between synthesis and degradation of cellular products. In our results we also found a decrease in lysosomal viability and an increase in autophagy, through the accumulation of monodanzilcadaverina, a fluorescent selective marker for vacuoles (Biederbick et al., 1995), aspects related to the induction of autophagy.

In the pathogenesis of cancer, autophagy may present a double role, and may be supra-regulated to provide tumor cell survival and proliferation in conditions of lack of oxygen and nutrients (Tang et al., 2010). However, some authors have supported the idea of autophagy induction as a mechanism of cell death, since autophagy characteristics have been observed in dead cells (Kanzawa et al., 2005; Shao et al., 2004), in situations of prolonged stress, when the turnover of proteins and organelles overwhelms the capacity of the cell. In renal cell carcinoma cells, a novel synthetic molecule (STF-62247) has been shown to promote cell death by inducing autophagy (Turcotte et al., 2008).

It is also known that in some solid and hematological malignancies, oxidative stress (ROS production) can induce apoptosis, and this can be explored in selective therapy strategies for cancer (Liu and Wang, 2015)

It is well established that the cytotoxicity of the chemotherapeutic agents Tamoxifen and Paclitaxel is associated with the accumulation of ROS and NO (nitric oxide) (Liu and Wang, 2015; Ramanathan et al., 2005). It is possible that the GFA-induced cytotoxicity had a behavior similar to that observed in the usual chemotherapeutic agents, since in fact we also observed alteration in mitochondrial permeability, ROS production, and increased fluorescence associated with apoptosis in all investigated cells.

Further confirming our data that altered mitochondrial permeability would induce apoptosis induction via ROS formation, it was soon discovered that the TRAP1 chaperone, a

protein restricted to mitochondria (Bekele et al., 2016), specifically in the matrix and associated with the inner membrane (Felts et al., 2000), is responsible for the regulation of mitochondrial dynamics (Cechetto and Gupta, 2000), and in tumor progression, it is believed that this protein protects tumor cells from treatments against various antineoplastic agents (Gesualdi et al., 2007; Takamura et al., 2012).

It is also known that in tumor cells, an increase in these reactive oxygen species increases the risk of oxidation and consequent lethal opening of the PTP (transition pore of mitochondrial permeability); therefore, malignant cells should keep PTP closed to avoid apoptotic events (Cairns et al., 2011).

In this long-running endeavor, one of the most important advances in the search for new therapies against cancer is the recognition that apoptosis is an essential phenomenon in the elimination of tumor cells. Thus a compound will only be considered an excellent anticancer drug candidate if it kills and / or incapacitates cancer cells without causing excessive damage to normal cells.

To that end, we can suggest that the diterpenoid tested is a promising candidate because it was able to promote several morphological and biochemical alterations related to apoptosis, including the production of oxidative stress constituents (ROS), fundamental molecules that actively participate in the destruction of tumor cells.

CONTRIBUTIONS OF THE AUTHOR

Tatiane Renata Fagundes (Study concepts, study design, bibliographic research, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript revision), Vírgínia Márcia Concato (experimental studies, data acquisition, data analysis, statistical analysis), Bruna Taciane da Silva Bortoleti (experimental studies), Aedra Carla Bufalo Kawassaki (experimental studies, data analysis, statistical analysis), Fernanda Tomiotto-Pellissier (review of the manuscript, data analysis, statistical analysis), Manoela Daiele Gonçalves (extraction of the compound, experimental study), Taylon Felipe Silva (preparation of the manuscript), Carolina Panis (study design, manuscript revision), Nilton Syogo Arakawa (handwriting review), Ivete Conchon-Costa (handwriting review), Wander Rogério Pavanelli (handwriting review). All authors read and approved the final manuscript.

ABBREVIATIONS

CaCl2	Calcim chloride
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
COX-2	Cyclooxygenase-2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
GFA	Grandiflorenic acid
H2O2	hydrogen peroxide
IC50	inhibitory concentration
LPS	Lipopolysaccharide
MTT	3-(4,5-dimethylthi azol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
PGE2	prostaglandin E(2)
ROS	Reactive Oxygen Species
TMRE	Tetra-Methylrhodamine Ester
WHO	World Health Organization

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INTEREST CONFLICTS

All authors declare no conflict of interest.

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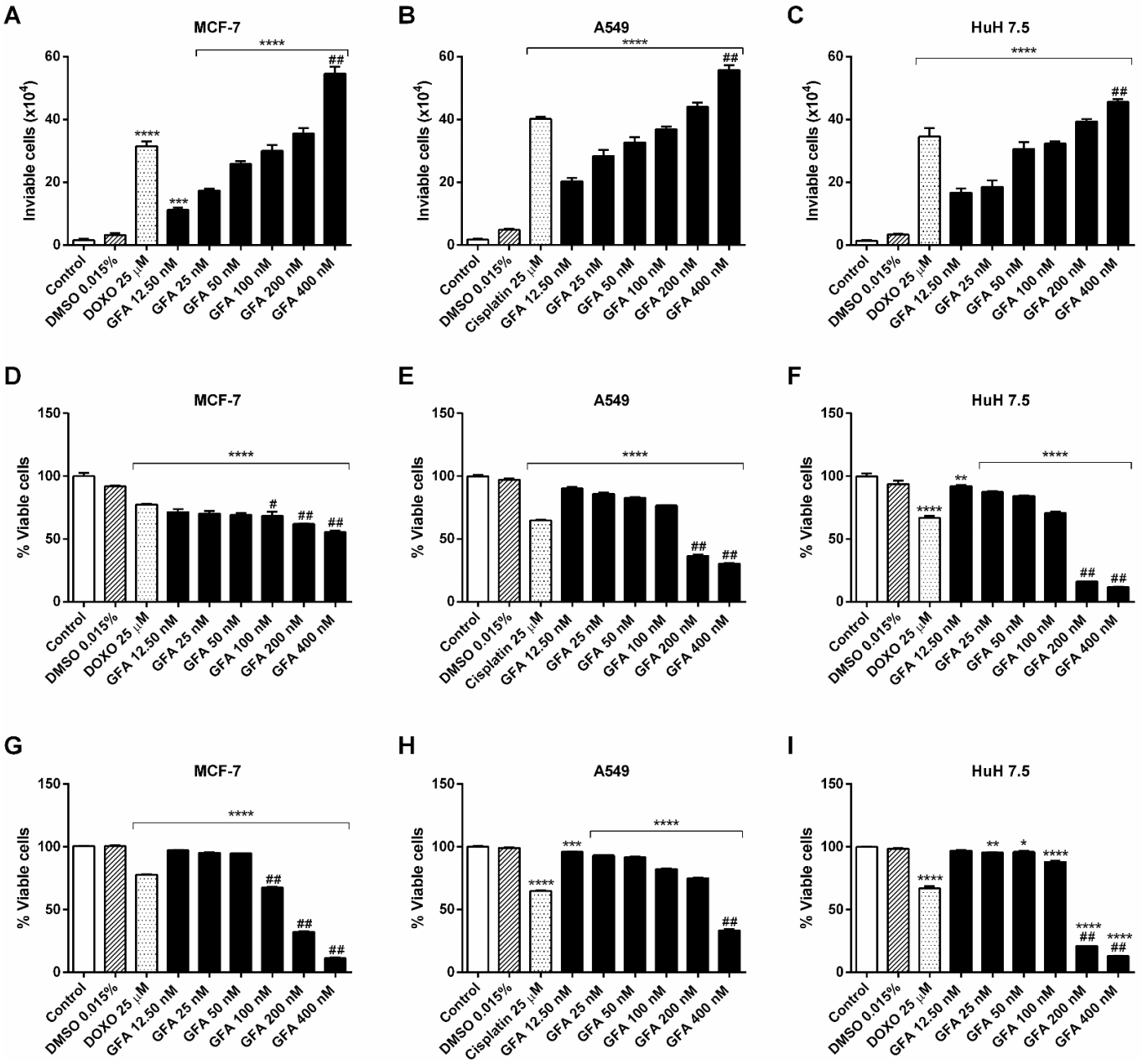

Figure 1. Viability of GFA-treated MCF-7, A549 and HuH 7.5 tumor cells. Cells were treated with GFA at concentrations of 12.50; 25; 50; 100; 200 and 400 nM for 24 h and analyzed by tripan blue counting (A, B and C), MTT viability test (D, E and F) and neutral red (G, H and I). Control (DMEM medium), 0.015% DMSO (vehicle) and 25 μM doxorubicin or cisplatin (positive control). The values represent the mean ± SEM of three different experiments performed in duplicate. * Significant difference in relation to control ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$). #Significant difference in relation to the positive control ($p \leq 0.05$) and ## ($p \leq 0.0001$).

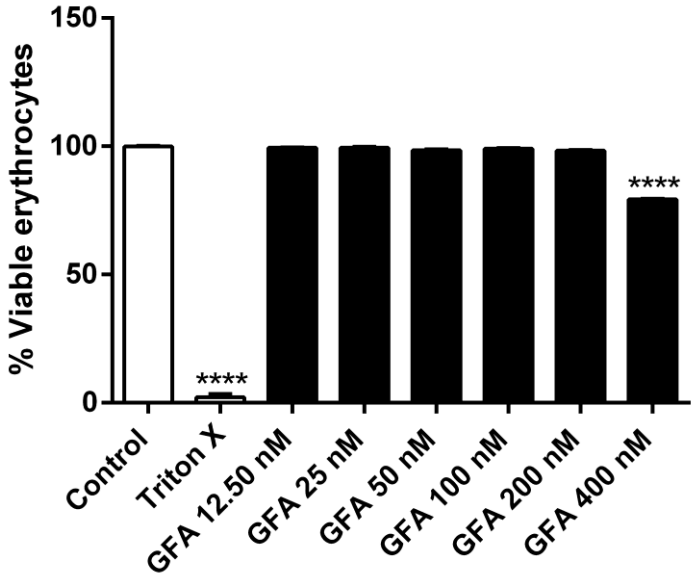

Figure 2. GFA cytotoxicity in ovine erythrocytes. The erythrocytes were treated with GFA at the concentrations of 12.5, 25, 50, 100, 200 and 400 nM for 3h to determine the percentage of viable erythrocytes. Control (erythrocytes + PBS), 0.1% DMSO (vehicle) and Triton X (positive control) for hemolysis. Values represent the mean ± SEM of three independent experiments performed in triplicate. ****Significant difference in relation to the negative control ($p \leq 0.0001$).

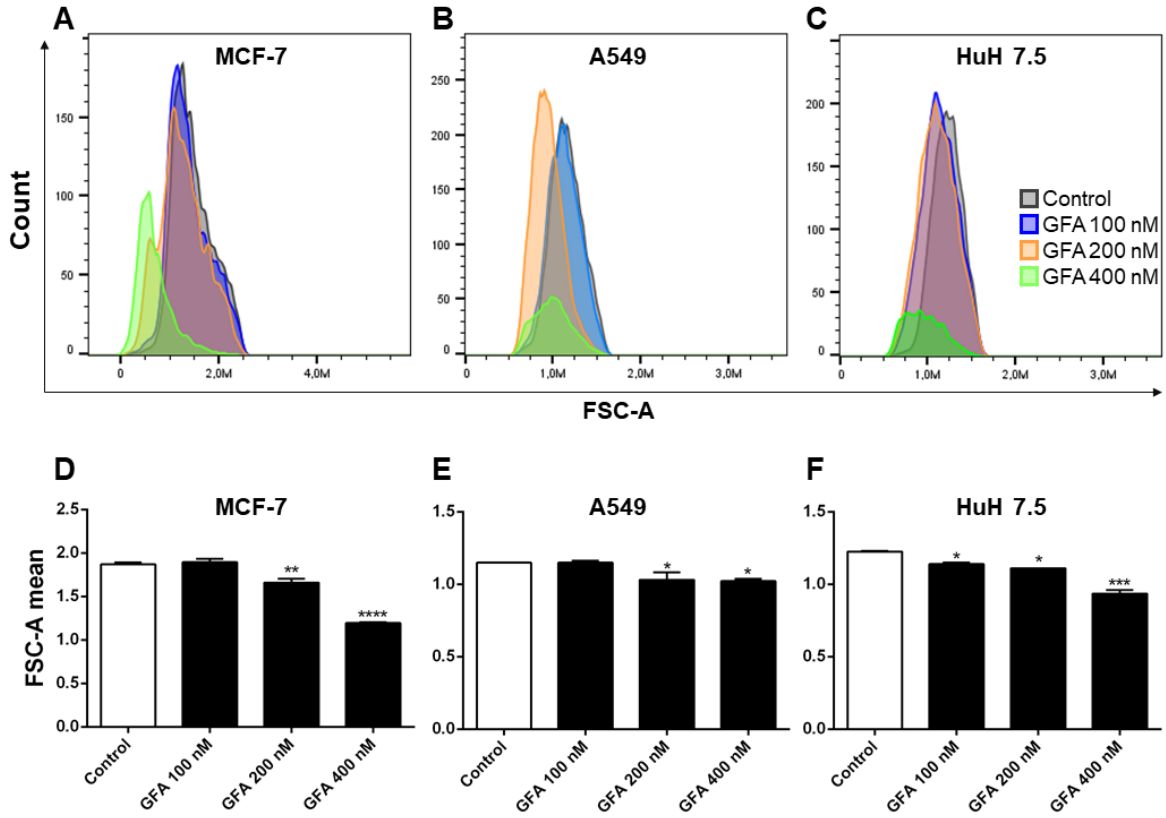

Figure 3. Cell volume of MCF-7, A549 and HuH 7.5 tumor lines after GFA treatment. Cells were treated with GFA at concentrations of 100, 200 and 400 nM and the cell volume was analyzed by flow cytometry. The FSC-A parameter was considered a function of cell size. Tumor line MCF-7 (A), A549 (B) and HuH 7.5 (C). Typical histograms of at least three independent experiments are shown. Mean ± SEM of the fluorescence intensity of FSC-A are presented with respect to lineage MCF-7 (D), A549 (E) and HuH 7.5 (E). *Significant difference in relation to control ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$).

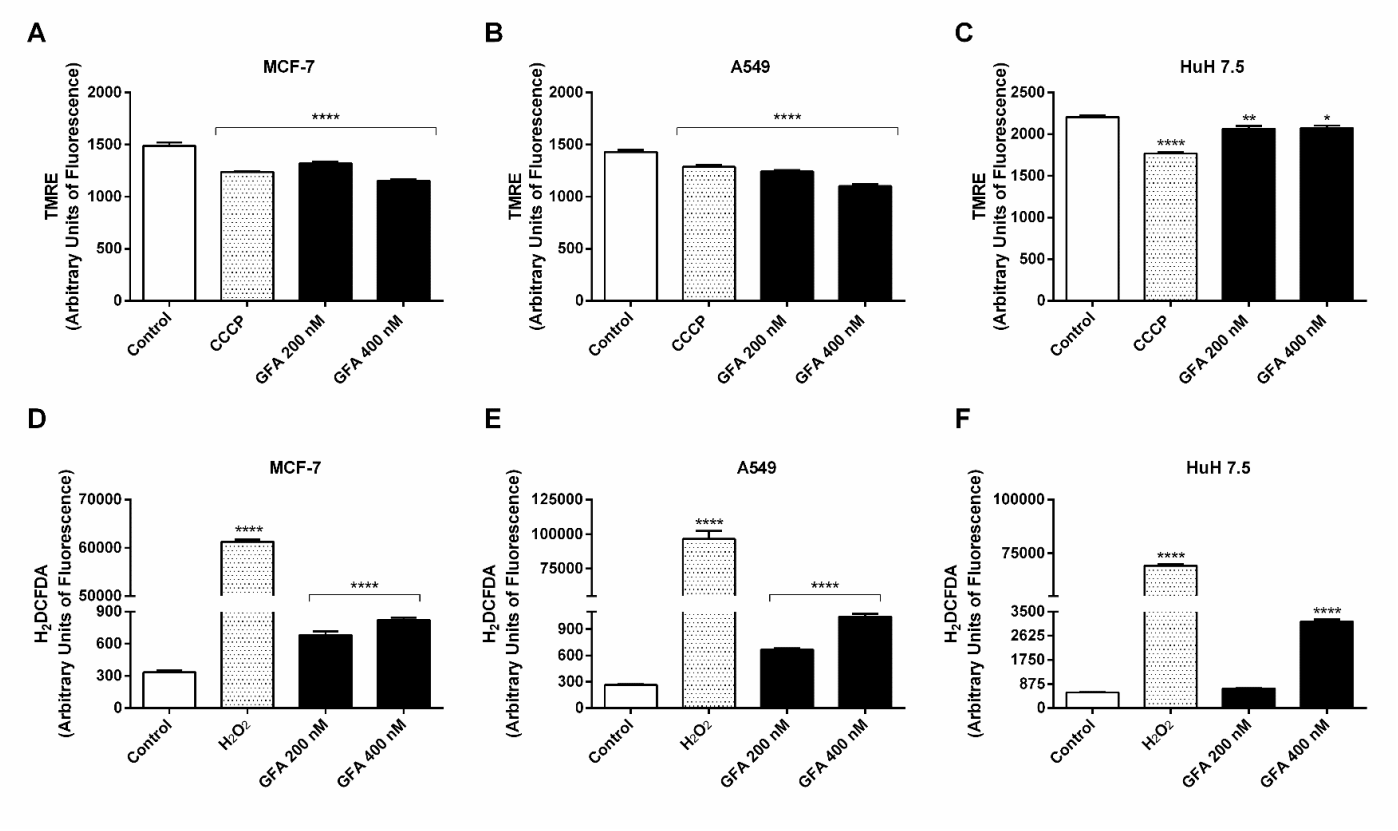

Figure 4. Mitochondrial depolarization and production of reactive oxygen species produced by GFA-treated MCF-7, A549 and HuH 7.5 tumor cells. Cells were treated with GFA at concentrations of 200 and 400 nM for 24 h and analyzed by the TMRE probe to verify the mitochondrial membrane (A) and H2DCFDA potential for analysis of reactive oxygen species (B) levels. CCCP and H2O2 were used as positive controls. The values represent the mean ± SEM of three different experiments performed in duplicate. * Significant difference in relation to control ($p \leq 0.05$), ** ($p \leq 0.01$) and **** ($p \leq 0.0001$).

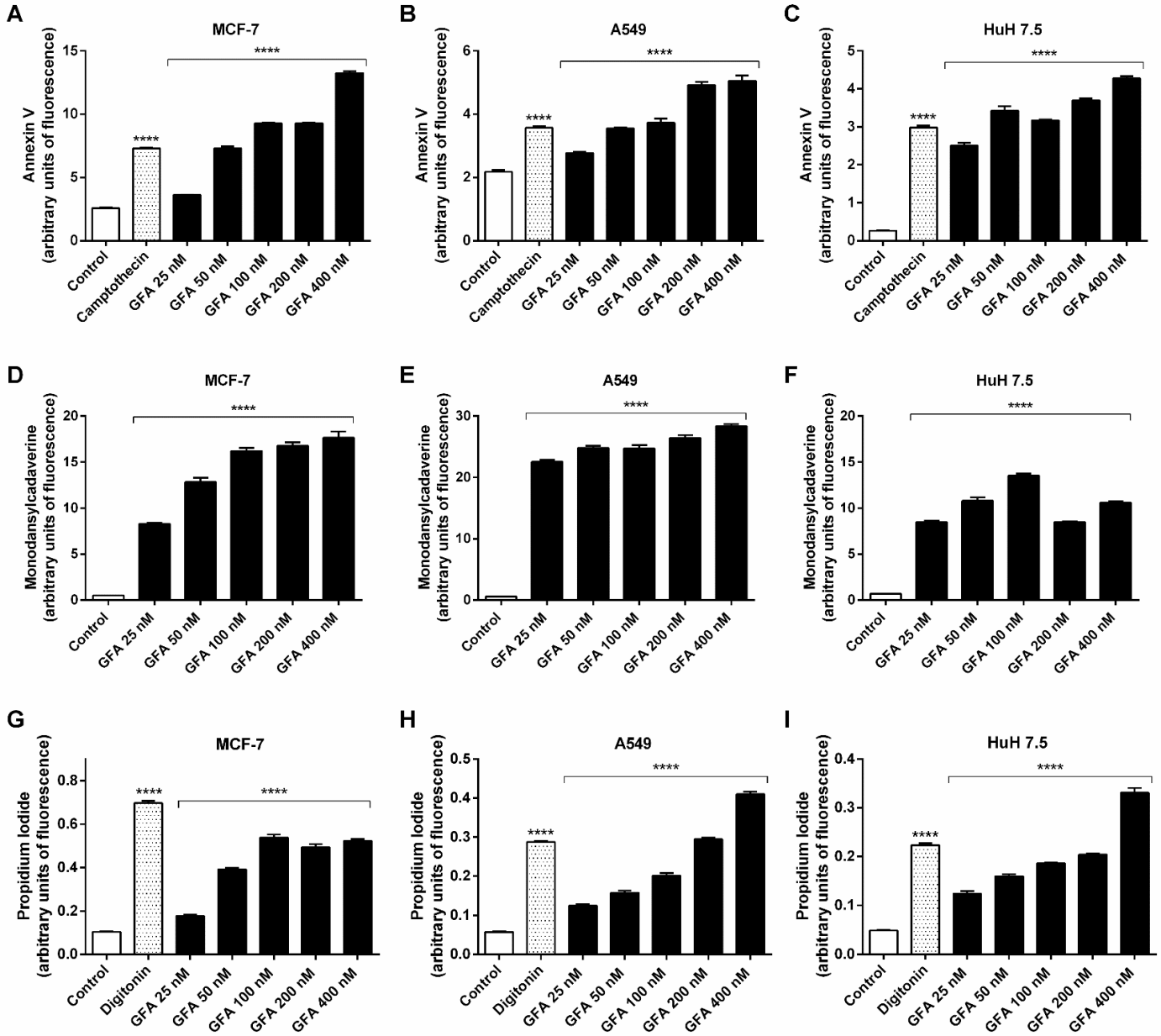

Figure 5. GFA-induced death mechanisms in MCF-7, A549 and HuH 7.5 tumoral lines. Cells were treated for 24 h with GFA at concentrations of 25, 50, 100, 200 and 400 nM and analysis of phosphatidylserine exposure by annexin V (A, B and C) labeling, autophagic vacuole formation by monodansilcadaverine labeling (D, E and F) and plasma membrane integrity analysis by propidium iodide (G, H and I). The data represent the mean ± SEM of three independent experiments performed in duplicate. **** Significant difference in relation to control ($p \leq 0.0001$). Camptothecin and digitonin were used as positive controls.

CONCLUSÃO

- As tioidantoínas diminuíram a proliferação celular da linhagem MCF-7 de câncer de mama, através da diminuição da viabilidade mitocondrial, lisossomal e azul de tripan. Houve aumento da produção de ROS, aumento da exposição de fosfatidilserina, perda de integridade de membrana, parada do ciclo celular em G1, além da formação de vacúolos autofágicos, apontando seu efeito como possível inibidor de reparo de DNA, causado por ROS. Nossos resultados mostram que as tioidantoínas devem ser melhores investigadas para melhor elucidação das vias de sinalização ligadas à autofagia e apoptose.
- O tratamento com ácido grandiflorênico sob as linhagens celulares MCF-7, A549 e HuH7.5 induziu citotoxicidade dose dependente, aumento da produção de ROS, despolarização de membrana mitocondrial, aumento da exposição de fosfatidilserina e diminuição do volume celular, aspectos característicos da morte celular apoptótica. Além disso, observamos eventos relacionados ao aumento da formação de vacúolos autofágicos, uma via que precisa ser pesquisada em relação ao seu caráter antitumoral.
- A busca por compostos que sejam eficazes contra as células tumorais e causem menos efeitos colaterais as células normais é o foco de pesquisas para terapias antitumorais. Assim, podemos concluir que os compostos usados devem ser investigados detalhadamente, devido ao efeito indutor de produção de ROS, que pode ser um importante mecanismo de morte às células tumorais na criação de terapias quimioterápicas efetivas para o câncer.

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