Universidade Estadual de Londrina

LARISSA JULIANI SANCHES

AVALIAÇÃO DO EFEITO CITOTÓXICO DO CITRAL SOBRE CÉLULAS DE MELANOMA MURINO METASTÁTICO B16F10

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RESUMO

O melanoma representa 4% dos cânceres de pele registrados no Brasil. Quando diagnosticado precocemente pode ser removido cirurgicamente, mas quando a doença encontra-se disseminada é comum ser refratária às terapias disponíveise as terapias oferecidas são apenas paliativas. A resistência observada muitas vezes está associada a uma menor suscetibilidade à apoptose e alterações nas vias de proliferação e sobrevivência celular. As espécies vegetais são fontes de compostos naturais que apresentam atividades de interesse na preservação da saúde e no tratamento de doenças, varias classes de compostos químicos já foram descritos em literatura, mas alguns compostos como o citral foram pouco estudados. O citral é um aldeído alifático de ocorrência natural e é encontrado na forma de mistura isomérica de neral (cis) e geranial (trans). Por apresentar aroma de limão é utilizado como aromatizante de alimentos, bebidas e doces, assim como na produção de fragrâncias para artigos cosméticos e de higiene. Apesar de existirem poucos estudos com o citral, os existentes exibem atividades antiproliferativa e/ou citotóxicas sobre algumas linhagens de células neoplásicas. O presentre trabalho avaliou o efeito do citral sobre células murinas de melanoma metástatico (B16F10), cultivadas a $2x10^5$ em placas de 24 poços, em meio de cultura Dulbecco's Modified Eagle Medium (DMEM, Gibco), suplementado com 5% de soro fetal bovino (SBF, Gibco) e 0,1% de solução antibiótica e antimicótica (Santa Cruz) e incubadas em estufa (Sanyo) a 37º C, com 5% de CO_2 com umidade controlada de 95%. As células foram expostas ao citral por 24 horas nas concentrações0,1; 0,5; 1,0e 2,5μM, todos os experimentos foram realizados juntamente como grupo controle com DMSO na concentração de 1% em cultura.Após o período de tratamento, observamos redução na viabilidade célular a partir da concentração 0,5 μM. O IC_{50} foi estimado em 1,04 μM para 24 horas de tratamento e em 0,77 μM para 48 horas.Foram realizados ensaios de citotoxicidade e proliferação celular através do teste por exclusão com azul de Tripan. O citral foi capaz de reduzir a viabilidade a partir da concentração 0,5 μM para 24 e 48h, enquanto a proliferação celular reduziu a partir da concentração 0,5 μM para 24h e 0,05 μM para 48h, sugerindo efeito antiproliferativo nas concentrações 0,05 e 0,1 μM para 48h. Para determinar os padrões de morte celular estavam envolvidos, realizamos o teste de diferencial de morte com brometo de etídio/larajado de acridina(BE/LA), o teste de TUNEL para confirmar apoptose, o Lactato desidrogenase (LDH) liberado no meio de cultura para avaliar a necrose. No teste de coloração diferencial BE/LA o citral foi observado padrão apoptótico de morte celular a partir da concentração 0,5 μM e foi confirmado pelo teste TUNEL. O padrão necrótico foi observado a partir da concentração 1,0 μM, confirmado pela dosagem de LDH. Avaliamos a formação de vacúolos autofágicos através da coloração com monodansilcadaverina (MDC) e observamos indução de autofagia na concentração 1,0 μM. A indução de lesão de DNA foi avaliada pelo teste do Cometa em pH alcalino, onde foi observada indução de lesão a partir de 0,5 μM. Para determinar os parâmetros de estresse oxidativo realizamos a dosagem de glutationa reduzida (GSH), de malondialdeído (MDA), sendo observado consumo de GSH a

partir da concentração 0,5 μM e aumento de MDA para concentração 2,5 μM. Para avaliar as espécies reativas envolvidas dosamos os níveis de óxido nítrico (NO) e utilizamos conhecidos sequestradores de espécies reativas de oxigênio (Tempol, trolox e L-histidina), onde observamos uma redução significativa dos níveis de NO e proteção contra o efeito citotóxico do citral para todos os sequestradores utilizados. Para verificar se o citral apresenta atividade oxidante direta realizamos o teste de consumo de oxigênio induzido por T-butil hidroperóxido em eritrócitos, onde não observamos efeito oxidativo direto do citral. Para elucidar a capacidade do citral de interferir em vias de sinalização intracelular, realizamos ensaios de imunocitoquimica para a proteínas ERK1/2, Pi3K,Akt, p53 e NFkB. O citralfoi capaz de diminuir os níveis de marcação imunocitoquímica para ERK1/2 celular na concentração 1,0 μM e nuclear nas concentrações 0,5 e 1,0 μM. Para as marcações de Pi3K e a Akt também observamos diminuição nas concentrações 0,5 e 1,0μM, enquanto para a p53 observamos aumento da marcação nas concentrações 0,5 e 1,0 μM. O citral foi capaz de aumentar a marcação de NFkB celular na concentração 1,0μM e diminuir a marcação nuclear do NFkB nas concentrações 0,5 e 1,0 μM. Finalmente para verificar a citotoxicidade do citral sobre células normais, cultivamos e tratamos as células de fibroblastos murino NIH-3T3nas mesmas condições que as células B16F10 e observamos que citral também induz citotoxicidade com IC_{50} 24h estimado em 2,5 μM. Nossos resultados concordam com resultados obtidos por outros autores para outras linhagens de células tumorais. Confirmamos que o mecanismo de ação do citral envolve aapoptose e as proteínas p53 e NFkB, e acrescentamos o envolvimento de autofagia, do estresse oxidativo e das proteínas ERK 1/2 e Pi3K. O citral não apresentou seletividade, uma vez que apresente citotoxicidade sobre as células normais NIH-3T3, no entanto estas apresentam uma resistência maior ao citral (IC_{50}de 2.5 μM 24h). Os resultados promissores *in vitro*apontam que o citral deve ser investigado *in vivo* em modelos experimentais de melanoma para averiguar seu efeito antitumoral no melanoma.

Palavras-chave: Melanoma. B16F10. Citral. Apoptose. Autofagia. Estresse oxidativo. Imunocitoquímica.

SANCHES, Larissa Juliani. **Evaluation citral-induced cytotoxicity in murine metastatic melanoma cells, B16F10.** 2014. 68p. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2014.

ABSTRACT

Melanoma represents 4 % of skin cancers recorded in Brazil. When early diagnosed, melanoma can be surgically removed, but when the disease is spread it is common to be refractory to available therapies and offered therapies are often palliative. The observed resistance is associated with decreased susceptibility to apoptosis and changes in the process of cell proliferation and survival. The plant species are sources of natural compounds which have interesting activities in health preservation and disease treatment, various classes of chemical compounds have been described in the literature, but some compounds such as citral, have a fewnumber of scientific studies. Citral is a naturally occurring aliphatic aldehyde and it is found in an isomeric mixture ofneral (cis), and geranial (trans). As citral has peculiar lemon scent, it is used as a food flavoring as well as in the production of fragrances for cosmetics and toilet products. Although there are few studies, antiproliferative and / or cytotoxic activity against cancer cells lineages have been described for citral. The presente study evaluated the effect of citral on murine cells metastatic melanoma (B16F10), cultured at $2x10^5$ in 24 well plates inDulbecco 's Modified Eagle Medium (DMEM , Gibco) supplemented with 5% fetal bovine serum (FBS , Gibco) and 0.1% antibiotic and antimycotic (Santa Cruz), and incubated in an incubator (Sanyo) at 37 ° C with 5 % CO_2 with controlled humidity of 95 %. Cells were exposed for 24 hours to citral at concentrations 0.1; 0.5; 1.0 and 2.5 µM, a control group with DMSO at a concentration of 1 % in culture, was also performed. After the treatment period, we observed a reduction in cell viability at concentrations higher than 0.5 µM. The IC_{50} was estimated at 1.04 µM for 24 h, and 0.77 µM for 48 h. Citral was able to reduce cell viability (by Trypan blue exclusion assay) at concentrations higher than 0.5 µMfor 24 and 48h, while reduced cell counting was observed for concentrations 0.5 µMfor 24h and 0.05 µM for 48 hours. To determine the patterns of cell death, we performed a differential colorimetric staining withethidium bromide/acridine orange (EB/AO), TUNEL assay to confirm apoptosis, measurement of lactate dehydrogenase (LDH) released into the culture medium to confirm necrosis. In differential staining EB/AO citral induced apoptotic pattern of cell death at concentrations higher than 0.5 µM, confirmed by TUNEL assay. Necrotic pattern was observed at concentrations higher than 1.0 µM, confirmed by LDH measurement. We evaluated the formation of autophagic vacuoles by monodansilcadaverina (MDC) staining, and we observed the induction of autophagy in citral 1.0 µM. The induction of DNA damage was evaluated by Comet assay in alkaline pH, citral induced DNA lesions in concentrations higher than 0.5 µM. To determine the parameters of oxidative stress we performed the measurement of reduced glutathione (GSH), and malondialdehyde (MDA) levels. GSH consumption was observed at concentrations higher than 0.5 µM and increased MDA levels were observed for citral 2.5 µM. To evaluate the reactive species involved in observed oxidative stress, we measured nitric oxide (NO) levels and used well-established scavengers of oxygenreactive species (Tempol, trolox and L-histidine). We observed a significant reduction in NO levels and protection against the cytotoxic effect of citral used scavengers. Direct oxidant activity was evaluated by

t-butyl hydroperoxide-induce oxygen uptake in red blood cells test, and we observed no direct oxidative effect of citral. To elucidate the ability of citral to interfere with intracellular signaling pathways, we performed immunocytochemistry assays for ERK1/ 2, PI3K, Akt, p53, and NFkB proteins. Citral decreased cytoplasmatic ERK 1/2 labeling in concentrationshigher than 1.0 μM and nuclear ERK 1/2 labeling in concentrations higher than 0.5 and 1.0 μM. PI3K and Akt labelling were also decreased in the concentrations 0.5 and 1.0 μM, p53 labelling was increased. Citral also increasedcytoplasmatic NFkB labelling in the concentration of 1.0 μM, but decreased nuclear NFkB labelling at 0.5 and 1.0 μM. Finally, to check the cytotoxicity of the citral on normal cells and treated normal murine fibroblast NIH-3T3 in the same conditions of B16F10 cells, and observed that citral was also able to induce cytotoxicity in NIH-3T3, citral was not selective since it presented cytotoxicity againstNIH-3T3 cells, however these cells showed a higher resistance against citral (IC_{50} 2.5 mM 24h). Our results agree with scientific literature for other tumor cell lines. We confirmed that the mechanism of action of citral involves apoptosis as well as p53 and NFkB proteins, but also contributed to the knowledge of its mechanisms by including the involvement of autophagy, oxidative stress and ERK1/2 and PI3K proteins.The promising resultsobserved *in vitro* indicate that citral should be investigated in*in vivo* experimental models evaluate its antitumor effect against melanoma.

Keywords: Melanoma. B16F10. Citral. Apoptosis. Autophagy. Oxidative stress. Immunocytochemistry .

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LISTA DE ABREVIATURAS E SIGLAS

Akt	Proteína quinase B
BE/LA	Brometo de etídio/larajado de acridina
B16F10	Células de melanoma murino metástatico
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Ácido desoxirribonucleico
ERK1/2	Quinase regulada por sinais extracelulares
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
IC_{50}	Concentração inibitória 50
INCA	Instituto Nacional do Câncer
LDH	Lactato desidrogenase
MCF-7	Células de câncer de mama
MDA	Malondialdeído
MDC	Monodansilcadaverina
MTT	(brometo de (3 -. (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio)
NB4	Células de leucemia promielocítica aguda
NFkB	Fator nuclear de transcrição kB
NIH-3T3	Células de fibroblastos murino
NO	Óxido nítrico
Pi3K	Fosfatidilinositol-3,4,5-trisfosfato
p53	Supressor tumoral
ROS	Espécies Reativas de Oxigênio
SBV	Soro bovino fetal

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APÊNDICE B – Spearman's correlation matrix of citral 1μM (24 h) effects against B16F10 cells. All variables were log (X+1) transformed before analysis. Values shown are the

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1 INTRODUÇÃO

1.1. Melanoma

Os melanócitos são células localizadas na camada basal da epiderme, responsáveis pela produção de melanina. Embora se localizem na pele, estas células alcançam o tecido epitelial durante o período embrionário. Neste processo, células de alta mobilidade da crista neural migram para o mesênquima da derme e posteriormente migram para a junção dermo-epidérmica diferenciando-se nos melanócitos. A produção da melanina inicia durante o período fetal e após o nascimento esta produção passa a ser estimulada pela irradiação ultravioleta (UV). Devido à produção de melanina, os melanócitos tem uma importante função na pele que é a proteção contra a luz UV, por este motivo estabelecem homeostasia com os queratinócitos. Por outro lado, os melanócitos estão sujeitos à falhas na integridade de seu genoma e ao sofrerem processos de mutação gênica e malignização originam o melanoma maligno cutâneo, um tipos de câncer com maior capacidade de metastização (KADEKARO *et al.*, 2003; KIERSZEMBAUM, 2004; HAASS *et al.*, 2005).

O câncer de pele corresponde a 25% dos tipos de câncer que acometem a população brasileira. Dentre este, o melanoma cutâneo é considerado o mais letal, sua incidência corresponde a 4% dos casos de câncer de pele e e sua incidência vem aumentado rapidamente desde a década de 60 (MEIER *et al.*, 1998; BALDI *et al.*, 2003; LAHN *et al.*, 2004; LEVERKUS *et al.*, 2006; INCA, 2012).

De um modo geral, o melanoma é um exemplo de doença multifatorial com envolvimento de fatores ambientais e genéticos. Do ponto de vista ambiental, a exposição aguda à radiação UV na infância e/ou adolescência, tem sido apontados como importantes fatores de risco, pois aumentam em cerca de 40% a 50% de risco de desenvolvimento de melanoma (MILLER; MIHM Jr, 2006). Esta relação existe, pois a irradiação ultravioleta B (UVB), pode ser absorvida pelo DNA celular resultando em lesões denominadas dímeros de pirimidina, ao mesmo tempo a UVB e a irradiação ultravioleta A (UVA) também geram radicais livres no tecido epitelial que induzem danos em diversas estruturas celular, incluindo o DNA. Na tentativa de reparar estas lesões a célula pode fixar uma mutação em um importante gene

regulador de ciclo celular desencadeando o processo de iniciação do câncer (ARMSTRONG; KRICKER, 2008; NARAYANAN; SALADI; FOX, 2010).

Com relação aos fatores genéticos, podemos salientar: (i) fatores associados ao fenótipo da pele, onde pessoas com pele clara (tipos de pele I e II de Fitzpatrick e Path) são mais susceptíveis ao melanoma; (ii) fatores genéticos que favoreçam o surgimento de nevos melanociticos, também são considerados fatores de risco, uma vez que os nevos podem evoluir para o melanoma (GARCIA; McLAREN; MEYSKENS, 2011; PAEK et al., 2008). Especificamente em relação ao genes, as mutações mais comumente observadas envolvem os genes das proteínas: BRAF (aparece em 81% dos casos sem exposição solar) e NRAS; receptoras c-KIT; quinases ativadas por mitógenos (MAPKs); quinase ERBB4, reguladora de expressão gênica TRRAP, receptoras de glutamato GRIN2A e GRM3, metaloproteinase MMP8, ciclinas CDKN2A e CDK4; entre outras (MILLER; MIHM Jr, 2006; PAEK et al, 2008; KUNZ, 2013).

Embora haja envolvimento de genes no desenvolvimento do melanoma, os exames de biologia molecular não são utilizados para seu diagnóstico. Geralmente a identificação do melanoma se inicia da suspeita de uma lesão de pele, onde o paciente se queixa do surgimento de uma nova lesão pigmentada ou observa modificações de tamanho, formato ou cor de um nevo melanocítico pré-existente. Durante a avaliação o critério ABCDE é bastante importante, pois avalia a presença de assimetria (A), bordas irregulares ou mal definidas (B), coloração mista (C), diâmetro superior a 5 mm (D) e evolução da lesão com aumento de tamanho ou sangramento (E). O resultado positivo para estes critérios é indicativo para a realização de uma biopsia para confirmação do diagnóstico (DOHERTY; WAY, 2004).

Durante o processo de avaliação diagnóstica, o melanoma pode ser avaliado quanto às características macroscópicas e microscópicas do tumor primário. Clinicamente o melanoma é dividido em quatro tipos de acordo com a localização anatômica e o padrão de crescimento do tumor (LEDERMAN; SOBER, 1985):

(i) Melanoma expansivo superficial (MES) é o mais frequente (70% dos casos), aparece principalmente entre a quarta e quinta décadas da vida, preferencialmente no tronco dos homens e membros inferiores das mulheres, o que sugere que esta forma clinica esteja associada à

exposição solar. Aparece junto a um nevo pré-existente, e tende a apresentar um crescimento radial, com invasão da derme e metástases mais tardias.

(ii) Melanoma nodular (MN) é o segundo mais comum (15 a 30% dos casos), ocorre mais frequentemente entre 50 e 60 anos de idade, na proporção de 2:1 para o sexo masculino, apresenta-se como lesão papulosa ou nodular, elevada, de cor castanha, negra ou azulada, com crescimento vertical, metástases precoce, sendo freqüentes os processos de ulceração e sangramento.

(iii) Melanoma lentiginoso acral (MLA) ocorre nas regiões palmoplantares, extremidades digitais, mucosa e semimucosas; é mais freqüente em indivíduos não-caucasianos (35 a 60%) e é o mais freqüente na faixa etária dos 70 anos de idade. Nas extremidades digitais o MLA apresenta-se como uma lesão tumoral acastanhada subungueal, melanoníquia estriada, fragmentação longitudinal da lâmina ungueal, além de paroníquia crônica e persistente. Do ponto de vista histológico o MLA é o tipo o mais agressivo dentre os melanomas.

(iv) Melanoma lentigo maligno (MLM), considerado o menos frequente (apenas 5% dos casos) e ocorre geralmente em idosos; surge em áreas de foto exposição crônica, apresenta-se como mancha acastanhada ou enegrecida de limites nítidos e irregulares, alcançando vários centímetros de diâmetro, localizada na face (90% dos casos), em mãos e membros inferiores (10% dos casos)

O estadimanto do tumor pode ser realizado através de parâmetros histológicos e clínicos. Do ponto de vista histológico pode ser determinado pelo nível de Clark e pelo índice de Breslow, sendo o segundo mais utilizado no Brasil. O índice de Breslow avalia a profundidade do tumor em milímetros sendo indispensável para o prognóstico. Já do ponto de vista clínico, o estadiamento é realizado com base no: (i) tamanho do tumor em relação à profundidade na derme (T, variando de 1 a 4), acometimento linfonodal (N, variando de 0 a 3) e na presença de metástases à distância (M, variando de 0 a 1). Desta forma, para o melanoma, os resultados encontrados na biopsia são extremamente importantes no prognóstico e

escolha do tratamento mais apropriado (FREEDBERG *et al.,* 2003; TSAO; ATKINS; SOBER, 2004; THOMPSON; SCOLYER; KEFFORD, 2005).

Quando diagnosticado precocemente, sem evidência de metástase, o tratamento preconizado é a remoção cirúrgica. Porém, quando encontra-se disseminado para linfonodos ou há presença de metástases, o tratamento envolve processos cirúrgicos associados à quimioterapia, radioterapia e/ou imunoterapia. Quando o melanoma já iniciou seu processo de disseminação, é comum ser refratário às terapias disponíveis, e na maioria das vezes é incurável (EMMETT; DEWING; PRITCHARD-JONES, 2007; KORN *et al*., 2008).

No Brasil, o quimioterápico mais utilizado é a dacarbazina (BECHARA *et al.* 2013), um agente alquilante de DNA e inibidor dos mecanismos de reparo, que tem como objetivo induzir lesões de DNA e como resultado induzir a apoptose nas células neoplásicas (TEMERK; IBRAHIM, 2014). No entanto, células de melanoma costumam apresentar resistência extraordinária à apoptose (GRAY-SCHOPFER; WELLBROCK; MARAIS, 2007), dentre os mecanismos de resistência podemos citar: (i) expressão dos membros das famílias de proteínas inibidoras da apoptose (IAPs) que têm sido associados com a progressão tumoral, e sua expressão pode ser observada já no início da disfunção melanocítica (nevos melanocíticos), e em maior quantidade em melanomas metastáticos altamente invasivos (SOENGAS; LOWE, 2003); (ii) expressão da cFLIP (proteína inibidora da FLICE), um inibidor da ativação de caspases (especialmente da caspase 8), também tem sido associado à resistência quimoterápica (BULLANI *et al*., 2001).

Devido à alta agressividade e baixa resposta terapêutica deste tipo de tumor, o melanoma é foco de estudos de prospecção de novos fármacos que apresentem uma melhor resposta terapêutico. Nestes estudos vários modelos são utilizados, mas um modelo que ganha bastante destaque é o uso das células metastáticas de melanoma murino B16F10.

1.2. Células B16F10

Isaias J. Fidler foi o pioneiro em realizar estudos com as células B16. Várias formas B16 foram isoladas, e algumas diferenças como o potência metastática foram encontradas entre as sub-linhagens da B16 (FIDLER; NICOLSON, 1976; FIDLER; BUCANA, 1977). As células B16F10 foram obtidas por FIDLER (1973) selecionando

as células com maior capacidade de colonizar os pulmões a partir de uma variante parental menos metastática. A linhagem B16F10 é amplamente utilizada nos laboratórios como modelo para melanoma murino, por ser altamente agressiva, crescer em frascos de cultura, possuir capacidade de gerar tumores subcutâneos ou metástase pulmonar de acordo do método de inoculação utilizado (MENON, KUTTAN; LUTTAN, 1995; HART, 1979). Para se ter uma idéia da importância deste modelo, somente nos primeiros três meses de 2014, já foram publicados 41 trabalhos utilizando essa linhagem celular (site utilizado: PubMed, palavra-chave: B16F10).

Alguns trabalhos utilizam outros tipos celulares para comparação de resultados *in vitro*. Um dos tipos utilizados é NIH-3T3, um fibroblasto de embrião de Swiss albino isolado em 1962, na Faculdade de Medicina da Universidade de Nova Iorque por Geroge Todaro e Howard Green. Trata-se de um tipo celular imortalizado por hipertriploidia, mas apresenta comportamento fenotípico normal e é classificada internacionalmente como linhagem não tumoral e bastante utilizada em transformação e malignização celular (GREIG *et al.*, 1985).

1.3. Citral

As plantas são conhecidas fontes de compostos naturais que apresentam atividades de interesse na preservação da saúde humana e no tratamento de doenças. Muitos compostos farmacêuticos foram descobertos através da triagem de produtos naturais, como é o caso dos quimioterápicos alcaloides da vinca, a vincristina e a vimblastina (NEWMAN; CRAGG; SNADER, 2000; ROCHA; LOPES; SCHUWARTSMANN, 2001). Apesar de haver grande quantidade de estudos com compostos químicos em relação ao câncer, o citral é um composto pouco estudado, havendo ausência de pesquisas em relação ao melanoma.

O citral é composto por uma mistura de isômeros neral (cis) e geranial (trans). Sua característica mais marcante é seu forte aroma de limão, razão pela qual é utilizado como aromatizante de alimentos, bebidas e doces, assim como na produção de fragrâncias para artigos cosméticos e de higiene (LORENZI; MATOS, 2002; LEAL *et al.*, 2003; DUDAI *et al.*, 2005). O citral é naturalmente encontrado em algumas plantas como: capim-limão (*Cymbopogon citratus*), verbena (*Verbena*

bonariensis) e melissa (*Melissa officinalis* L.) (NAKAMURA *et al*., 2003; DUDAI *et al*., 2005).

Embora várias atividades biológicas tenham sido descritas para o citral, tais como ação antibacteriana, antifungica, antiparasitária e inseticida (VINITKETKUMNUEN; PUATANCHOKCHAI; KONGTAWELERT., 1994), com relação ao câncer existem poucos estudos.

Em 2005, Dudai *et al.* observaram que o citral induz apoptose em células neoplásicas através da ativação da caspase 3. Em 2009, Chaoki *et al.* detectaram inibição da proliferação celular em células de câncer de mama MCF-7, com parada do ciclo na fase G2/M e indução de apoptose. Mais recentemente, Xia *et al.* (2012) relataram atividade antiproliferativa do citral sobre células de leucemia promielocítica aguda NB4, sendo que esta atividade foi relacionada com a ativação da caspase 3 por via intrínseca, com o aumento da expressão da proteína pró-apoptótica bax e com redução da expressão de da proteína anti-apoptótica bcl-2.

Para a prospecção de novos fármacos para o tratamento do câncer, a identificação dos efeitos citotóxicos é de suma importância, bem como a elucidação dos mecanismos envolvidos.

1.4. Aspectos avaliados na prospecção de novos fármacos

1.4.1 – Citotoxicidade: Padrões de Morte celular

Os processos de morte celular podem ser classificados, de acordo com suas características morfológicas e bioquímicas, havendo basicamente dois padrões de morte celular, o padrão necrótico e o padrão apoptótico (RICCI; ZONG, 2006). No padrão necrótico, a lesão celular extrapola as capacidades de homeostasia celular, desencadeando processos celulares que resultam em alterações nucleares (cariopicnose, cariorexe e cariólise) e citoplasmáticas irreversíveis, geralmente induzindo processo inflamatório tecidual (MAJNO; JORIS, 1995).

Já no padrão apoptótico, a célula entra em um programa de morte celular, desta forma as alterações morfológicas observadas são conseqüência de uma cascata de eventos moleculares e bioquímicos específicos e regulados (SARASTE; PULKKI,

2000). Células apoptóticas apresentam: inversão de fosfolipídios de membrana, cariorexe com condensação periférica da cromatina, embolhamento de membrana, fragmentação da cromatina e formação de corpos apoptóticos. De maneira resumida, este processo é intermediado pela ativação da cascata de proteínas denominadas de caspases, que podem ser ativadas por sinais extrínsecos (externos às células) ou intrínsecos (internos), e são subdivididas em caspases iniciadoras (tipos 2, 8 e 9) e caspases efetoras (tipos 3, 6 e 7). Neste processo é importantíssima a participação de eventos mitocondriais, onde proteínas como a bax, podem desestabilizar a membrana mitocondrial liberando, o citocromo C, um ativador da caspase 9 (BOATRIGHT; SALVESEN, 2003).

Uma das maneiras de se induzir a apoptose é através do aumento intracelular da proteína p53, uma importante sinalizadora de lesões de DNA. Esta proteína é capaz de induzir o aumento da expressão de proteínas pró-apoptoticas, induzindo a apoptose (SHILOH, 2006). A p53 também é capaz de induzir outros tipos de comportamentos celulares como a autofagia (LEVINE;ABRAM, 2008).

Autofagia é um processo adaptativo conservado evolutivamente e controlado geneticamente, ela ocorre em resposta a um estresse metabólico que resulta na degradação de componentes celulares, associado a um processo catabólico lisossomal (DANIAL; KORMEYER, 2004; LUM, DeBERARDINI; THOMPSON, 2005; CHECINSKA; SOENGAS, 2011). A autofagia pode ser classificada em três tipos, a mais comum é a macroautofagia, onde um componente celular a ser degradado é englobado no autofagossomo, que posteriormente se funde a um lisossomo formando o autolisossomo (KROEMER;JÄÄTTELÄ, 2005; CHECINSKA; SOENGAS, 2011).O processo de autodegradação não está envolvido apenas na remoção de agregados protéicos ou organelas danificadas, mas também em respostas adaptativas a uma variedade de sinais de estresse intra e extracelular (KROEMER; MARIÑO; LEVINE, 2010).Em células neoplásicas, a autofagia parece promover o crescimento, uma vez que otimiza o uso de nutrientes e degrada organelas danificadas com papel chave na indução de morte celular, como mitocôndrias despolarizadas e estruturas oxidadas. Por outro lado, a autofagia também está associada à supressão do crescimento tumoral, limitando o tamanho das células e levando-as à morte. Desta forma, a indução da autofagia é um importante alvo na terapia antitumoral (BAEHRECKE, 2005).

1.4.2 – Vias de sinalização celular

Outros alvos importantes de estudo na terapia antitumoral são proteínas sinalizadoras e reguladores do ciclo celular. Por se tratar de um processo extremamente complexo, boa parte das pesquisas focam suas atenções em famílias de proteínas reguladoras de ciclo celular. Dentre as famílias estudadas podemos citar a via da Raf/MEK/ERK (ou simplesmente via MAPK) e da Akt/Pi3K, mais recentemente outras vias, como a NFkB, também tem sido avaliadas.

A Raf-MEK-ERK é uma via envolvida em processos de proliferação e diferenciação celular. Nesta via, receptores de tirosina quinase ou receptores acoplados à proteína G promovem a ativação da MAPKKK (Raf), que por sua vez, ativam a MAPKK (MEK1 e MEK2). Quando ativada, a MAPKK fosforila as proteínas ERK1 e ERK2 (MAPK), ativando-as (CHENG; ZHANG;LI, 2013). Após sua ativação, a p-ERK1/2 se transloca para o núcleo e ativa fatores de transcrição, alterando a expressão de genes que promovem crescimento celular ou diferenciação (KONDOH; TORRI; NISHIDA, 2005). Trata-se de uma via de sinalização bastante envolvida com a transformação celular, incluindo o melanoma (GOLLOB *et al*., 2006; PANKA *et al*., 2008).

A proteína Pi3K pertence à família de quinases intracelulares relacionadas com a transdução de sinais, capaz de fosforilar o anel inositol grupo 3' - OH em fosfolípidos de inositol. A Pi3Kestá envolvida em muitas respostas celulares, como a progressão do ciclo celular e o crescimento. PI3K é recrutada para a membrana , o que leva à ativação da subunidade catalítica, resultando na produção do segundo mensageiro do fosfatidilinositol - 3 ,4,5 - trifosfato (PIP3) . PIP3 recruta um subconjunto de proteínas de sinalização, incluindo a Akt . Uma vez ativada, a Akt medeia a ativação e inibição de diversos alvos, resultando na sobrevivência celular, crescimento e a proliferação através de vários mecanismos (VANA *et al*., 2004).

O NF-kB (fator nuclear kappa B) é uma importante família de proteínas reguladoras de processos imunulógicos e inflamatórios em resposta a lesões e infecções. No estado latente, o NF-κB permanece no citosol associado à uma proteína inibidora, o IkB. Após a estimulação de receptores de membrana, como os receptores Toll-like e receptores de citoquinas (ex. receptores do fator de necrose tumoral – TNF), uma série de eventos proximais da membrana resultam na ativação

fosforilação do IkB, o que libera o NF-kB para translocação e ativação da transcrição de genes alvo no núcleo da célula (NAPETSCHNIG; WU, 2013). O NF-kB tem sido considerado um elemento importante na conexão entre o processo inflamatório e o câncer, uma vez que estimula a expressão de citocinas promotoras de neoplasias, como a IL-6 e o TNF-α, bem como genes de sobrevivência celular como o Bcl-XL (KARIN*et al.*, 2002). Além disso, o NF-kB também é um elemento responsivo ao estresse oxidativo, uma vez que o estresse oxidativo induz modificações no IkB, que promovem sua proteólise via ubiquitinação, o que libera o NF-kB para que transloque para o núcleo, de forma independente de sinais exteriores à célula (VAN DEN BERG *et al.*, 2001).

1.4.3 – Estresse oxidativo

O estresse oxidativo é considerado o desbalanço entre a geração de espécies reativas de oxigênio (e/ou de nitrogênio) e as defesas antioxidantes da células, tecido ou sistema, sendo melhor definido como um processo de interrupção das sinalizações redox e processos de controle associados (JONES, 2006; HALLIWELL, 2007). O estresse oxidativo é um processo conhecidamente relacionado com o câncer (GADJEVA; DIMOV; GEORGIEVA, 2008), pois está envolvido na inflamação crônica e ativa diversos fatores de transcrição como NF-kB, AP-1, p53, HIF-1α, dentre outros, que levam à expressão de aproximadamente 500 genes diferentes responsáveis por fatores de crescimento, citocinas, quimiocinas e reguladores de ciclo celular. Desta forma o estresse oxidativo está envolvido na proliferação celular, na resistência à quimioterapia e radioterapia, na angiogênese tumoral e na invasão e metástase (REUTER*et al.*, 2010). Além disto, o estresse oxidativo também está envolvido no processo de iniciação do câncer, pois espécies reativas podem induzir lesões no DNA e modular as respostas celulares à estes danos (BARZILAI; YAMAMOTO, 2004).

Os radicais livres, na maioria das vezes, são derivados do metabolismo do oxigênio e são encontrados em todos os sistemas biológicos. As espécies reativas de oxigênio (EROs) mais importantes em meios biológicos são: i) oxigênio singlet ($^1O_2$): forma excitada do oxigênio molecular e não possui elétrons desemparelhados

em sua última camada (HALLIWELL; GUTTERRIDGE, 1990); ii) radical superóxido (O_2^-): formado em todas as células aeróbicas, cerca de 5% do oxigênio consumido é reduzido a radical ânion superóxido, sua formação é especialmente induzida durante a ativação de neutrófilos, monócitos, macrófagos e eosinófilos no "*burst*" respiratório. (HALLIWELL; GUTTERIDGE, 1985) iii) peroxil ou hidroperoxila (HO_2): forma protonada do superóxido, sendo considerado mais reativo que o radical superóxido, por apresentar maior facilidade em iniciar lesões em membranas biológicas (HALLIWELL ; GUTTERIDGE, 1990). Além das ROS também podemos observar entre os radicais livres, as espécies reativas de nitrogênio, que incluem o óxido nítrico e o peroxinitrito. O óxido nítrico (NO), é uma importante molécula sinalizadora e é sintetizado pelas enzimas óxido nítrito sintases (NOS) a partir da L-arginina. Existem três isoformas de NOS,a endotelial (eNOS) e a neuronal (nNOS) as duas dependentes de cálcio e a (iNOS) independente de cálcio (ALDERTON; COOPER; KNWLES, 2001). Quando a produção de NO é excessiva, esta molécula passa ser a causa, ou contribui, para condições patológicas, incluindo o câncer (CHOUDHARI *et al*., 2013). A reação entre o O_2^- e o NO produz o peroxinitrito ($ONOO^-$) (ou a forma protonada (ONOOH), que é considerado um forte agente oxidante, induzindo nitração de proteínas, lesão de DNA e lipoperoxidação (PATEL *et al*., 1999). Como visto anteriormente tanto espécies reativas de oxigênio, quanto de nitrogênio, podem atacar estrutura biológicas, dentre elas os lipídeos. Uma da moléculas resultantes deste ataque, e que representa o processo de peroxidação lipídica originado por estresse oxidativo, é o malondialdeído (MDA), que por sua vez também apresenta atividades tóxicas, como a lesão de DNA, e pró-inflamatórias (RAGHAVAN; SUBRAMANIYAM; SHANMUGAM, 2012; MARNETT, 1999).

Existem vários mecanismos enzimáticos e não enzimáticos naturais que constituem o sistema de defesa antioxidante. Uma importante defesa antioxidante não enzimática é a glutationa. A glutationa (GSH) é composta de três aminoácidos (L-glutamina, L-cisteína e glicina) e é encontrada em praticamente todas as células do corpo humano. Sua capacidade redutora é determinada pelo agrupamento tiol-SH, presente na cisteína. Quando a GSH é oxidada ocorre uma interligação de duas moléculas do tripeptídeo por uma ponte dissulfeto, dando origem à glutationa oxidada (GSSG), uma reação mediada pela enzima glutationa peroxidase (GSH-Px). A depleção dos níveis de GSH pode prejudicar as defesas celulares contra a ação

tóxica dos radicais livres. As células integras mantêm uma razão GSH/GSSG alta, para isso, a GSSG formada é novamente reduzida em GSH, pela ação da enzima glutationa redutase (GSH-Rd) (HALLIWELL; GUTTERIDGE, 1989).

Com relação ao estresse oxidativo, o melanoma é uma neoplasia com perfil relativamente único quando comparado com outros tipos de câncer. O melanoma apresenta elevado níveis de estresse oxidativo, tanto no microambiente tumoral primário (SANDERS *et al.* 2003; FRUEHAUF; TRAPP, 2008), quanto nas células tumorais e até mesmo ao nível sistêmico (GADJEVA; DIMOV;GEORGIEVA, 2008; PICARDO *et al*., 1999; PICARDO *et al*., 1996). Diferentemente dos melanócitos normais, as células de melanoma maligno possuem alta capacidade antioxidante e usam essa habilidade para favorecer sua proliferação e invasão/metástase (WITTGEN; VAN KEMPEN, 2007; MEYSKENS *et al*., 2003).

2 OBJETIVO

2.1. Objetivo geral

Avaliar, *in vitro*, o efeito antiproliferativo e citotóxico do citral em células de melanoma murinho metastático B16F10, e elucidar os mecanismos envolvidos.

2.2. Objetivos específicos

Nossos objetivos específicos foram:

- Determinar o tipo de morte celular induzido (apoptose, necrose e autofagia).
- Verificar a participação de espécies reativas de oxigênio e nitrogênio nos efeitos do citral.
- Avaliar a interferência do citral sobre importantes vias de sinalização celular.
- Verificar a citotoxicidade em células murinas normais (NIH-3T3).

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"CITRAL INDUCES APOPTOSIS AND AUTOPHAGY IN B16F10 MELANOMA CELLS BY GENERATING OXYGEN REACTIVE SPECIES AND REDUCING THE EXPRESSION OF CELL GROWTH PROTEINS"

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ABSTRACT

Citral is a natural compound with antiproliferative and antitumoral effects. In this study, we investigated the cytotoxic effect of citral in murine melanoma cells, B16F10. Citral reduced cell viability and proliferation (IC_{50} 24h: 1.04 μM), by inducing apoptosis, and autophagic vacuoles. Scavengers of oxygen species revealed a mechanism dependent on oxidative stress generation. Immunocytochemistry labeling showed increased p53, decreased Pi3K, and positive Akt, and ERK 1/2 labeling, with decreased nuclear labeling of NFkB. Our findings suggests that citral is a cytotoxic compound, which effects are related to oxidative stress and intricate modulation of cell growth proteins.

Keywords: melanoma, B16F10, citral, apoptosis, autophagy, oxidative stress, immunocytochemistry.

1.Introduction

A melanoma is a malignant tumor that arises from melanocytes, dendritic cells that produce melanin, a pigment that protects the body from damaging ultraviolet (UV) radiation. Melanocytes use tyrosine to synthesize melanin. A cluster of melanocytes form nevi (pigmented lesions or moles), and melanoma results when these melanocytes undergo a malignant transformation [1,2].

Although melanoma represents the least common form of skin cancer (accounting for only about 5% of all skin cancer cases), it is the deadliest form of skin cancer claiming about 75% of skin cancer-related deaths, and the incidence of melanoma is increasing worldwide. According to an estimative from the American Cancer Society one person dies every hour from melanoma [3].

Surgical excision of the primary skin lesion can be curative for those patients that have only localized disease, standard treatment for patients with thick (≥2.0 mm) primary melanoma with or without regional metastases to lymph nodes is surgery followed by adjuvant therapy [4].In patients with disseminated disease or who developed distant metastasis post resection, treatment aims prolonging survival and improving quality of life [5].Patients who progress to stage IV metastatic melanoma have a median survival of less than 1 year. Standard treatment with chemotherapy yields low response rates, of which few are durable. Cytokine therapy with IL-2 achieves durable benefits in a greater fraction, but it is accompanied by severe toxicities that require the patient to be hospitalized for support during treatment [4].

The use of plant-derived natural products for medicinal benefits has played an important role in almost all the people on earth. Since 1961, several anticancer drugs have been made available on the market that trace their origins to plants such as Taxol, oncovin, navelbine and vumon [6-8]. In cancer therapy, the focus is on strategies that suppress tumor growth through cell cycle disruption and activate the apoptotic program in cell [9,10].

Citral (3,7-dimethyl-2,6-octadienal) is a naturally occurring aliphatic aldehyde of the terpene series and is an isomeric moisture of geranial and neral, is a key component of essential oils extracted from several herbal plants such as lemon grass (*Cymbopogon citrates*), Melissa (*Melissa officinalis*), and verbena (*Verbena officinalis*), is used as a food additive and as a fragrance in cosmetic industry [11,12].The toxicity studies indicate that citral is devoid of major toxicity and

carcinogenic potencial in both mice and rats [13,14].It was also reported that citral is devoid of mutagenic effect on *in vitro*models [15].Substantial antibacterial, antifungal, antiparasitic, and insecticidal effects of citral on different organisms have been described in several studies [16, 17].

The aim of the present work was to evaluate the antiproliferative and/or cytotoxic effect of citral on murine melanoma cells B16F10, and also elucidate the mechanisms involved in this effect.

2. Materials and methods

2.1. Cell culture and treatment

The murine melanoma cell line (B16F10) was seeded in tissue culture flasks and grown in Dulbecco's modified Eagle's Medium (DMEM, Gibco), supplemented with 5% fetal bovine serum (FBS, Gibco) and 1% penicillin/ streptomycin mixture (Santa Cruz). The culture was maintained in a humidified atmosphere with 5% CO_2 at 37°C (Sanyo CO_2 Incubator; Sanyo, Japan).

The cytotoxicity, proliferation and genotoxicity assays were performed by seeding 2 x10^5 cells in 24 well plates and culturing cells with fresh culture medium for 24h prior to citral exposure (Sigma – Aldrich, Citral C.A.S. 5392-40-5, mixture of *cis* and *trans*, >96%), indifferent concentrations. Citral was diluted in DMSO (maximum 1% final concentration). In the control group, 1% of DMSO was added in the culture medium.

For the inhibition of 50% (IC_{50}) of cell proliferation and the lethal dose that produce 50% of cell death (LD_{50}) which represents the cytotocicity. For Ethidium Bromide (EB) and Acridine Orange (AO) staining, TUNEL and Autophagy assay the cells were seeded on circular coverslips. The experiments were performed in triplicate and three independent repetitions.

NIH-3T3 murine fibroblast cells was used to verify the cytotoxicity of citral against normal cells. NIH-3T3 were cultured and treated under the same conditions of B16F10 cells.

2.2. Determination of IC_{50} concentration of drug in melanoma murine cells B16F10

The determination of the inhibitory concentration 50 (IC_{50}) was obtained using the MTT assay ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), performed according to Mosmann (1983) [18]. A regression analysis was performed on MTT assay proliferation data and the resultant equation was used to compute the inhibition concentration required to produce a 50% reduction in cell proliferation. The MTT assay was also used to determine the involvement of reactive oxygen species in citral cytotoxicity by using different specific reactive oxygen species scavengers.

2.3. Cytotoxicity and cell proliferation assay

After 24h of treatment, the cells were washed with phosphate-buffered saline (PBS) and trypsinized. Cells were suspended in Trypan Blue (0.05%) and counted using a Neubauer chamber. For cell viability a total of 300 cells were counted, distinguishing the inviable cells (stained in soft blue).

2.4. Ethidium Bromide (EB) and Acridine Orange (AO) staining

The EB/AO staining enables the differentiation between viable cells from those under going death by apoptosis or necrosis. After treatment, the adhered cells on coverslips were washed with phosphate-buffered saline (PBS) and incubated with 10 µl PBS containing 10 µg/ml of EB and 3 µg/ml of AO as described by Sun et al. (2011). The cells stained by EB/AO were immediately visualized under the Olympus Fluorescence System Microscope BX3-URA (Olympus Corporation, Tokyo, Japan). Cells were classified as: viable (green nuclei), apoptotic (green or orange nuclei with condensed chromatin presenting or not nuclear fragmentation, and cell membrane bubbling), and necrotic (orange cells, with extensive nuclear modifications). For each treatment, 600 cells were analyzed to estimate the percentage of viable, apoptotic and necrotic cells.

2.5. TUNEL assay

After treatment cells were washed and fixed with formaldehyde 10%. The apoptosis were evaluated using the Dead End Colorimetric TUNEL System (Promega, WI, USA). The experimental procedures were carried out according to the manufacturer's protocol and the coverslips were photographed utilizing the Olympus Fluorescence System Microscope BX3-URA (Olympus Corporation, Tokyo, Japan), multiple photos were taken randomly.

2.6. LDH release assay

The *in vitro* assay kit for lactic dehydrogenase quantification (Doles, Goiás, BR) was used for the measurement of LDH released from the cytosol of damaged cells into the supernatant, to estimate cellular membrane damage associated to necrosis. The assay was based on the reduction of nicotinamide adenine dinucleotide (NAD) by LDH. Absorbance was read at 490 nm using a microplate spectrophotometer (Multiscan Go; Thermo Scientific, USA).

2.7. Autophagy assay – Monodansylcadaverine (MDC)

After treatment, cells were washed with PBS and incubated with 0.05 mM MDC in fresh culture medium at 37°C for 10 minutes. Cells were immediately analyzed using a fluorescence microscope Olympus Fluorescence System Microscope BX3-URA (Olympus Corporation, Tokyo, Japan) with 380 and 525 nm wavelength excitation and emission filters, respectively.

2.8. Comet assay

Single-cell gel electrophoresis (SCGE; Comet assay) was performed according to the procedure described by Tice *et al.,* (2000) [19]. After treatment, cells were harvested and suspended in low-melting-point agarose (0.5%) and deposited on pregelatinized slides (agarose: 1.5%). Subsequently, the slides were placed in an electrophoresis buffer (pH: 13) for 20 min for DNA desnaturation. Then, the slides were subjected to alkaline electrophoresis (pH: 13.0; 25 V; 300 mA; 4°C). All steps

were performed under undirected light. Finally, the slides were treated with pH-neutralizing buffer, fixed with ethanol, and stained with Gel Red (33%). A total of 100 random nucleoids from each slide were blindly examined at 400× magnification by using a fluorescence microscope (excitation filter was set at 420–490 nm, while emission filter was set at 520 nm) connected to an image capture system. For the determination of tail moment of each nucleoid, Comet ScoreTM Freeware (version: 1.5; Tritek, USA) was used. Apoptotic/necrotic nucleoids with extensive DNA damage were not included in the analysis. The mean value of the tail moment was considered as an index of DNA damage [20].

2.9. Oxidative stress parameters

2.9.1. Determination of reduced gluthatione levels (GSH)

Glutathione is considered the main regulator of redox balance in the cellular milieu due to its capacity for detoxifying deleterious molecules. The oxidative stress, induced as a result of a variety of stimuli, promotes protein oxidation usually at cysteine residues, leading to changes in their activity. GSH levels were measured as described by Locatelli (2009) [21]. The assay involved spectrophotometric (Multiscan Go; Thermo Scientific, USA) measurement of reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid by GSH at 412 nm; the principle of the assay is based on the determination of change in absorbance due to the formation of yellow-colored 5-thio-2-nitrobenzoic acid. Results were expressed as amount of GSH (µM)/g of total protein.

2.9.2. Determination of malondialdehyde (MDA) levels

After treatment cells were washed and trypsinized. Cellular suspension were centrifuged 60 xg (4ºC, 3 min). The cellular pellets were suspended in cold PBS buffer and frozen (-80ºC) until analyses. The cellular suspension were used to measure MDA levels. The measurement was performed as described by Victorino *et al.,* (2012) [22] by using a high-performance liquid chromatography (HPLC) system (HPLC-Shimadzu 20AT) equipped with a LC20AT pump, an absorbance detector (UV SPDM20A diode-array), and a reverse phase C18 column. Results were

expressed in nM MDA/g of total protein. The total protein content was determined based on the modified Lowry Miller method (1959).

2.9.3. Determination of the levels of nitric oxide (NO)

The determination of the levels of cellular NO was realized as previous described Panis *et al.,* (2012) [23]. Aliquots cells plus supernatants (60 µL) were desproteinized by adding 50 µL of $ZnSO_4$ 75 mM solution (Merck), shaked and centrifuged at 10000 rpm, 2 minutes, 25 °C and then 70µL of NaOH 55mM (Merck), shaked and centrifuged at 10000 rpm, 5 minutes, 25 °C. The limpid supernatant was recovered and diluted in glycine buffer solution (45g/L pH 9.7, Merck) in a proportion of 5:1. Cadmium granules (Fluka) stored in H_2SO_4 100mM solution (Merck) were rinsed 3 times in distilled sterile water and added to a $CuSO_4$ 5mM solution in glycine-NaOH buffer (15 g/L, pH 9.7, Merck) during 5 minutes and the copper-coated cadmium granules were used within 10 minutes. Activated granules (600-1000 mg, approximately 1-2 granules) were added to glycine buffer diluted supernatant and stirred during 10 minutes. Aliquots of 200 µL were recovered in appropriated tubes to nitrite determination and the same volum of Griess reagent was added (Reagent I: 50 mg of *N*-naphthylethylenediamine in 250 mL of distilled water; reagent II: 5 g of sulfanilic acid in 500 mL of 3 M HCl, Sigma). After an incubation of 10 minutes at room temperature, tubes were centrifuged at 10000 rpm, 2 minutes, 25°C and 100 µL were added to 96 wells microplates in triplicate. To determine sample nitrite concentration, calibration curve was prepared by dilution of $NaNO_2$ (Merck) in distilled sterile water to concentrations of 125 to zero µM and 100 µL of the curve point + 100 µL of Griess reagent was added in triplicate to the microplate wells. The absorbance was measured at 550 nm in a microplate reader. The final results were expressed in µM.

2.9.4. Scavengers of reactive species of oxygen

To evaluate the involvement of reactive species of oxygen in citral cytotoxicity we performed a MTT assay (24 hours) with simultaneous treatment of citral 0.5 or 1.0 µM and free radical scavangers (L-histidine 100 mM, Trolox 50 mM, and Tempol 50

mM,). L-histidine is a oxygen single quencher [24], Trolox is a water-soluble analogue of alfa-tocoferol and is a scavenger of singlet oxygen [25], and peroxyl radical (Naquib, 1998), while Tempol is a superoxide scavenger [26].

2.9.5. T-butyl hydroperoxide-induced oxygen uptake by red blood cells, as a parameter of direct induction of oxidative stress

Erythrocytes exposed to t-butyl hydroperoxide (t-BHP) delay in oxygen uptake, and this delay can be reduced by antioxidants [27], or increased in pro-oxidantive situations [28]. The assay was performed according to Lissi et al. [27]. Summorized, the heparinized blood sample was obtained from a healthy donator, after that blood was centrifuged and washed 3 times to obtain isolated red blood cells.Aliquots of erytrocytes were incubated with citral (0.1; 0.5; 1.0 or 2.5μM) diluted in $NaH_2PO_4$10mM pH 7.4 during 5 min at 37°C. Control group was incubated with NaH_2PO_4buffer at same conditions of citral treatment. After incubation time, the erythrocytes were transferred to oxygen consumption chamber of the Oxymeter (Clark) equipped with a polarographyc eletrod for oxygen levels measurement. Red blood cells suspensions were treated with t-butyl hydroperoxide 2 mM 37ºC and oxygen levels were monitored during 10 min. Data obtained from curves were converted to area under the curve graphic using numerial integration.

2.10. Immunocytochemistry labelling for p53, ERK1/2, AKT, PI3K and NFkB proteins

After treatment cells were washed and fixed in commercial mixture of polyethylene glycols - Citofix® (Doles,Goiás, BR). Immunocytochemistry analysis was performed on coverslip-adherent cells using the labelled streptavidin biotin method using a LSAB KIT (DAKO Japan, Kyoto, Japan). The sections were incubated with 10% Triton X-100 solution during 1 hour, washed 3 times in PBS and treated for 40 min at room temperature with 10% BSA. In addition, coverslips were incubated overnight at 4°C with the primary antibodies (anti-p53, anti-NFkB p65, anti-ERK 1-2, anti – PI3K and anti-AKT, diluted 1:200, Santa Cruz Biotechnology). After secondary antibody treatment (2 hours, room temperature), horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3'-diaminobenzidine (DAB) for 5 min. At the last step, the sections were weakly counterstained with Harry’s

hematoxylin (Merck). For each case, negative controls were performed by omitting the primary antibody. Intensity and localization of immunoreactivities against primary antibody used were examined on all coverslips using a photomicroscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan). For the image analysis study, photomicroscopic colour slides of representative areas (400x magnification) were digitally acquired. After positive pixels for DAB were thresholded and processed by Image J software. Positive immunostained area was calculated as positive labelled area. Nuclear labelling for p53, ERK1/2 and NFkB images were also analyzed using Image J software, for each image the total number of cells were counted and the number of cells with nuclear labelling were used to calculate the percentage of cells with nuclear labelling (%). For all analyses we use at least 10 representative images per treatment.

2.12. Statistical analysis

Data were expressed as mean ± standard error of the mean and analyzed using one-way analysis of variance (ANOVA). Intergroup differences were analyzed by Dunnett's test, and $p < 0.05$ was considered statistically significant. Data analysis was conducted using GraphPad Prisma (version 5.0; California, USA). Data obtained from 24 h of treatments with citral were also used to calculate Spearman's correlation using GraphPad Prisma, and correlations with $p<0.05$ were considered significant.

3. Results

3.1. Effects on cell proliferation and viability

The concentrations of citral required to produce a 50% reduction in cell proliferation (IC_{50}) were computed by regression analysis using data from MTT assay results, the values of IC_{50} were estimated in 1.04μM for 24h and 0.77μM for 48h. Citral 0.5μM reduced the the percentage of viable cells for 24h (85.75±2.4μM) and 48 h of treatment (84.94±3.4μM), when compared with the respective controls (24 h: 90.75±1.909μM; 48 h: 94.22±2.487μM) (Figure.1A). Cell proliferation was reduced in concentrations equal or higher thancitral 0.1μM(4.1± 0.3 x 10^5) for 24h and 0.05μM(5.8 ±0.4 x 10^5) for 48 h, when compared to control (24 h: 4.5±0,3 x 10^5; 48 h: 7.4± 0.1 x 10^5), as showed in figure 1B. For 48 h of treatment, citral 0.05 and 0.1 μM reduced the number of cells/ml but did not reduce cell viability.

3.2. Cell death patterns

Cell death patterns were evaluated using the EB/AO staining. Citral 0.5 (1.9 ± 0.05μM), 1.0 (4.83 ± 0.12μM), and 2.5 μM (2.16 ± 0.06μM) were able to increase the percentage of apoptotic cells compared to control (0.86 ± 0.2μM). Necrotic pattern of cell death increased in citral 1.0 (11.27 ± 1.20μM) and 2.5 μM (24.79 ± 0.65μM) compared to control(7.44 ± 0.91μM) (Fig. 2A).

Apoptosis was confirmed by TUNEL assay. In this assay, citral 0.5 (1.53 ± 0.59μM), 1.0 (4.45 ± 0.92μM) and 2.5 μM (1.86 ± 2.05μM) were able to induce apoptosis (control 0.33 ± 0.48μM) (Fig. 2B). The measurement of LDH released into culture medium demonstrated the increase of cell membrane damage associated to necrosis in citral 1.0 (0.51 ± 0.03μM) and 2.5μM (0.60 ± 0.02μM) (control 0.43 ± 0.03μM), confirming the results observed with EB/AO staining (Fig. 2C).

To better characterize the cell death patterns of citral we evaluated the autophagy, using the monodansylcadaverine (MDC). In this test, autophagic vacuoles accumulate MDC emitting fluorescence. In our conditions of treatment, only

citral 1.0 µM 29.5 ± 9.52µM) significantly induced autophagy compared to control (8.7 ± 3.91µM) (Fig 2 D).

3.3. Genotoxicity.

To identify the involvement of genotoxicity in the cytotoxic effect of citral, we performed the Comet assay in alkaline conditions. After 24 h of treatment, citral0.5 (42.8 ± 8.01µM), 1.0 (76.6 ± 4.92µM) and 2.5µM (91.8 ± 3.61µM) were able to induce DNA damage (control 35.91 ± 3.78µM)(Fig. 3).

3.4. Oxidative stress parameters

To demonstrate of there was the involvement of oxidative stress in the citral effects we performed the analyses of reduced gluthatione and malondiahyde (MDA) levels. Citral 0.5 (5.05 ± 0.21µM), 1.0 (5.09 ± 0.08µM) and 2.5µM (4.84 ± 0.04µM) induced the consumption of reduce gluthatione (control 5.36 ± 0.18)µM (Fig. 4A). Citral 2.5 µM (23.53 ± 6.29µM) (control 15.98 ±1.4µM)(Fig. 4B) was also able to increase MDA levels, demonstrating the oxidation of cell membrane lipids. To identify the reactive species involved we performed the measure of NO levels, and a MTT assay with simultaneous treatment with free radical scavangers. Citral was able to significantly decrease NO levels in a dose dependent manner (0.1µM 34.38 ± 2.64µM; 0.5 µM 28.36 ± 1.49µM; 1.0 µM 24.92 ± 0.68µM; 2.5 µM 18.42 ± 1.0µM; control (37.33 ± 0.78µM). All tested radicalar scavangers were not cytotoxic on B16F10 cells, but they were able to reduce citral cytotoxicity (Fig. 4D). For citral 0.5 µM histidine (90.03 ± 2.96µM), trolox (92.68 ± 1.14µM) and tempol (93.8 ± 2µM) reverted cell viability to control levels (91.63 ± 5.41µM). For citral 1.0 µM histidine (61.58 ± 3.65µM), trolox (54.51 ± 3.2µM), and tempol (57.76 ± 6.44µM) protected the cells against citral cytotoxicity, but were not able to revert cell viability to control levels. All tested concentrations of citral were did not change oxygen uptake by red blood cells (Fig. 4E).

3.5. Immunocytochemistry

In the present work we also investigated the interference of citral on proteins involved in cell signaling pathways. For immunocytochemistry only the concentrations 0.1, 0.5, and 1.0 µM were used). Citral 1.0 µM decreased the ERK1/2 labelled area/cell (14.1 ± 1.76µM) (control 53.88 ± 0.95µM) and citral 0.5 (11471 ± 601.7µM) and 1.0 µM (9503 ± 397.0µM) (control 11997 ± 455.6µM) also decreased the nuclear labelling for this protein labelling (Fig. 5A).Citral 0.5 and 1.0 reduced the expression of Akt (control 8846 ± 512.8µM; citral 0.5µM 6080 ± 620.3µM; citral 1.0 µM 5197 ± 640.3µM) and Pi3K(control 10260 ± 109.3µM; citral 0.5µM 9582 ± 520.7µM; citral 1.0 µM 3529 ± 460.7µM) proteins (Fig. 5B and 5C). For p53 protein citral 0.5µM (2.63 ± 0.33µM) and 1.0 µM (6.62 ± 1.86µM) (control 0.94 ± 0.26µM) increased the percentage of labelled cells (Fig. 5D). Citral1.0 µM increased NFkB labelled area/cell, but all tested concentrations (control 9245 ± 1936µM) reduced the nuclear labelling of this protein (Fig.5E).

Table 1 shows the Sperman's correlation matrix obtained from citral 1µM 24h treatments in B16F10 cells. The most significant correlations ($p<0.05$) allowed to realize that: (i) reduced cell counting was associated to decreased NO levels; (ii) diminished cell viability was associated to increased percentage of necrosis, increase LDH release, increased p53 labeling and decreased Pi3K and ERK 1/2 labeling; (iii) apoptosis percentage was correlated to increased p53 labeling; (iv) increased DNA damage was associated to increased p53 labeling, decreased GSH levels, and decreased Pi3K and ERK 1/2 labeling; (v) autophagy was correlated to increased p53 labeling and decreased Pi3K and ERK 1/2 labeling.

3.6. Determination of IC_{50} in NIH-3T3 murine fibroblast cells

To determine de specificity of citral we investigated the cellular viability of normal cells, NIH-3T3 murine fibroblast cells (3T3). 3T3 cells were treated under the same concentrations and conditions of B16F10 cells. The IC_{50} 24 h, determined by MTT assay, was estimated in 2.5µM. Citral 1.0µM (79.59 ± 8.08µM) and 2.5 µM (51.08 ± 3.23µM) reduced cell viability compared to control 91.20 ± 5.77µM (Fig. 6).

4. Discussion

Citral is an important compound of many essential oils, especially lemongrass. It has been related to antibacterial, antifungal, antiparasitic, and insecticidal effects on different organisms [16,17;29,30]. More recently citral demonstrated to have antiproliferative effects against human breast cancer cells MCF-7 [31]. In this context, this is the firs study that evaluated the antineoplastic of citral on melanoma cells. The main goal of this work was to evaluate the antiproliferative and/or cytotoxic effect of citral on murine melanoma cells B16F10 and to identify the mechanisms involved in this effect.

We first estimated the IC_{50}-24h citral at 1.04μM, revealing its cytotoxic effect on B16F10 cells. For 48h of treatment citral 0.05 and 0.1 μM reduced the cell counting but did not reduce cell viability, suggesting that citral also has antiproliferative activities. This result agree with previous works revealing antiproliferative effects of citral on human leukemia cells (HL-60) [32], and MCF-7 cells [31]. The IC_{50} value represents the concentration of citral required to inhibit cell viability in 50% at a time point. Previously,Tatman & Mo *et* al., (2002) [33] estimated the IC_{50} 48h in 30±10 μM of citral in B16F10 cells (1.25 x 10^5 cells/ml) using a cell counting approach, but in our work IC_{50} 48h was estimated in 0.77μM.

Regarding the mechanism enrolled in cell death triggering, citral-induced cytotoxicity was evaluated by EB/AO staining, TUNEL assay and LDH release. Citral was able to induce apoptotic and necrotic patterns of cell death according to tested concentration. Using EB/AO staining, morphologic aspects of necrotic pattern were recognized for citral 1.0 and 2.5μM, and confirmed by the increase of LDH release to the culture medium. EB/AO also revealed the induction of apoptosis for citral 0.5, 1.0 and 2.5 μM, that were further confirmed by TUNEL assay. Dudai *et al.,* (2005) [12] observed that citral induced apoptosis in cancer cells (22.25 μM) and suggest that this pro-apoptotic depends on α,β-unsaturated aldehyde group. In our work we also observed apoptosis in B16F10 cells in much lower concentrations of citral (1.0μM). The apoptosis induction is correlated to the genotoxic effect observed by comet assay at same concentration. This result is further supported by the increased p53 levels, that was also previously reported by Duerken-Hughes (1999) [32] for citral treatments. Hata *et al.,* (2003) [32] also described the induction of apoptosis in MCF-7 cells (IC_{50}-24h: 266μM). European Food Safety Autority concluded that for citral

genotoxicity is not of concern [35], but consider valid the ability of citral to induce sister chromatid exchanges on Chinese hamster ovary cells (NTP, 2003), and consider the increase of p53 levels on mouse fibroblast cells [34], as an indicator of genotoxicity.

We also observed that citral was able to induce the generation of autophagyc vacuoles in B16F10 cells. Autophagy is considered a non-selective process for bulk degradation, and has been associated with physiological processes of adaptation to starvation, cell differentiation and development, the degradation of aberrant structures, among others [36]. Increased autophagy offers a distinct advantage in various physiological and stress conditions suggesting that this process represents an adaptive mechanism to rescue cells from death [37]. Many anticancer treatments including novel targeted therapies stimulate autophagy, which can lead to increased cytotoxicity as well as to therapeutic resistance [38]. The presence of autophagic vesicles in dying cells may reflect an adaptive response that failed to maintain cell survival under stress conditions rather than a reflection of death induced by autophagy [39]. In the present study, citral 1.0μM was able to induce autophagic vesicles, suggesting that B16F10 cells shifted cellular metabolism, trying to recycle damaged structures under treatment with citral.

Many anticancer drugs (or a metabolite) are able to generate oxygen reactive species usually by electron transfer processes [40], which damage several cellular structures such as DNA, leading to cell death. For this reason we investigated the ability of citral to alter redox balance of B16F10 cells. To reach this goal, we evaluated the antioxidant content of cells by measuring GSH content, as well as investigated the pro-oxidant profiling by analyzing MDA and NO levels. GSH is a tripeptide involved in non-enzymantic antioxidant defenses, usually depleted during xenobiotic biotransformation and enhanced oxidative stress conditions. GSH depletion has been recently implicated in cell death [41].

Malondialdehyde (MDA) is a product of polyunsaturated fatty acid peroxidation, and it should be considered as more than just a marker of lipid peroxidation, due its interaction with DNA and proteins [42]. In the present work citral was able to slightly reduce GSH levels, revealing the consumption of antioxidant defenses. Only the highest tested concentration (2.5 μM) was able to increase MDA levels, revealing that citral, at highest concentrations, generates reactive species that overcome antioxidant defense mechanisms of B16F10 cells. The citral-induced

oxidative stress is probably associated to citral metabolism, once we did not observed modification in oxygen uptake measurement in red blood cells treated with citral 5 min (37ºC). The same result was observed for 30 min of treatment with citral (data not shown). These findings implicate the GSH consumption as a putative mechanism enrolled in the cell death mechanism induced by citral treatment on melanoma cells. This network may be associated with the augmented p53 labeling induced by citral, once p53 is both an apoptotic and redox sensor protein, activated under oxidative stress conditions [43].

To understand the role of ROS in the mechanism of action of citral the levels of nitric oxide (NO) were measured. NO has a variety of different biological roles. Despite the beneficial functions, this molecule has also a pivotal role in cell death. NO is synthesized endogenously by a family of enzymes called nitric oxide synthases (NOS). Lee *et al.,* (2008) [44] observed reduced iNOS expression in RAW 264.7 cells (Mouse leukemic monocyte macrophage cell line) submitted to treatment with citral. Our results are in accordance to Lee *et al.*, (2008) [44], since we observed that citral was able to significantly reduce NO levels.

To understand the involvement of oxidative species in the cytotoxic effect of citral we used three well established ROS scavengers (L-histidine, Tempol and Trolox). All tested scavengers interfered on cytotoxicity observed for citral 0.5 an 1.0 μM, revealing that many oxygen species may be involved in citral effect. These results demonstrate that cytotoxic effect of citral on B16F10 cells can be partially explained by the generation of oxidative stress burst, without the involvement of a particular oxidative specie. For citral 1.0 μM, all scavangers were not able to revert cell viability to control levels, showing that oxidative stress generation is only one of the mechanisms of citral effects. High levels of reactive oxygen species (ROS) can induce oxidative damage in lipids, and proteins. ROS are also associated with DNA damage and subsequent activation of signal transduction pathways [45]. In addition to this, ROS can elicit autophagy as well as apoptotic cell death [46], and it is important to emphasize that autophagy and apoptosis are closely interrelated because major players of both pathways cross-talk to each other [47]. ROS generation would thus partially explain the presence of autophagic vesicles and induction of apoptosis in B16F10 cells. Although we did not detected any alteration in MDA levels during the treatment of melanoma cells with citral 1.0 μM, it is possible that the reactive species enrolled in citral cytotoxicity interacted whith cellular GSH,

resulting in observed GSH depletion. This hypothesis helps to understand why the reactive species did not appears as oxidative-derived metabolites such as MDA. On the other hand, it is also probable that citral-generated reactive species also promoted DNA oxidative injury with increased DNA damage (observed in Comet assay), and induction of apoptosis and autophagy during citral exposition.

In the present work we also evaluated the ability of citral to interfere in cell signaling pathways: ERK 1/2, Akt, Pi3K, p53 and NFkB. Erk1/2 (Extracellular signal-regulated protein kinases 1 and 2) are members of the mitogen-activated protein kinase (MAPK) super-family that mediate cell proliferation and apoptosis, and participate in the Ras-Raf-MEK-ERK signal transduction cascade. During activation processes MEK 1/2 (MAPK K) phosphorylate and activate the ERK 1/2 MAPKs. Activated ERKs can translocate to the nucleus, where they phosphorylate and regulate many transcription factors [48]. Citral was able to reduce ERK 1/2 labelled area/cell and also reduced nuclear labelling of this protein, demonstrating that citral reduces ERK 1/2 protein expression and also inhibits the translocation of this protein to the nucleus, blocking further cellular signaling.

Phosphatidylinositol-3 kinases, PI3Ks, constitute a lipid kinase family characterized by their ability to phosphorylate inositol ring 3′-OH group in inositol phospholipids, and is involved in many cellular responses, including cell cycle progression and cellular growth. Activation of growth factor receptor protein tyrosine kinases results in autophosphorylation on tyrosine residues. Pi3K is recruited to the membrane, this leads to allosteric activation of the catalytic subunit, resulting in production of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 recruits a subset of signaling proteins, including Akt. Once activated, Akt mediates the activation and inhibition of several targets, resulting in cellular survival, growth and proliferation through various mechanisms [49]. Citral 0.5 and 1.0 μM were able to reduce Pi3K and Akt labelled area/cell, revealing that citral is also able to reduce the expression of both proteins, inhibiting Pi3K/Akt/mTOR pathway.

The p53 has been rediscovered as a tumor suppressor gene in 1989 and became one of the most profoundly studied proteins to date [50]. Cellular stress (including genotoxic damages), oncogene activation, and hypoxia stabilize p53 and lead to an increase in its transcriptional activity through posttranslational modifications by phosphorylation, acetylation, ubiquitination, and methylation. Once activated p53 has been mainly recognized as a transcription activator that also inhibit

the activity of some genes. The p53 pathway mediate cellular stress responses initiating DNA repair, cell-cycle arrest, senescence and, importantly, apoptosis [51]. The p53 protein is also able to induce autophagy through the induction of lysosomal proteins such as DRAM (damage-regulated autophagy modulator) [52] or through negative regulation of mTOR signaling is certainly consistent with an observed role for autophagy in tumor suppression [53]. Oxidative stress is a potent inducer of the tumour-suppressor p53, thought the activation of p66 protein [54]. In our work we observed that citral 0.5 and 1.0 μM increased the percentage of p53 labelled cells, in response to genotoxic effect associated to oxidative stress.

We also observed that citral increased NF-kappa B (NF-kB) levels in cytoplasm, but reduced nuclear labelling of this protein, suggesting that citral inhibited the translocation of this signaling molecule to the nucleus. NF-κB is a dimeric transcription factor that is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response. It is activated by a variety of stimuli, including oxidative stress [55]. NF-kB is composed of two proteins p50 and p65. During resting state NF-kB is blocked in the cytosol because it is bound to the inhibitory protein, IkB. A variety of agents are able to induce IkB phosphorylation that triggers its proteolysis. The released NF-kB is phosphorylated and translocate into the nucleus [56]. As a nuclear transcription factor, NF-kB regulates inflammatory and cell growth responses by interacting with responsive elements that includes iNOS [57] and cyclin D1 [58] genes, among others. Previously, Lee *et al.,* (2008) [44] reported that citral was able to suppress the activation of NF-kB in RAW264.7 (macrophage) cells, reducing the LPS-induced NF-kB DNA binding activity. In our work, citral slightly increased NF-kB labelled area/cell, this result can be explained by the generation of oxidative stress observed by the comsumption of cellular GSH. Simultaneously, citral reduced the percentage of cell with nuclear labelling, revealing that the inhibitory effect of citral includes the inhibition of NFkB translocation into the nucleus, what could also explain in part the decreased NO levels and the interference on cell growth. Previously, citral demonstrated the capacity inhibit the interaction of activated NFkB with cellular DNA, via unknown mechanism [44], but our results suggests that this inhibition is associated to decreased arrive of NFkB in the nucleus.

Finally, to observe the specificity of citral, we used the same conditions of treatment on NIH-3T3 cells (murine fibroblasts). We observed that NIH-3T3 cells

seems to be more resistant to citral (IC50 24h 2.5μM), compared to B16F10 cells (IC50 24h 1.04μM).

Taken together, all results allowed to obtain the interactome of citral effect on tested pathways (Fig. 7), revealing that Akt seems to be an important key to mediate citral effects. Further investigations need to be performed to elucidate the involvement of other signaling pathways that could result in regulation of tumorigenesis, energy storage, cell growth and survival, protein synthesis, and agiogenesis. These findings support that citral presents a huge potential as antineoplastic drug to be tested in vivo models of melanoma, with a tumor-driven mechanism of action enrolling the depletion of NO and interference in signaling pathways pivotal to cell proliferation and survival.

5. Conflict of Interest

We declare that we have no conflict of interest.

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CONCLUSÃO

O presente trabalho científico avaliou as atividades biológicas do citral em células de melanoma murino B16F10. O citral apresentou atividade citotóxica com IC_{50} 24h estimado em 1,04 μM, sendo capaz de induzir padrão necrótico de morte celular a partir da concentração 1μM, que foi observada pelo método de coloração com brometo de etídio/laranjado de acridina (BE/LA) e liberação de lactato desidrogenase em cultura. O citral foi capaz de induzir apoptose em concentrações iguais ou superiores a 0,5 μM, observada tanto no BE/LA, quanto no teste TUNEL. Pela primeira vez em literatura, descrevemos também a capacidade do citral em induzir autofagia. O citral também foi capaz de induzir lesões de DNA (a partir da concentração 0,5 μM), observadas pelo teste do cometa. Quanto ao estresse oxidativo, o citral promoveu depleção da glutationa GSH nas concentrações 0,5; 1,0 e 2,5 μM, e induziu a geração de malondialdeido na concentração 2,5 μM. Em contrapartida os níveis de NO sofreram redução acentuada com as concentrações testadas de citral, sugerindo que o estresse oxidativo observado seja derivado das espécies oxidativas de oxigênio. O uso de sequestradores de espécies reativos de oxigênio, apontaram que várias espécies (peroxil, oxigênio singlet e superóxido) estão envolvidas no estresse induzido pelo citral nas células B16F10. A avaliação da capacidade pró-oxidante do citral revelou que esta molécula não é capaz de gerar estresse oxidativo de forma direta. Com relação aos mecanismos de sinalização celular, o citral foi capaz de: (i) reduzir os níveis citoplasmáticos e nucleares de ERK1/2; (ii) reduzir os níveis citoplasmáticos de Pi3K e Akt; (iii) aumento dos níveis de p53; (iv) aumento dos níveis citoplasmáticos de NFkB, porém com forte redução da marcação nuclear deste marcador. Estes resultados sugerem que além da geração de estresse oxidativo, o citral também interfere em importantes vias de sinalização favoráveis a divisão e sobrevida celular. Em comparação as células B16F10, células normais murinas NIH-3T3 apresentaram efeito citotóxico com um IC50 24h superior de 2,5 μM, sugerindo que o citral não apresenta atividade seletiva, mas apresenta atividade citotóxica mais acentuada nas células B16F10. Podemos concluir que o citral apresentou resultados promissores *in vitro*, apontando que esta molécula deve ser estudada em sistemas *in vivo* para averiguar seu efeito antitumoral sobre o melanoma.

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APÊNDICES

APÊNDICE A

Figuras

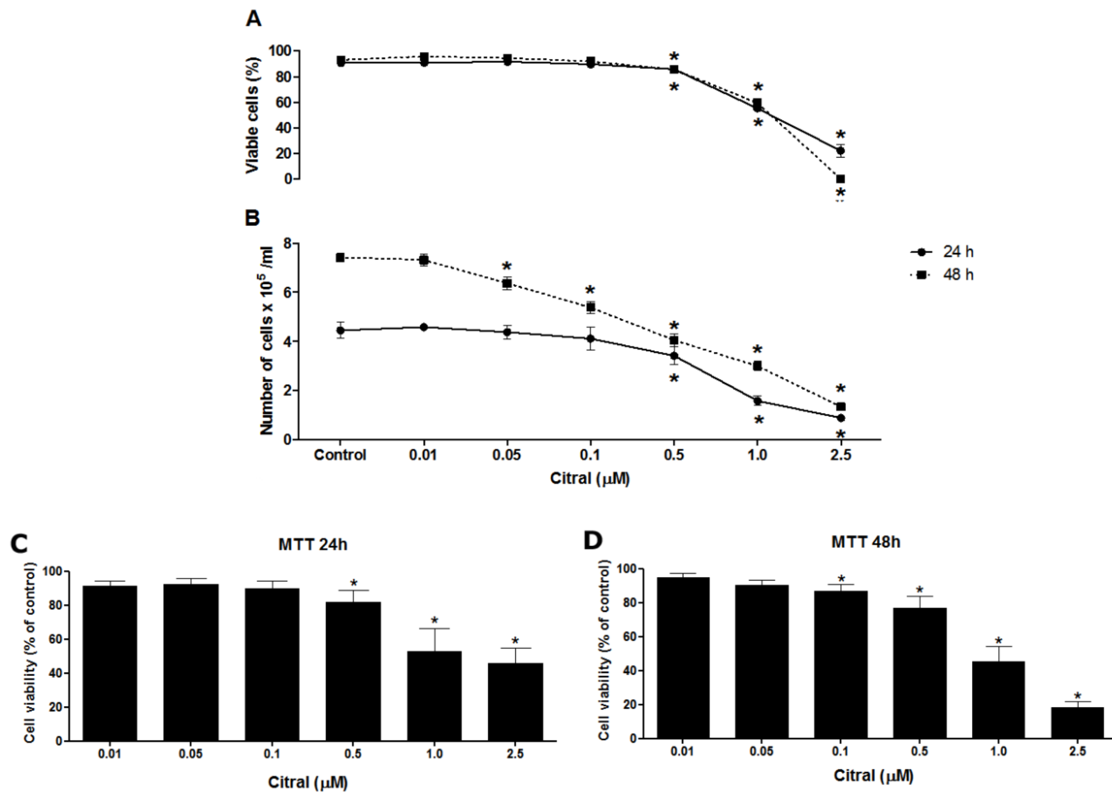

Fig.1. Citral effect in proliferation and viability in metastatic murine melanoma cells B16F10. The cells were treated for 24h or 48h with increasing concentrations ofcitral (0.01-2.5μM). (A) Cell viability by trypan blue exclusion assay. (B) Cell proliferation by cell counting. (C) Cell viability (% of control) obtained from MTT assay after 24 h of citral treatment. (D)) Cell viability (% of control) obtained from MTT assay after 48 h of citral treatment. * Statistically different from control group ($p<0.05$).

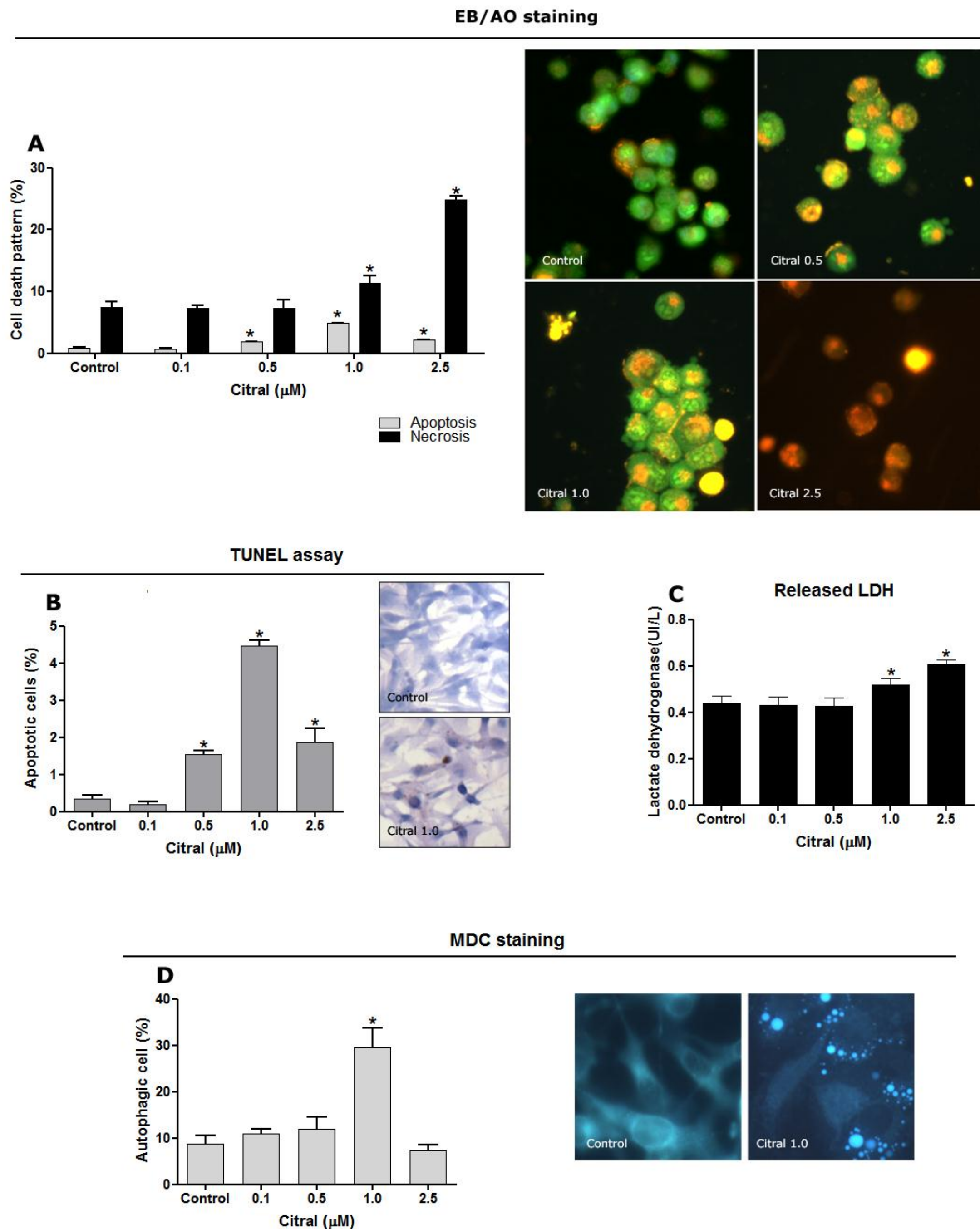

Fig. 2. Analysis of citral induced cell death patterns in metastatic murine melanoma cells B16F10. (A) Percentage of cell death patterns obtained from Ethidium Bromide and Acridine Orange staining(EB/AO), with representative photomicrographs demonstrating membrane bleebing and nuclear fragmentation typical of apoptosis, and intensive orange fluorescente typical of necrotic cells.(B) Percentage of apoptotic cells revealed by Tunel assay, with representative photomicrographs with the nucleus of apoptotic cells labelled in dark brown. (C) Measument ofLactate dehydrogenase (LDH)(UI/L) released into culture medium. (D) Percentage of cells with autophagic vacuoles revealed by monodansyldacaverine assay, with representative images. * Statistically different from control group (p<0.05).

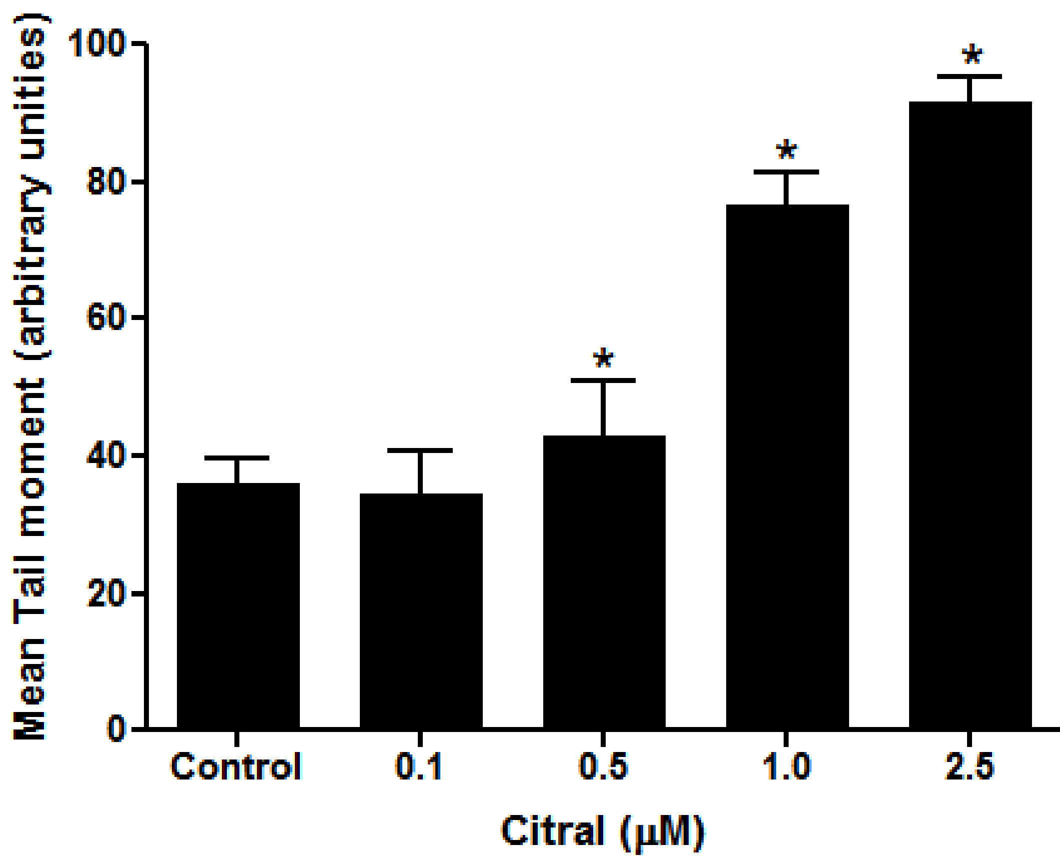

Fig. 3. Induction of DNA damage observed after 24 h of citral treatment. Mean tail moment (arbitrary unities) was obtained from comet assay in metastatic murine melanoma cells B16F10.*: Statistically different from control group ($p<0.05$).

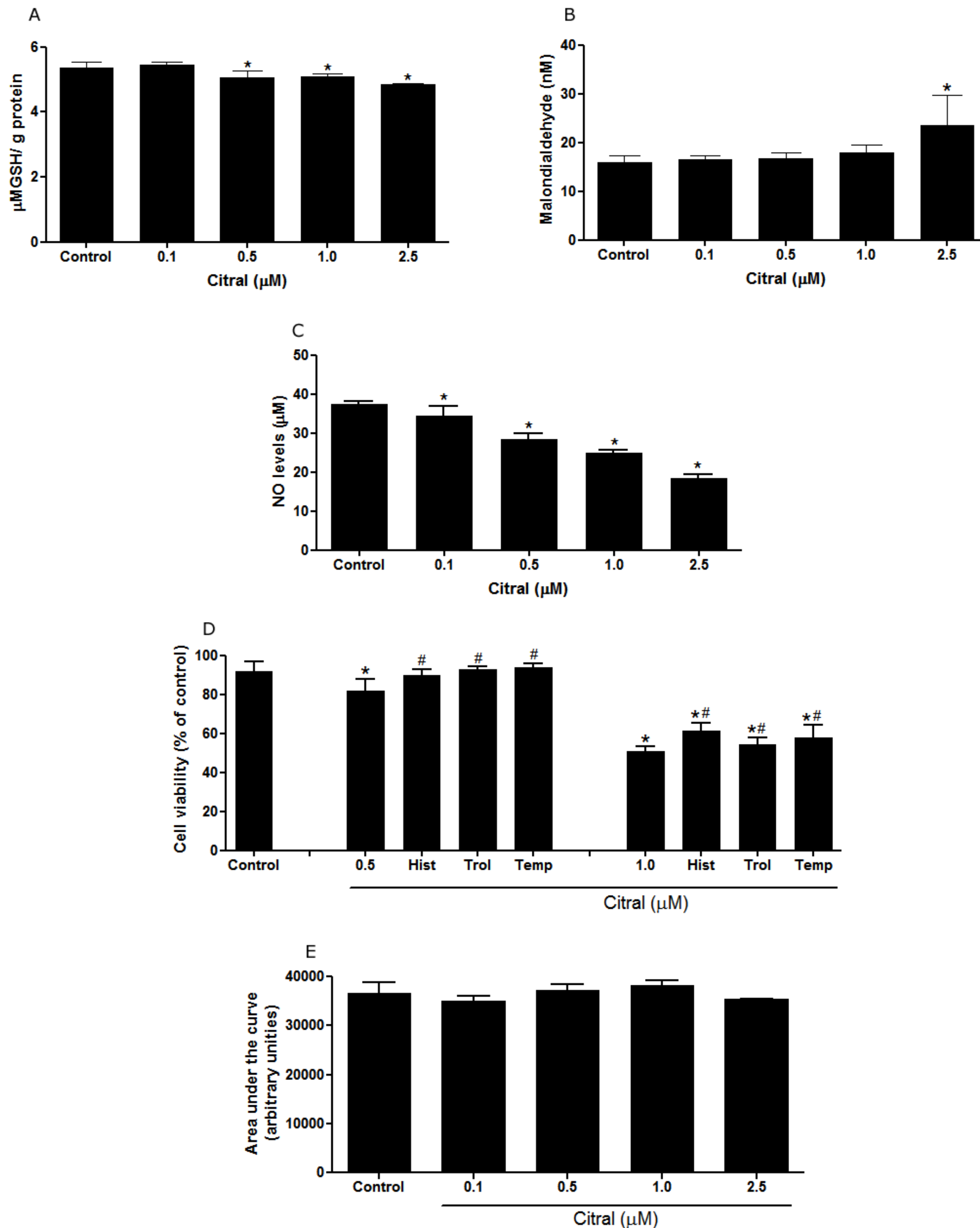

Fig.4. Oxidative stress parameters in metastatic murine melanoma cells B16F10 treated with citral.(A) Reduced glutathione (GSH)levels (μM of GSH/g of protein). (B) Malondealdehyde levels (nM).(C) Nitric oxide (NO) levels (μM) obtained from nitrite levels measured by Griess/Cadmium assay.(D) Evaluation of citral cytotoxicity by the MTT assay in the presence and in the absence of specific scavengers of oxygen reactive species: L-histidine 100 mM (Hist), Trolox 50mM (Trol) and Tempol 50mM (Temp) (E) Area under the curve of data obtained from oxygen uptake by red blood cells treated with citral 5 min (37°C).* Statistically different from control group ($p<0.05$).

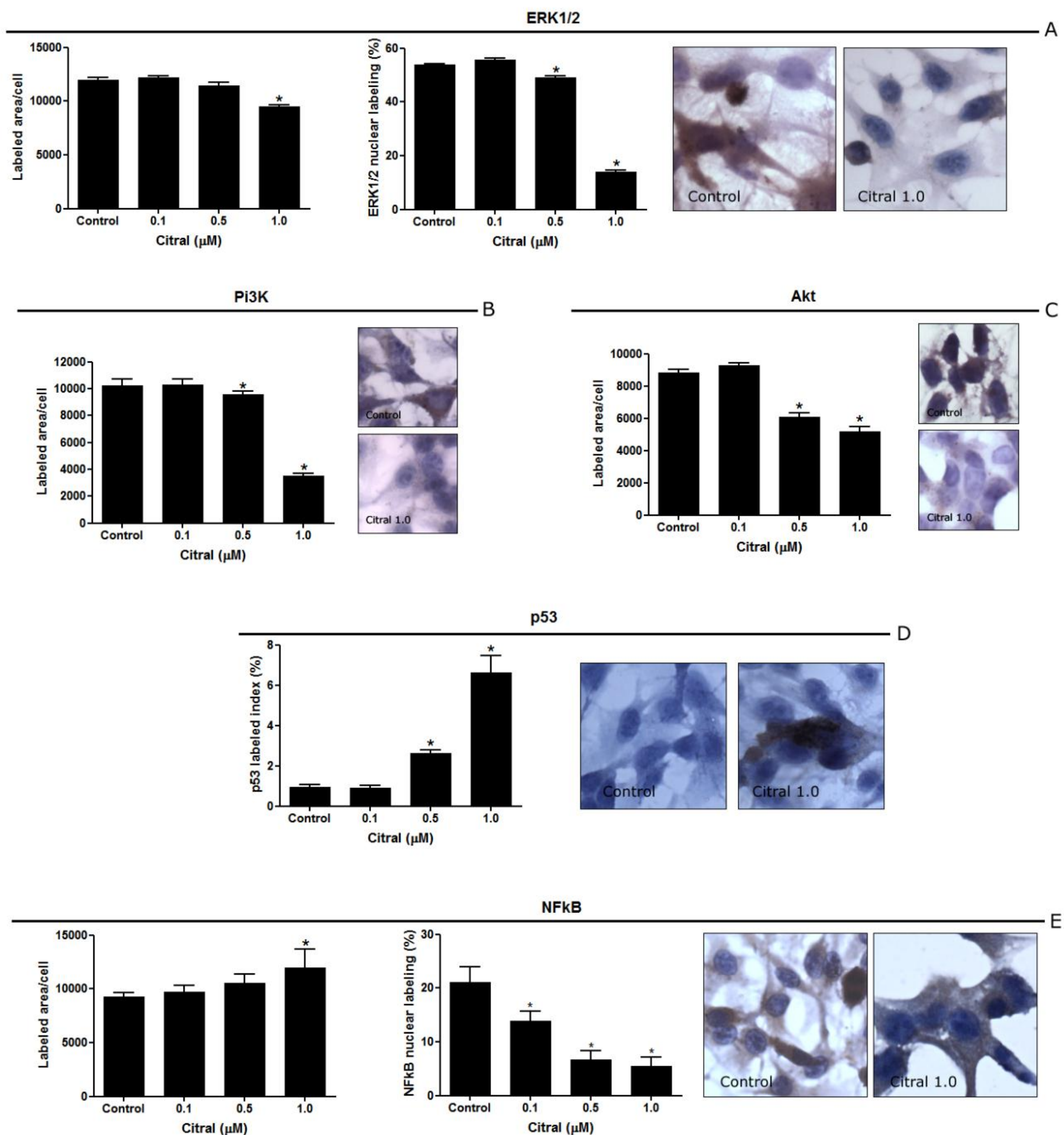

Fig. 5. Immunocitochemical labelling in metastatic murine melanoma cells B16F10 treated with citral. (A) ERK 1/2 labelled area/cell and percentage of cells with nuclear labelling for this protein. (B) Pi3K labelled area/cell. (C) Akt labelled area/cell. (D) Percentage of cell labelled with p53 antibody. (E) NFkB Labelled area/cell and percentage of cells with nuclear labelling for this protein. Images are representative photomicrographs for each protein. * Statistically different from control group ($p<0.05$).

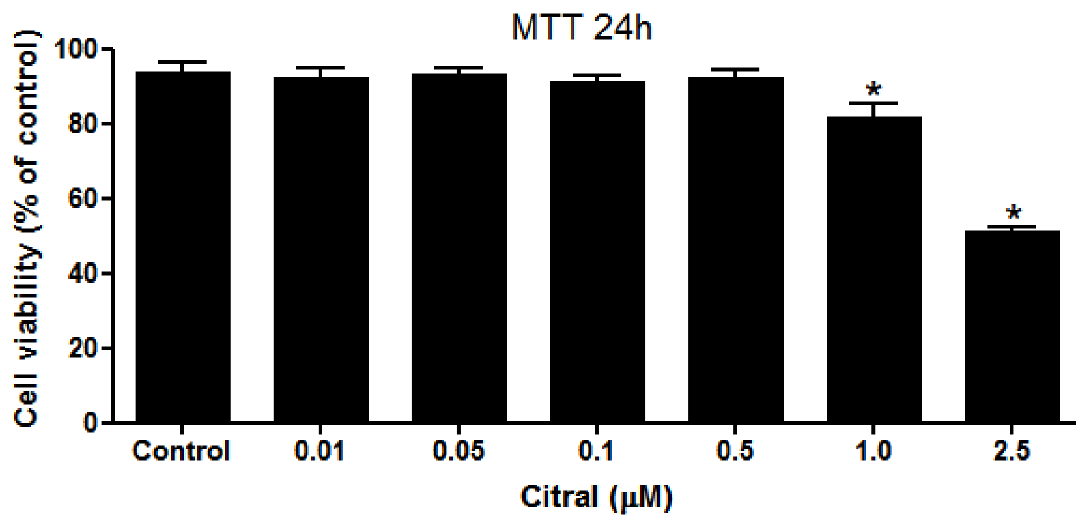

Fig. 6. Citral effect in cellular viability in NIH-3T3 murine fibroblast cells.The cells were treated for 24h with increasing concentrations of citral (0.01-2.5μM)*Statistically different from control group ($p<0.05$).

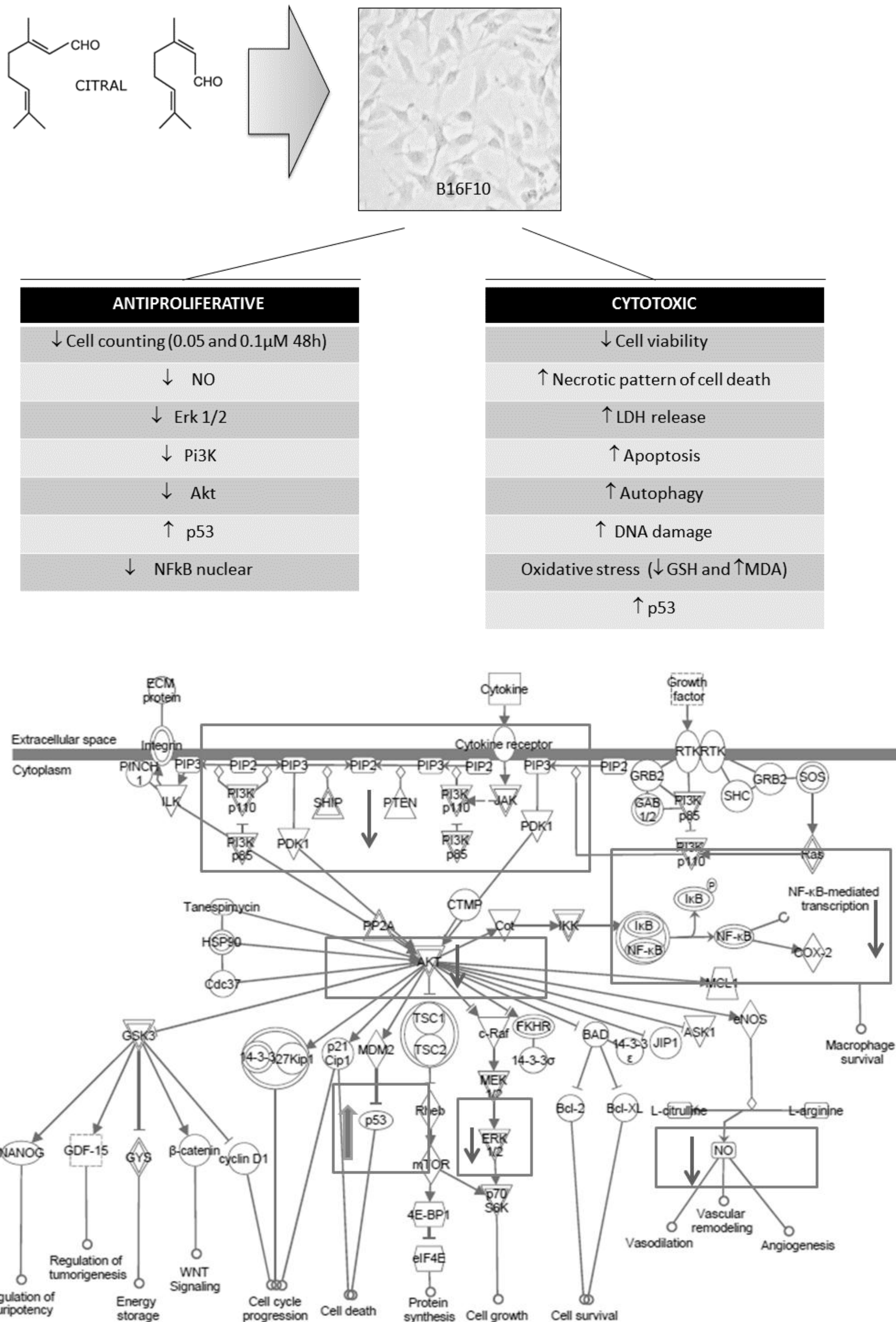

Fig. 7. Overall view of citral-induced effects in B16F10 cells (A) and interactome analysis of all evaluated pathways (the protein interaction network was obtained from the Ingenuity Pathway Analysis software (IPA) available at http://www.ingenuity.com).

APÊNDICE B

Tabela

Variables	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Cell counting (1)	-	**0.921**	**0.973**	-0.737	**-0.894**	-0.864	-0.403	**-0.953**	**0.946**	-0.847	**0.988**	0.881	0.891	0.859	**0.968**	-0.942	**-0.965**	0.911
Cell viability (2)	**0.921**	-	**0.979**	-0.542	**-0.996**	**-0.988**	-0.182	**-0.977**	**0.946**	**-0.963**	**0.939**	**0.996**	**0.998**	**0.992**	0.785	**-0.987**	-0.944	0.773
MTT assay (3)	**0.973**	**0.979**	-	-0.694	**-0.958**	**-0.937**	-0.358	**-0.996**	**0.984**	**-0.902**	**0.974**	**0.980**	**0.984**	**0.970**	0.854	**-0.991**	**-0.967**	0.827
Apoptosis (%) (4)	-0.737	-0.542	-0.694	-	0.466	0.411	**0.915**	0.707	-0.774	0.315	-0.684	**-0.986**	**-0.990**	**-0.979**	-0.833	**0.991**	**0.960**	-0.810
Necrosis (%) (5)	**-0.894**	**-0.996**	**-0.958**	0.466	-	**0.998**	0.095	**0.954**	**-0.916**	**0.981**	**-0.922**	**-0.996**	**-0.994**	**-0.989**	-0.779	**0.993**	**0.956**	-0.797
LDH levels (6)	-0.864	**-0.988**	**-0.937**	0.411	**0.998**	-	0.038	**0.934**	**-0.892**	**0.986**	**-0.898**	**-0.996**	**-0.988**	**-0.990**	-0.717	**0.983**	0.937	-0.766
Autophagy (%) (7)	-0.403	-0.182	-0.358	**0.915**	0.095	0.038	-	0.387	-0.483	-0.081	-0.341	**-1.000**	**-0.999**	**-0.997**	-0.750	**0.983**	0.933	-0.751
Comet assay (8)	**-0.953**	**-0.977**	**-0.996**	0.707	**0.954**	**0.934**	0.387	-	**-0.991**	**0.886**	**-0.955**	**-0.997**	**-0.999**	**-0.994**	-0.779	**0.985**	0.940	-0.765
GSH levels (9)	**0.946**	**0.946**	**0.984**	-0.774	**-0.916**	**-0.892**	-0.483	**-0.991**	-	-0.829	**0.951**	**0.996**	**0.991**	**0.989**	0.761	**-0.992**	**-0.954**	0.794
MDA levels (10)	-0.847	**-0.963**	**-0.902**	0.315	**0.981**	**0.986**	-0.081	**0.886**	-0.829	-	**-0.880**	-0.910	-0.918	-0.891	-0.949	**0.962**	**0.976**	-0.905
NO levels (11)	**0.988**	**0.939**	**0.974**	-0.684	**-0.922**	**-0.898**	-0.341	**-0.955**	**0.951**	**-0.880**	-	0.877	0.876	0.848	0.942	**-0.954**	**-0.990**	**0.965**
ERK1/2 labeling (12)	0.881	**0.996**	**0.980**	**-0.986**	**-0.996**	**-0.996**	**-1.000**	**-0.997**	**0.996**	-0.910	0.877	-	**0.998**	**0.998**	0.733	**-0.980**	-0.928	0.742
ERK1/2 nuclear labeling (%) (13)	0.891	**0.998**	**0.984**	**-0.990**	**-0.994**	**-0.988**	**-0.999**	**-0.999**	**0.991**	-0.918	0.876	**0.998**	-	**0.998**	0.749	**-0.976**	-0.922	0.732
Pi3K labeling (14)	0.859	**0.992**	**0.970**	**-0.979**	**-0.989**	**-0.990**	**-0.997**	**-0.994**	**0.989**	-0.891	0.848	**0.998**	**0.998**	-	0.703	**-0.966**	-0.904	0.700
Akt labeling (15)	**0.968**	0.785	0.854	-0.833	-0.779	-0.717	-0.750	-0.779	0.761	-0.949	0.942	0.733	0.749	0.703	-	-0.831	-0.892	0.912
p53 labeling (16)	-0.942	**-0.987**	**-0.991**	**0.991**	**0.993**	**0.983**	**0.983**	**0.985**	**-0.992**	**0.962**	**-0.954**	**-0.980**	**-0.976**	**-0.966**	-0.831	-	**0.983**	-0.860
NFkb labeling (17)	**-0.965**	-0.944	**-0.967**	**0.960**	**0.956**	0.937	0.933	0.940	**-0.954**	**0.976**	**-0.990**	-0.928	-0.922	-0.904	-0.892	**0.983**	-	-0.938
NFkB nuclear labeling (5) (18)	0.911	0.773	0.827	-0.810	-0.797	-0.766	-0.751	-0.765	0.794	-0.905	**0.965**	0.742	0.732	0.700	0.912	-0.860	-0.938	-

Tab.1 – Spearman's correlation matrix of citral 1µM (24 h) effects against B16F10 cells. All variables were log (X+1) transformed before analysis. Values shown are the correlation coefficients between variables. Bold coefficient were statistically

significant ($p<0.05$). Positive and negative coefficients indicate positive or negative correlations, with 1.0 or -1.0 being the strongest relationship.

ANEXOS

ANEXO A

Submissão do artigo

Dear Dr. Larissa Sanches,

You have been listed as a Co-Author of the following submission:

Journal: Cancer Letters

Corresponding Author: Rodrigo Luiz

Co-Authors: Larissa J Sanches, Master; Poliana C Marinello, PH.D. student; Carolina Panis, PH.D.; Tatiane R Fagundes, Master student; José Andrés M Diaz, Doctor; Julio Cesar M Freitas Jr., PH.D.; Rubens Cecchini, PH.D.; Alessandra L Cecchini, PH.D.;

Title: CITRAL INDUCES APOPTOSIS AND AUTOPHAGY IN B16F10 MELANOMA CELLS BY GENERATING OXYGEN REACTIVE SPECIES AND REDUCING THE EXPRESSION OF CELL GROWTH PROTEINS

If you did not co-author this submission, please contact the Corresponding Author of this submission at rc.luiz@gmail.com;rc.luiz@yahoo.com.br; do not follow the link below.

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