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Fabiana Ramos

Investigação do Mecanismo de Citotoxicidade do Óleo Essencial de *Piper gaudichaudianum* Kunth em Células V79

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Dissertação submetida ao Programa de Pós-Graduação em Patologia da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre

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Lista de abreviaturas

µg: micrograma

µL: microlitro

µm: micrometro

µM: micromolar

$^{1}O_2$: oxigênio singlete

8 oxo G: 8-oxo-7,8-dihidro-2'-desoxiguanosina

A: adenina

AIF: fator indutor de apoptose

AKt: proteína serina treonina cinase B

Anexina V (PE)-7 AAD: anexina cinco ficoeritrina - 7 aminoactinomicina

AP-1: Proteína Ativadora 1

Apaf-1: fator ativador de protease apoptótica

ATM: proteína Ataxia Telangiectasia mutada

ATP: adenosina trifosfato

ATR: proteína Ataxia Telangiectasia relacionada

BAK: BCL-2 *homologous antagonist killer*

BAX: proteína BCL-2-associada à proteína X

BCL-2: proteína linfoma de célula B 2

BER: reparo por excisão de bases

BSA: albumina sérica bovina

C: Citosina

CAT: catalase

CDK: proteína cinase dependente de ciclina

CIT-C: citocromo C

COX-2: ciclo-oxigenase 2

DCF: Diclorofluoresceína

DCFH-DA: 2’7’-diclorofluoresceína

DMEM: *Dubelcco’s Modified Eagle Medium*

DMSO: dimetilsulfóxido

DNA: ácido desoxirribonucleico

DOX: doxorrubicina

DSB: quebras nas duas fitas de DNA

ERN: espécies reativas de nitrogênio

ERO: espécies reativas de oxigênio

eV: elétron volt

FACS: citometria de fluxo

FBS: soro fetal bovino

FID: detector de ionização de chama

G: guanina

G0: fase de repouso do ciclo celular

G1: fase intermediária 1 do ciclo celular

G2: fase intermediária 2 do ciclo celular

GC/MS: cromatografia gasosa e espectrometria de massa

GC: cromatografia gasosa

GC-FID: cromatografia gasosa com detecção por ionização em chama

GC-IRMS: cromatografia gasosa correlacionada à espectrometria de massa derelação de isótopos

ɣ-H2AX: histona gama H2AX

GPx: glutationa peroxidase

GSH: gama-L-glutamil-L-cisteínil-L-glicina (glutationa reduzida)

GSSG: glutationa dissulfeto (glutationa oxidada)

HPLC: cromatografia líquida de alta eficiência

HR: reparo por recombinação homóloga

IAP: proteína inibidora de apoptose

IC50: concentração inibitória em 50%

IgG: imunoglobulina G

INCA: Instituto Nacional do Câncer

iNOS: enzima óxido nítrico sintase induzida

kDa: quilodalton

M: mitose

MAPK: proteína cinase ativada por mitógeno

Mre11: *Meiotic Recombination 11 Homolog*

MRN: complexo de proteínas Mre11 - Rad50 - Nbs1

MS: espectrometria de massa

mTOR: *mammalian Target of Rapamycin*

MUTYH: DNA glicosilase

NAC: N-acetilcisteína

NAD+: dinucleotídeo de nicotinamida e adenina oxidada

NADH: dinucleotídeo de nicotinamida e adenina reduzida

NADP+: fosfato de dinucleotídeo de nicotinamida e adenina oxidada

NADPH: fosfato de dinucleotídeo de nicotinamida e adenina reduzida

NAM: nicotinamida mononucleotídeo

NER: reparo por excisão de nucleotídeos

NHEJ: reparo por recombinação não-homóloga

OE: óleo essencial

OGG1: 8-oxoguanina-DNA glicosilase

Omi: serina peptidasa HtrA 2 mitocondrial

OMS: Organização Mundial da Saúde

ONOO-: radical peroxinitrito

p21: oncogene p21

p38: oncogene p38

p53: oncogene p53

PADPR: polímero de poli (ADP-ribose)

PAGE: eletroforese em gel de poliacrilamida

PARP 1: poli (ADP-ribose) polimerase 1

PARP 2: poli (ADP-ribose) polimerase 2

PARP: gene codificador da poli (ADP-ribose) polimerase

PARP: poli (ADP-ribose) polimerase

PARPi: inibidor da poli (ADP-ribose) polimerase

pB: pares de base

PBS: solução salina tamponada com fosfato

PGE2: prostaglandina E2

pH: potencial hidrogeniônico

PLE: extração com líquidos pressurizados

Prx- peroxirredoxina

PS: fosfolipídeo de membrana citoplasmática

Rad-50: *DNA repair protein RAD-50*

Rho 123: rodamina 123

RI: índice de retenção

ROS: *reactive oxygen species*

S: fase de síntese do ciclo celular

SDS: dodecil sulfato de sódio

SIRT-1: sirtuína 1

Sítios AP: sítio apurínico/apirimidínico

Smac: segundo ativador mitocondrial de caspase

SNC: sistema nervoso central

SOD: superóxido dismutase

SOD-CuZn: superóxido dismutase cobre-zinco

SOD-Mn: superóxido dismutase manganês

TB: *Trypan Blue*

Trx: tiorredoxina

TrxR: tiorredoxina redutase

Tween 20: polysorbate 20

UV: ultravioleta

V79: células de fibroblasto de hamster chinês

VIT A: vitamina A (betacaroteno)

VIT C: vitamina C (ácido ascórbico)

VIT E: vitamina E (tocoferol)

Resumo

Introdução: O óleo essencial (OE) das folhas de *Piper gaudichaudianum* Kunth possui propriedades biológicas, tais como anti-inflamatória, antimicrobiana e antitumoral. Previamente, foi demonstrado que este OE possui pronunciada ação citotóxica, mutagênica e genotóxica em células V79, como consequência do potencial oxidativo do mesmo. O presente trabalho pretende ampliar o conhecimento sobre o seu mecanismo de citotoxicidade.

Objetivos: Avaliar a citotoxicidade e o mecanismo de ação do OE em células V79, por meio da análise da composição química do OE, viabilidade celular, função mitocondrial, formação de radicais livres, proteínas envolvidas na resposta celular, indução de morte celular e ação da N-acetilcisteína, após o tratamento com diferentes concentrações do OE, durante 3 h e 24 h.

Material e Métodos: O OE foi extraído por hidrodestilação e analisado por GC-FID e GC-MS. A viabilidade celular e o efeito da N-acetilcisteína foram verificados com Azul de Trypan; a formação de espécies reativas, com DCFH-DA; a função mitocondrial com Rodamina 123; o tipo de morte celular, com Anexina V-PE e 7-AAD; a atividade da superóxido dismutase e catalase por espectrofotometria; e, a expressão de PADPR e SIRT-1 por Western Blotting.

Resultados: Foram identificados como componentes majoritários (E)-nerolidol (31,13%), α-humuleno (14,83%) e (E)-cariofileno (6,79%). O OE diminuiu a viabilidade celular, com IC_{50} de 4,15 µg/mL (3 h) e 4,49 µg/mL (24 h); aumentou a despolarização mitocondrial e a produção de espécies reativas; reduziu a atividade da superóxido dismutase; aumentou a expressão de PADPR e diminuiu a de SIRT1; induziu a morte celular por necrose e apoptose

tardia; O pré-tratamento celular com N-acetilcisteína restabeleceu a viabilidade celular.

Conclusão: O OE exerce efeito citotóxico, concentração-dependente, pela indução de estresse oxidativo celular com envolvimento de dano mitocondrial e modulação da resposta antioxidante e de reparo no DNA.

Palavras-chave: *Piper gaudichaudianum* Kunth, óleo essencial, citotoxicidade.

Abstract

Introduction: The essential oil (EO) of *Piper gaudichaudianum* Kunth leaves has biological properties such as anti-inflammatory, antimicrobial and antitumor activities. Previously, it was shown that EO showed pronounced cytotoxic, mutagenic and genotoxic effects in V79 cells as a result of its oxidative potential. This work aims to increase the knowledge about EO cytotoxic mechanism.

Purpose: To evaluate the cytotoxicity and the mechanism of action of EO in V79 cells, by analyzing the chemical composition of EO, as well as cell viability, mitochondrial function, free radicals formation, proteins involved in cellular response, cell death induction and N-acetylcysteine action, after treatment with different concentrations of EO, for 3 h and 24 h.

Material and Methods: EO was extracted by hydrodistillation and analyzed by GC-FID and GC-MS. Cell viability and the effects of N-acetylcysteine were checked with Trypan Blue; ROS formation with DCFH-DA; mitochondrial function with Rhodamine 123; cell death with Annexin V-PE and 7-AAD; superoxide dismutase and catalase activities by spectrophotometry; and the expressions of SIRT-1 and PADPR by Western Blotting.

Results: The molecules identified as major components of EO were (E)-nerolidol (31.13%), α-humulene (14.83%) and (E)-caryophyllene (6,79%). EO decreased cell viability, with IC50 of 4.15 μg/ml (3 h) and 4.49 μg/ml (24 h); increased mitochondrial depolarization and ROS production; decreased superoxide dismutase activity; increased PADPR levels and decreased levels of

SIRT1; induced cell death by necrosis and late apoptosis. Cellular pretreatment with N-acetylcysteine restored cell viability.

Conclusion: EO exerts cytotoxic effect in a concentration-dependent manner, by the induction of cellular oxidative stress with involvement of mitochondrial damage and modulation of antioxidant response and DNA repair.

Keywords: *Piper gaudichaudianum* Kunth, essential oil, cytotoxicity.

1. Introdução

O Brasil possui uma rica biodiversidade de plantas naturais, sendo, há muitos anos, alvo de pesquisa de inúmeros botânicos e estudiosos nacionais e internacionais. Como exemplo, podem ser citados os manuscritos de Auguste de Saint-Hilaire, um naturalista francês que identificou cerca de 7000 espécies de plantas na América do Sul, das quais 4500 espécies pouco conhecidas até o momento. Ele publicou dois livros em 1824 denominados *Plantes usuelles des Brasiliens* e *Histoire des plantes plus remarcables du Brésil et du Paraguay.* Sua coleção está depositada no Museu Nacional de História Natural em Paris e tem dados de 283 plantas nativas brasileiras dos estados de Minas Gerais, Espírito Santo, Rio de Janeiro e Goiás, identificadas quando a flora desta região ainda era preservada e inexplorada (Brandão *et al.*, 2012).

Outro botânico taxonômico que dedicou 50 anos estudando a família Piperaceae, com ênfase nos gêneros *Peperomia* e *Piper*, foi Truman George Yuncker. Este pesquisador descreveu 839 novas espécies, 211 novas variedades e teve como sua última grande obra, *The Piperaceae of Brazil* (1972-1975), completada com o auxílio de Ethel Claflin Yuncker. Sua coleção está depositada no *New York Botanical Garden Herbarium* (Yuncker, 1958; Yuncker, 1961; Holmgren *et al*., 1996).

O Brasil possui 20% da flora mundial e a habitação humana de áreas florestais, antes preservadas, conduziu ao desmatamento, comércio irregular e consequente extinção de várias espécies vegetais nativas. Este fato, aliado ao pouco conhecimento sobre muitas outras espécies ainda existentes, ressalta a necessidade de implantação de políticas públicas eficazes no intuito de

preservar e identificar esta imensa biodiversidade vegetal brasileira (Brandão *et al*., 2013).

A identificação de espécimes vegetais é essencial, pois medicamentos a base de plantas medicinais vêm sendo utilizados ao longo do tempo (Rates, 2001; Thomas *et al*., 2011; Petrovska, 2012), sempre com crescente interesse na pesquisa e no desenvolvimento de novos fármacos contendo produtos de origem vegetal (Cordell, 1995; Shu *et al*.,1998; Simões *et al*., 2007; Chen *et al*., 2011; Groussin e Antoniotti, 2012; Sireeratawong *et al*., 2012a; Sireeratawong *et al*., 2012b). São exemplos de alcaloides derivados de plantas medicinais, utilizados como medicamentos: a quinina, antimalárica, e a quinidina, utilizada no tratamento de arritmias cardíacas (oriundas da casca da *Cinchona spp*); além de tantos outros, como a atropina, que possui ação anticolinérgica (derivada das folhas e flores da *Atropa belladonna*); a fisostigmina, com atividade parassimpaticomimética (obtida das sementes da fava-de-calabar), utilizada no tratamento do glaucoma; e a morfina (derivada do ópio obtido do látex da flor da papoula, *Papaver sominiferum*), ainda considerada um dos analgésicos opioides de ação central mais utilizados (Phillipson, 2001; Gurib-Fakim, 2006). Cabe ressaltar que o mercado da indústria farmacêutica é um dos mais lucrativos (Lobo, 2013) e a inovação tecnológica aliada à união entre universidade, empresa e governo são consideradas impulsionadoras do conhecimento, na busca por medicamentos para o tratamento de diversas doenças (Santos e Siani, 2012).

Com o aumento da expectativa de vida também se elevaram os índices de doenças crônicas degenerativas e tumorais. Com o propósito de amenizar

sintomas e buscar a cura de doenças infecciosas, neurodegenerativas e tumorais, diversos estudos, aliados ao desenvolvimento biotecnológico, visam identificar novas moléculas, derivadas de plantas medicinais, que tenham interações com alvos moleculares específicos (Cragg e Newman, 2013; Phillipson, 2007; Mishra e Tiwari, 2011; Ansari *et al*., 2013; Lee *et al*., 2013). Como exemplo de agentes antitumorais comercializados, oriundos de plantas medicinais, podemos citar os alcaloides derivados da *Vinca rósea* Linn, Vimblastine e Vincristine; o quimioterápico Paclitaxel (Taxol), originário da casca da árvore do Teixo do Pacífico (*Taxus brevifolia*); e o Topotecan, antitumoral procedente da *Camptotheca acuminata* (Prakash *et al*., 2013; Sisodiya, 2013).

A busca por novas drogas antitumorais, com eficácia superior às já utilizadas ou com mecanismos de ação diferentes, que tenham resultados significativos, no que se refere à resistência aos medicamentos atuais, aliada a menor toxicidade para o paciente (Alfaro *et al*., 2013; Yousef e Hussiem, 2015) são fundamentais, especialmente quando se considera que, somente em 2012, ocorreram 14,1 milhões de novos casos de câncer e 8,2 milhões de mortes por câncer no mundo. A previsão para 2030 é de uma incidência de 21,4 milhões de casos de câncer, com 13,2 milhões de óbitos (Ferlay *et al*., 2012). Nos Estados Unidos, os tipos de tumores mais prevalentes foram os de pele, pulmão, colon e reto, em ambos os sexos. Os homens foram mais acometidos por câncer de próstata e as mulheres por câncer de mama (Siegel *et al*., 2014). No Brasil, em 2014, a estimativa foi de 576 mil novos casos, portanto um problema de saúde pública. Os tumores de pulmão, colon e reto também acometeram os brasileiros de ambos os sexos. Os canceres de próstata,

estômago e da cavidade oral foram mais prevalentes nos homens. Já os tumores de mama, colo do útero e glândula tireoide apresentam uma frequência maior em mulheres (Instituto Nacional de Câncer José Alencar Gomes da Silva, 2014).

A correta identificação e preservação de plantas medicinais, aliada ao conhecimento sobre a composição química, efeitos biológicos, segurança e interação medicamentosa de produtos de origem vegetal são indispensáveis para a comercialização e utilização destes compostos na pesquisa científica, indústria farmacêutica e pela população (Hu *et al*., 2005; Bhattarai, 2012; Rodrigues e Barnes, 2013).

1.1. *Piper gaudichaudianum* Kunth

Piper gaudichaudianum Kunth é uma planta medicinal da família das angiospermas, pertencente à ordem Piperales e à família Piperaceae, que compreende aproximadamente três mil espécies distribuídas em oito gêneros, dos quais se destacam os gêneros *Piper*, *Peperomia* e *Pothomorphe*. A família Piperaceae se destaca por produzir substâncias com grande potencial comercial na produção de novas drogas terapêuticas, sendo as pertencentes ao gênero *Piper* as mais estudadas e conhecidas quanto a sua composição química e suas propriedades biológicas. Destas, se destacam espécies como a pimenta, *Piper nigrum*, e outras de grande utilização medicinal, como a *Piper betle*, *Piper longum*, *Piper angustifolium* e *Piper methysticum*, de grande importância na medicina chinesa e indiana (Di Stasi e Hiruma-Lima, 2002). Azevedo e Silva (2006) destacam também o uso destas plantas por motivo

religioso, contribuindo de forma negativa para o extrativismo vegetal na Mata Atlântica.

As espécies do gênero *Piper* são as mais abundantes, com localização pantropical, podendo ser encontradas nas Américas Central e do Sul e em outros continentes, como a Ásia (Yuncker, 1958; Yuncker, 1961; Callejas, 1989; Callejas e Johnson, 1989; Callejas, 1990; Meurer-Grimes, 1989; Luziatelli *et al*., 2010). No Brasil, comumente localizam-se na região da Mata Atlântica, compreendendo em torno de 460 espécies dispostas em cinco gêneros. Normalmente, predominam em locais úmidos abaixo das copas de árvores, mas também ocupam locais secos nas beiras de florestas (Carvalho-Okano e Alves, 1998; Figueiredo e Sazima, 2000; De Lima e Dos Reis, 2004; Medeiros, 2006; Bardelli, 2008; Cavalheiro *et al*., 2013; Sarnaglia Junior *et al*., 2014).

As plantas do gênero *Piper* caracterizam-se como arbustos, lianas, epífitas, ervas e pequenas árvores aromáticas, por possuírem células produtoras de óleos essenciais (Carvalho-Okano e Alves, 1998; Figueiredo e Sazima, 2000, Bratti *et al*., 2013). Apresentam caule nodoso, folhas pecioladas ou raramente sub-sésseis e estípulas adnatas ao pecíolo ou ausentes (Carvalho-Okano e Alves, 1998; Albiero *et al*., 2005).

A análise química de espécies de *Piper* tem sido amplamente investigada em diversas partes do mundo com a identificação de vários compostos biologicamente ativos, como alcaloides (Gutierrez *et al*., 2013), fenilpropanoides, lignanas, terpenos, esteroides, flavonas, derivados do ácido benzoico, benzopirenos, dentre outros (Parmar *et al*., 1997; Figueiredo e Sazima, 2000; Lopes *et al*., 2007, Puhl *et al*., 2011; Yamaguchi *et al*., 2011).

Estes compostos derivados das espécies de *Piper* se destacam como potencial terapêutico por apresentarem propriedades analgésicas, anti-inflamatórias, antifúngicas, antimicrobianas (Di Stasi e Hiruma-Lima, 2002; Lago *et al*., 2004; Colvard *et al*., 2006; Koroch *et al*., 2007; Péres *et al*., 2009; Schmit e Riffel, 2010; Puhl *et al*., 2011), antiprotozoárias (Batista Júnior *et al*., 2008, Batista Júnior *et al*., 2011) e antitumorais (Rai *et al*., 2011; Tak *et al*., 2011; Lai *et al*., 2012; Bezerra *et al*., 2013; Wang *et al*., 2014).

Piper gaudichaudianum é referida popularmente como iaborandi, jaborandi, murta ou pariparoba (Albiero *et al*., 2005). Esta planta assemelha-se ao jaborandi verdadeiro e casos de troca ou até de falsificação comercial podem ocorrer. Embasado neste fato, Albiero *et al*. (2005) realizaram o estudo morfo-anatômico do caule e da folha de *P. gaudichaudianum*, a fim de estabelecer caracteres estruturais como auxílio na identificação e avaliação do controle de qualidade durante a comercialização desta planta como droga vegetal.

Piper gaudichaudianum caracteriza-se como um arbusto de pequeno porte com folhas simples, alternas, curto-pecioladas, membranáceas, um pouco ásperas, acuminadas no ápice, assimétricas na base, estipuladas, com limbo inteiro de formato ovado-elíptico, de base oblíqua e de ápice cuspidado (Figura 1). A nervura principal apresenta, na base, oito feixes vasculares colaterais, que se reduzem a apenas um na região média e apical da folha. Na nervura, o colênquima está presente em ambas as faces da folha e, no parênquima, constam idioblastos cristalíferos, contendo ráfides e monocristais, bem como idioblastos oleíferos. Ainda, esta planta possui inflorescências do

tipo espiga, levemente curvadas, podendo atingir até oito centímetros de comprimento (Di Stasi e Hiruma-Lima, 2002; Albiero *et al*., 2005; Iwazaki *et al*., 2006; Medeiros, 2006).

Figura 1: Arbusto de *Piper gaudichaudianum* Kunth (Imagem do Laboratório de Palinologia da ULBRA).

Insetos e morcegos utilizam a *P. gaudichaudianum* como fonte de alimento (De Lima e Dos Reis, 2004; Ramos, 2009), contribuindo para a dispersão de suas sementes, bem como para o processo evolutivo e a diversidade das florestas (Mikich *et al*., 2003; Teixeira, 2003).

Na região da Mata Atlântica, popularmente se utiliza a infusão das folhas frescas de *P. gaudichaudianum* para aliviar a dor nos dentes e as raízes frescas são mastigadas produzindo efeito anti-inflamatório (Figueiredo e Sazima, 2000; Di Stasi e Hiruma-Lima, 2002; Di Stasi *et al*., 2002).

Péres *et al*. (2006a) estudaram a espécie *P. gaudichaudianum* comparando o método de extração clássico Soxhlet com o de extração por líquido pressurizado (PLE), relacionando a quantidade de material extraído com a sua composição química. Neste estudo foi utilizado éter de petróleo e etanol

na extração de terpenos, ácidos graxos e vitamina E das folhas de *P. gaudichaudianum*, a fim de verificar o efeito da polaridade do solvente no processo de extração. A análise dos extratos foi realizada por cromatografia gasosa (GC) com detecção por espectrometria de massas (MS). Como resultados, os autores identificaram que o processo de extração por PLE foi mais eficaz, simples e realizado em menor tempo. Os principais compostos identificados foram nerolidol, fitol e ácido palmítico. A maior concentração de nerolidol foi obtida na extração de Soxhlet com éter de petróleo, enquanto a maior concentração de ácido palmítico foi encontrada no extrato por PLE e éter de petróleo. Seguindo o estudo, no mesmo ano, estes autores investigaram a influência de alguns parâmetros experimentais relacionados com a extração de compostos das folhas de *P. gaudichaudianum* por PLE utilizando éter de petróleo como solvente extrator e identificando os compostos majoritários dos extratos deste espécime por GC-MS, encontrando como principais constituintes ácido palmítico, ácido esteárico e nerolidol. Além do extrato etanólico, outro componente biologicamente ativo muito estudado da espécie *P. gaudichaudianum* é o seu óleo essencial, objeto de estudo desta dissertação.

1.2. Óleo essencial de *P. gaudichudianum* Kunth

1.2.1. Aspectos gerais dos óleos essenciais

Óleos essenciais são compostos naturais voláteis complexos, decorrentes do metabolismo secundário das plantas, caracterizados por apresentar odor forte e característico (Bakkali *et al*., 2008). São sintetizados pelas plantas e costumam atrair insetos, contribuindo para a polinização, mas também atuam como fator de proteção do vegetal contra pragas e predadores

(Bakkali *et al*., 2008; Ramos, 2009; Müller e Buchbauer, 2011; Mithöfer e Boland, 2012; Tisserand e Young, 2014).

O teor dos compostos presentes no óleo essencial no vegetal é determinado geneticamente, mas a quantidade e a natureza dos constituintes ativos sofrem influência do ambiente, pela disponibilidade hídrica, temperatura, luz, radiação ultravioleta e nutrientes do solo. Neste sentido, torna-se importante respeitar a época em que o espécime vegetal é coletado, devendo ser observados a sazonalidade, bem como o período do dia ou da noite e a temperatura. Além disso, a idade e o desenvolvimento do vegetal também devem ser considerados, pois tecidos novos podem ter maior taxa biossintética, influindo diretamente na quantidade dos metabólitos produzidos e nas proporções relativas dos componentes da mistura (Gobbo-Neto e Lopes, 2007; Tisserand e Young, 2014).

Os óleos essenciais são líquidos apolares, lipossolúveis e solúveis em solventes orgânicos, apresentam cor límpida ou claro-amarelada. São sintetizados pelo vegetal e armazenados em células secretoras, células epidérmicas ou tricomas glandulares. Podem ser extraídos de várias partes das plantas, como raízes, brotos, caules, cascas, folhas, flores, frutos e sementes (Bakkali *et al*., 2008).

Geralmente, os óleos essenciais são obtidos pelo método da hidrodestilação por arraste de vapor d’água em aparelho do tipo Clevenger (Ferhat *et al*., 2006). Por ser uma mistura volátil e complexa, a análise de seus compostos requer a utilização de mecanismos precisos de alta resolução, tendo a combinação da cromatografia gasosa (GC) acoplada à espectrometria

de massas (MS) o método de escolha (Başer e Buchbauer, 2010). Ainda, a cromatografia gasosa correlacionada acoplada à espectrometria de massas de relação de isótopos (GC-IRMS) por meio de uma interface de combustão tem provado ser um método ideal para avaliar a autenticidade de óleos (Tisserand e Young, 2014). Além disso, a utilização da Cromatografia Líquida de Alta Eficiência com detector ultra violeta (HPLC-UV) pode representar um método alternativo ou complementar para a análise de óleos voláteis, devido à sua versatilidade, sensibilidade e seletividade, constituindo-se o método de escolha para os compostos menos voláteis (Pourmortazavi e Hajimirsadeghi, 2007; Adams e Dev, 2010; Başer e Buchbauer, 2010; Smelcerovic *et al*., 2013; Bagheri *et al*., 2014; Tisserand e Young, 2014).

Após a extração, Tisserand e Young (2014) preconizam que o óleo essencial deve ser corretamente armazenado, pois todos os compostos orgânicos estão sujeitos à degradação química, o que leva à perda das propriedades biológicas. Portanto, destacam que o óleo essencial deve ser armazenado em frasco âmbar fechado e refrigerado, ou seja, protegido da luz, do oxigênio e do calor, a fim de evitar a degradação decorrente da oxidação das suas moléculas. Ressaltam ainda que a presença de água provoca a deterioração, promovendo a oxidação e hidrólise de moléculas, tornando o óleo essencial opaco.

Os óleos essenciais são amplamente conhecidos pelas suas propriedades biológicas com utilização na indústria de alimentos (Frutuoso *et al*., 2013), na cosmética e na agricultura, como larvicidas e pesticidas (Ferraz *et al*., 2010; Oh *et al*., 2012). Na indústria farmacêutica são empregados no

tratamento de diversas doenças, sendo incorporados em formulações de medicamentos antiespasmódicos, analgésicos locais, antinflamatórios, antiparasitários, antimicrobianos, fungicidas e antitumorais (Bakkali *et al*., 2008).

A resistência de micro-organismos aos antibióticos existentes, a resistência de diversos tumores a várias drogas antitumorais e a toxicidade elevada para o paciente, na utilização de inúmeros medicamentos no tratamento do câncer, conduziu à necessidade de alavancar a pesquisa e o desenvolvimento de novos antimicrobianos e antitumorais para tratar diversas doenças, impulsionando a busca por novos medicamentos derivados de óleos essenciais e de seus componentes. Para tal, há a necessidade da elucidação do mecanismo de ação destes compostos para a identificação de novos alvos terapêuticos, a fim de atingir vias biossintéticas não inibidas pelas drogas atuais (Alfaro *et al*., 2013; Boire *et al*., 2013).

1.2.2. Característica química do óleo essencial de *P. gaudichaudianum*

A análise química do óleo essencial de espécies de *Piper* tem sido amplamente investigada, identificando como compostos majoritários hidrocarbonetos monoterpenos, sesquiterpenos e os derivados de fenilpropanóides (Martins *et al*., 1998; Mundina *et al*., 1998; Sumathykutty *et al*., 1999; Dos Santos *et al*., 2001; Mesquita *et al*., 2005; Péres *et al*., 2009; Ferraz *et al*., 2010; Morandim *et al*., 2010; Do Carmo *et al*., 2012; Santos *et al*., 2014).

Von Poser *et al*. (1994) investigaram o óleo essencial das folhas frescas de *P. gaudichaudianum* e *P. mikanianum*, coletadas no sul do Brasil, encontrando pequenas quantidades de monoterpenos, dentre os quais, β-

pineno e linalol foram os mais abundantes. Dos sesquiterpenos identificados, α-humuleno (37,5%) e β-cariofileno (17,4%) foram majoritários.

Andrade *et al*. (1998) verificaram duas amostras das folhas de *P. gaudichaudianun* denominadas A e B, encontrando como compostos majoritários do óleo essencial, respectivamente, 12,1% e 19,3% de β-cariofileno, 13,3% e 29,2% de α-humuleno, 15,7% e 3,7% de β-selineno e 16,6% e 8,9% de α-selineno. Calderari (2002) encontrou os mesmos compostos majoritários no óleo essencial das folhas de *P. gaudichaudianum*, porém em menores concentrações, α-humuleno (6%), β e α-selineno (ambos com 5%), além de outros como espatulenol (5%) e óxido cariofileno (6%). Seguindo, De Morais *et al*. (2007) identificaram como constituintes majoritários do óleo essencial das folhas de *P. gaudichaudianum,* β-selineno (15.77%) e óxido de cariofileno (16.63%).

Péres *et al*. (2009) também investigaram a composição química do óleo essencial extraído das folhas secas de *P. gaudichaudianum* pelo método de hidrodestilação durante 4 horas, utilizando um aparelho do tipo Clevenger, em conformidade com o método recomendado pela *European Pharmacopoeia* (1983), produzindo 0,51% (w/v) de óleo essencial. O óleo destilado foi seco, em uma coluna de sulfato de sódio anidro, armazenado em frascos escuros fechados, acondicionados a 4ºC até a caracterização química realizada por cromatografia gasosa com detector de ionização de chama (GC-FID) e GC-MS. Foram identificados 47 componentes no óleo essencial bruto, representando 92,3% do total de picos cromatográficos. Os compostos majoritários sesquiterpênicos totalizaram 87,6% da amostra, dentre os quais se destacaram

o *(E)*-nerolidol (22,4%), (E)-cariofileno (8,9%), α-humuleno (16,5%) e biciclogermacreno (7,4%). Monoterpenos e fenilpropanóides foram identificados em pouca quantidade (4,7%).

1.2.3. Atividades farmacológicas de óleos essenciais, do óleo essencial de *P. gaudichaudianum* e de seus componentes majoritários

Diversas propriedades biológicas decorrentes de óleos essenciais de plantas têm sido descritas na literatura, tais como anti-inflamatória, analgésica, anticonvulsivante, anti-ulcerativa, antiprotozoária, antimicrobiana, antifúngica, antioxidante, antitumoral, larvicida, pesticida e repelente (Crowell, 1996; Ruberto e Baratta, 2000; Sylvestre *et al*., 2005, Sylvestre *et al*., 2006; De Morais *et al*., 2007; Autran *et al*., 2008; Bakkali *et al*., 2008; Ferraz *et al*., 2010; Miguel, 2010; Morandim *et al*., 2010; Nerio *et al*., 2010; Prakash *et al*., 2010; De Almeida *et al*., 2011; Lang e Buchbauer, 2012; De Sousa, 2011; Afoulous *et al*., 2013; Frutuoso *et al*., 2013; Guimarães *et al*., 2013; Nogueira Neto *et al*., 2013; Nuzhat e Vidyasagar, 2013; Bagheri *et al*., 2014; Dias *et al*., 2014; Ghosh *et al*., 2014, Oliveira *et al*., 2014; Da Silva *et al*.; 2014). Ademais, o uso de compostos derivados de óleos essenciais como potencializadores da ação de antimicrobianos e outros agentes convencionais utilizados em quimioterapia e radioterapia tem se mostrado uma estratégia promissora no tratamento de doenças infecciosas e tumorais (Legault e Pichette, 2007; Hemaiswarya *et al*., 2008; Ravizza *et al*., 2008; Chiu *et al*., 2011; Gonçalves *et al*., 2011; Lesgards *et al*., 2011; Lesgards *et al*., 2014; Monteiro *et al*., 2014).

Bakkali *et al*. (2008) realizaram uma revisão sobre os efeitos biológicos dos óleos essenciais, referindo que a maioria dos óleos essenciais estudados

foi considerada antioxidante, esta propriedade também foi reportada no estudo de Anthony *et al*. (2012). Entretanto, estudos recentes em células eucarióticas revelaram que os óleos essenciais também poderiam agir como pro-oxidantes, afetando principalmente as membranas celulares internas e organelas, tais como as mitocôndrias. Destacam ainda que estes compostos não são considerados genotóxicos, mas que, mesmo assim, podem exibir efeitos citotóxicos, de forma dose-dependente em células vivas. Estes autores enfatizaram que, em alguns casos, mudanças no potencial redox intracelular e disfunções mitocondriais, induzidas por óleos essenciais, poderiam estar associadas à capacidade de exercer efeitos anti-genotóxicos.

As atividades biológicas dos óleos essenciais são consequência da sua composição, da ação e do sinergismo de seus componentes majoritários, agindo sobre diversos alvos moleculares celulares, como membranas e organelas, podendo interferir na síntese de energia celular (Bakkali *et al*., 2008; Tisserand e Young, 2014). Apesar dos benefícios da utilização de óleos essenciais, alguns de seus componentes podem comprometer a instabilidade genômica de células de mamífero, induzindo alterações como o aumento de células micronucleadas e multinucleadas, mitoses irregulares com fusos multipolares e mal localizados, bem como quebras cromossômicas, consequentes do aumento de espécies reativas de oxigênio intracelular (Catanzaro *et al*., 2012).

Péres *et al*. (2009) investigaram os efeitos citotóxicos, mutagênicos e genotóxicos do óleo essencial das folhas de *P. gaudichaudianum* em células V79. Os resultados obtidos neste estudo revelaram que os efeitos citotóxicos

foram dose-dependentes, e uma diminuição significativa na sobrevivência celular foi observada a partir das concentrações de 0,5 µg/mL. Além disso, foram verificadas quebras simples no DNA em concentrações acima de 2 µg/mL, bem como um aumento significativo na frequência de micronúcleos nas concentrações de 4 µg/mL, 6 µg/mL e 10 µg/mL. Os autores também referiram um aumento na peroxidação lipídica em doses crescentes a partir de 0,5 µg/mL, sugerindo que o potencial oxidante observado foi, provavelmente, o responsável pelo efeito citotóxico e pelos efeitos genotóxicos no modelo celular estudado. Confirmando estes achados, Pículo *et al*. (2011), ao testar a atividade do nerolidol, um dos compostos majoritários do óleo essencial de *P. gaudichaudianum*, em células de mamífero, demonstraram que houve uma fraca indução dose-dependente nos níveis de danos no DNA das células analisadas, com aumento do número médio de células micronucleadas, nas doses mais elevadas testadas, apoiando a visão de que o nerolidol induziu clastogênese e genotoxicidade muito fraca nas células de ratos testadas. Entretanto, ao analisar a citotoxicidade do óleo essencial de *P. gaudichaudianum* em *Saccharomyces serevisiae*, Sperotto *et al*. (2013) revelaram que tanto o óleo essencial quanto o nerolidol não foram mutagênicos e que a citotoxicidade estava relacionada com a formação de lesões oxidativas.

Arruda *et al*. (2005) avaliaram a atividade do nerolidol contra os protozoários *Leishmania amazonensis, L. braziliensis, L. chagasi* promastigotas e *L. amazonensis* amastigotas, causadores de Leishmaniose, demonstrando uma inibição no crescimento dos protozoários com IC-50 de 85 µM, 74 µM, 75 µM, e 67 µM, respectivamente. Com a dose de 100 µM ocorreu uma diminuição de 95% da infecção, mas em longo prazo, a infecção não foi curada. Os

autores atribuíram o efeito da droga ao bloqueio de um passo inicial na via do mevalonato, interferindo na síntese de isoprenóides e inibindo a biossíntese de dolicol, ergosterol e ubiquinonas, gerando danos às membranas celulares destes parasitas. Marques *et al*. (2011) consideraram o efeito do (E)-nerolidol sobre *L. amazonensis* decorrente da inibição da arginase, uma metaloenzima dependente de magnésio, essencial para a produção de ornitina e sobrevivência do protozoário. Monzote *et al*. (2010) mencionam a atividade do safrol (IC-50 de 22,3 ± 1,8 μg/mL) contra espécies de *L. donovani*. Do Carmo *et al*. (2012) também destacam a ação antiprotozoária do limoneno (IC-50 de 278 μM) e do cariofileno (IC-50 de 96 μM), referindo que estes compostos podem ser promissores coadjuvantes no tratamento da leishmaniose.

Fernandes *et al*. (2007) descrevem as propriedades anti-inflamatórias de dois sesquiterpenos isolados do óleo essencial de *Cordia verbenacea*, mas também presentes no óleo essencial de *P. gaudichaudianum*, α-humuleno e (E)-cariofileno, demonstrando que o tratamento oral com ambos os compostos reduziu o fator de ativação de plaquetas, a bradicinina e o edema induzido por carragenina nas patas dos camundongos utilizados no estudo, bem como diminuiu a produção de prostaglandina E2 (PGE2), a síntese de óxido nítrico e a expressão de ciclo-oxigenase 2 (COX-2). Os autores também consideraram estes efeitos anti-inflamatórios comparáveis aos observados em animais tratados com dexametasona.

Brehm-Stecher e Johnson (2003) inferem que os componentes sesquiterpenóides de óleos essenciais, nerolidol, farnesol, bisabolol, e apritone, poderiam agir como coadjuvantes no tratamento de doenças infecciosas,

potencializando o efeito de drogas como a ciprofloxacina, clindamicina, eritromicina, gentamicina, tetraciclina, e vancomicina contra *Staphylococcus aureus* e, da polimixina B contra *Escherichia coli*, por promoverem maior permeabilidade bacteriana aos agentes antimicrobianos.

Também, Gautam *et al*. (2014) expuseram a ação citotóxica do α-humuleno e do β-cariofileno sobre células tumorais em estudos *in vivo* e o efeito citotóxico do óleo essencial de *P.gaudichaudianum* sobre células V79. Os compostos β-cariofileno e α-humuleno demonstraram atividade contra células cancerígenas, sendo que o β-cariofileno otimizou o efeito antitumoral do α-humuleno, com ação superior quando utilizados juntos. β-cariofileno também facilitou a passagem de paclitaxel através da membrana mitocondrial, potencializando a ação deste medicamento antitumoral (Legault e Pichette, 2007).

Park *et al*. (2011) relataram que o β-cariofileno induziu a produção de espécies reativas de oxigênio na mitocôndria de células tumorais, sem aumentar o estresse oxidativo em células saudáveis. Este composto também apresentou propriedades antitumorais contra células de câncer de próstata e de mama, bem como em vários outros tipos de tumores sólidos (Kubo *et al*., 1996), além de induzir apoptose em células de linfoma (Amiel *et al*., 2012). Nerolidol e α-humuleno foram ativos contra câncer de pulmão e colon (Sylvestre *et al*., 2007). O α-humuleno ainda apresentou atividade antitumoral contra câncer de próstata (Loizzo *et al*., 2007) e de mama (Legault e Pichette, 2007). Em contrapartida, Amiel *et al*. (2012) inferem ao β-cariofileno atividade pró-apoptótica e antiproliferativa contra células tumorais, mas não contra as

células normais, revelando uma possível ação seletiva deste composto contra células tumorais.

1.2.4. Mecanismos celulares e moleculares envolvidos na citotoxicidade do óleo essencial de *P. gaudichaudianum* e de seus componentes majoritários

Os óleos essenciais e seus componentes majoritários são compostos lipofílicos e pró-oxidantes, o que lhes confere a capacidade de atravessar a membrana plasmática celular (Bakkali *et al*., 2008), induzir citotoxicidade, genotoxicidade e mutagenicidade (Péres *et al*., 2009; Park *et al*., 2011; Cha e Kim, 2012). Mendanha *et al*. (2013) avaliaram a toxicidade de terpenos, dentre eles o nerolidol, constatando o aumento na fluidez da membrana celular.

Buscando demonstrar o mecanismo antitumoral de diversos óleos essenciais de plantas, Gautam *et al*. (2014) realizaram uma revisão em aproximadamente 130 estudos disponíveis na literatura, destacando, em profundidade, vários mecanismos de ação dos diferentes óleos essenciais e seus constituintes reportados nas estratégias de tratamento para diferentes tipos de câncer. Este estudo verificou que os óleos essenciais e os seus constituintes agem por múltiplas vias e mecanismos que envolvem a indução de apoptose, alteração do ciclo celular, efeitos antimetastáticos e anti-angiogênicos, além de promover o aumento dos níveis de espécies reativas de oxigênio (ERO) e nitrogênio (ERN), modulação do reparo do DNA, dentre outros que demonstraram ter um efeito antiproliferativo em células tumorais, resumidos na Figura 2.

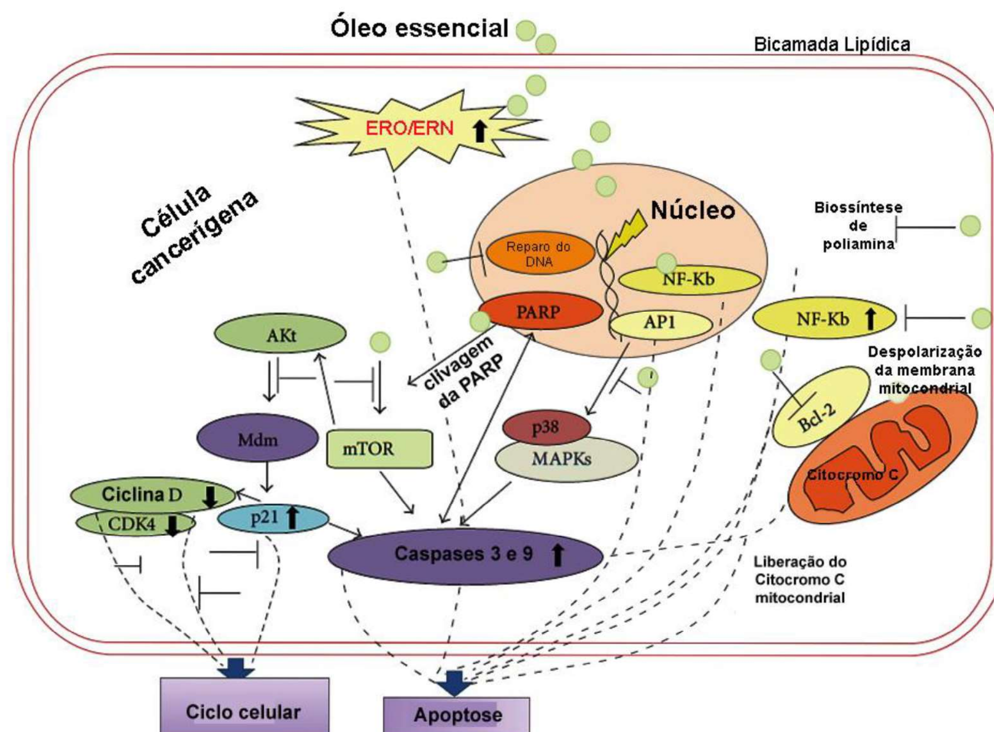

Figura 2: Mecanismo de ação dos óleos essenciais em células tumorais: OE podem permear a membrana celular e atuar em diferentes alvos celulares, induzindo a formação de ERO e ERN. O estresse oxidativo mitocondrial conduz à despolarização mitocondrial com a liberação do citocromo C para o citoplasma, clivagem da PARP, ativação de caspases e indução de apoptose em células cancerosas (adaptado de Gautam *et al*., 2014).

Cha e Kim (2012) também destacaram a ação de monoterpenos na indução de morte celular em células de carcinoma oral epidermóide. Os autores constataram que o estresse oxidativo mitocondrial desempenhou papel fundamental na morte celular por apoptose, correlacionando este fato com a diminuição mitocondrial de proteínas Bcl2, liberação do citocromo C mitocondrial para o citosol com consequente ativação da via das caspases 3 e 9, além da clivagem da proteína poli (ADP-ribose) polimerase (PARP) e alteração do ciclo celular com o aumento do número de células na fase sub-G1

(Jo *et al.,* 2012). Além do mais, Lesgards *et al*. (2014) inferem que estes efeitos poderiam ocorrer com pequenas modificações em células saudáveis e que, em células tumorais, também estariam relacionados com a superexpressão e regulação de enzimas de detoxificação do fígado, alterações no potencial de membrana mitocondrial, produção de radicais livres e modificação de genes indutores de tumor.

A indução de apoptose tem sido relatada como o principal mecanismo de ação de drogas sintetizadas de plantas medicinais e de medicamentos antitumorais existentes (Chen *et al*., 2011). Além disso, Ueta *et al*. (2008) destacam que sistemas de reparo do DNA mitocondrial estão associados com a susceptibilidade de células cancerosas a agentes antitumorais. Estudando células de carcinoma oral de células escamosas, estes autores verificaram que a inibição da capacidade de reparo do DNA mitocondrial e a baixa regulação da 8-oxoguanina-DNA glicosilase (OGG1) poderia ser uma estratégia útil no tratamento do câncer combinado com a radiação e a quimioterapia.

Diversos autores, como já mencionado, referem alterações em membranas celulares e em organelas como a mitocôndria, alterações no ciclo celular, modulação no processo de reparo do DNA, modificação em genes indutores de tumor, dentre outras que culminam na indução de morte celular, devido à ação do óleo essencial de *P. gaudichaudianum* e de compostos majoritários como o nerolidol, α-humuleno e β-cariofileno (Kubo *et al*., 1996; Legault e Pichette, 2007; Loizzo *et al*., 2007; Sylvestre *et al*., 2007; Amiel *et al*., 2012; Cha e Kim, 2012; Gautam *et al*., 2014), capazes de gerar espécies reativas de oxigênio (ERO).

Espécies reativas de oxigênio (ERO), incluindo os radicais livres, fazem parte do processo celular fisiológico e são resultantes do metabolismo do oxigênio, gerados principalmente na cadeia transportadora de elétrons mitocondrial, na peroxidação de lipídios e durante o processo inflamatório (Halliwell e Chirico, 1993; Shen *et al*., 2000; Barreiros e David, 2006; Burton e Jauniaux, 2011; Martin-Ventura *et al*., 2012). Fontes exógenas como a radiação ultravioleta (Farrukh *et al*., 2014) e agentes químicos, como os quimioterápicos, também são indutores de ERO intracelular (Campos *et al*., 2014). Radicais livres são átomos ou moléculas com um elétron desemparelhado em seu orbital de valência, conferindo alta reatividade química a estes elementos, dentre os quais se destacam, como os mais importantes, os radicais hidroxil ($HO^{\bullet}$), ânion superóxido ($O_2^{\bullet}$), óxido nítrico ($NO^{\bullet}$), alcoxil ($RO^{\bullet}$), peroxil ($ROO^{\bullet}$) e peroxinitrito ($ONOO^{-}$). Outros elementos, como o ácido hipocloroso (HOCl), o peróxido de hidrogênio (H_2O_2), o oxigênio singlete ($^1O_2$) e o ozônio (O_3) não são radicais livres, mas estão envolvidos em reações que geram estes produtos (Halliwell, 2001; Picada *et al*., 2003; Burton e Jauniaux, 2011; Da Silva e Ferrari, 2011; Halliwell, 2012).

Além de constituírem-se produtos inevitáveis, ERO fazem parte da regulação de funções celulares importantes, como no controle de fatores de transcrição, proteínas cinases e fosfatases (Mansfield *et al*., 2005; Jones, 2006; Bartosz, 2009), bem como podem elevar os níveis de enzimas envolvidas nos mecanismos de defesa antioxidante e metabolização de xenobióticos (Valko *et al*., 2007; Halliwell, 2008; Circu e Aw, 2010; Ma, 2010; Procházková *et al*., 2011; Altenhöfer *et al*., 2014; Bereiter-Hahn, 2014). Elmann *et al*. (2009) revelaram efeito protetor neuronal contra peróxidos de hidrogênio decorrente

do estímulo do sistema antioxidante no uso de alguns compostos encontrados em óleos essenciais, dentre os quais se destacam o α-humuleno e o β-cariofileno. Por outro lado, quando há excesso na produção de ERO, estas podem oxidar irreversivelmente lipídios (Halliwell e Chirico, 1993; Lima e Abdalla, 2001; Sultana *et al*., 2011; El-Beltagi e Mohamed, 2013), proteínas (Burton e Jauniaux, 2011) e o DNA (Berquist e Wilson, 2012; Alexeyev *et al*., 2013).

A oxidação de lipídios pode desencadear reações em cadeia, danificando diferentes polissacarídeos, ácidos graxos, alterando a permeabilidade da membrana celular e de diferentes organelas como a mitocôndria, retículo endoplasmático e peroxissomos (Bakkali *et al*., 2008; Cao e Kaufman, 2014; Chaudhari *et al*., 2014). O aumento da fluidez da membrana mitocondrial leva à perda de radicais, proteínas e íons, diminuindo o potencial de membrana mitocondrial e induzindo a sua despolarização (Kowaltowski *et al*., 2009; Brand e Nicholls, 2011). Estes fatores levam à redução na geração de ATP e consequente colapso bioenergético da célula (Chandel, 2014). Além disso, a passagem do citocromo C mitocondrial para o citosol conduz à ativação da morte celular por apoptose (Candé *et al*., 2002; Bogner *et al*., 2010; Cha e Kim, 2012). Ferreira *et al*. (2012) testaram a ação do nerolidol em células hepáticas de ratos e em células de carcinoma hepático humano, revelando a redução da produção de ATP mitocondrial e diminuição da bioenergética destas células, refletida na diminuição da viabilidade celular e morte por apoptose.

Ainda, a abertura de canais de íons induzida pelo estresse oxidativo, como ocorre no retículo endoplasmático com a abertura dos canais de cálcio, pode interferir no dobramento de proteínas e consequente perda de função, bem como o acúmulo de proteínas intracelulares, o que também pode levar à morte celular (Burton e Jauniaux, 2011).

Os danos no DNA estão relacionados com alterações nas bases nucleotídicas e na estrutura açúcar-fosfato do DNA, originando um amplo espectro de lesões não volumosas, como a oxidação da guanina (8-oxo-7,8-dihidro-2'-desoxiguanosina) e volumosas, como sítios abásicos, quebras simples (SSB) e duplas (DSB), pontes intra e inter cadeia e formação de adutos nas fitas de DNA (Hoeijmakers, 2001; Berquist e Wilson, 2012).

A oxidação da guanina, pela adição de um oxigênio na posição do carbono 8 e de um hidrogênio na posição do nitrogênio 7, gerando a 7,8-dihidro-8-oxoguanina (8-oxo-G), já é considerada um biomarcador estabelecido do efeito da oxidação no DNA. Seu potencial mutagênico consiste na formação de pareamentos 8-oxoG•C ou 8-oxoG•A; este último, uma transversão no pareamento entre as bases, pareando com adenina ao invés de parear com a base citosina, podendo passar despercebido durante a replicação do DNA e induzir mutações (Cooke *et al*., 2003; German *et al*., 2013; Kasymov *et al*., 2013).

Como forma de defesa contra os efeitos deletérios das ERO, as células desenvolveram sistemas antioxidantes, de adaptação e vias de reparo como o reparo por excisão de bases (BER), reparo por excisão de nucleotídeos (NER), recombinação homóloga (HR) e recombinação não homóloga (NHEJ)

(McCullough *et al*., 1999; Hoeijmakers, 2001; Picada *et al*., 2003; Sancar *et al*., 2004; Agnoletto, 2006; Jackson e Bartek, 2009; Ciccia e Elledge, 2010; Berquist e Wilson, 2012; Damasceno, 2013; German *et al*., 2013).

O sistema de defesa antioxidante visa neutralizar espécies reativas, evitando danos às estruturas celulares. Para Halliwell e Gutteridge (1995), caracteriza-se como antioxidante qualquer substância que, quando presente em baixas concentrações, em comparação com um substrato oxidável, impede os danos oxidativos de moléculas alvo. Segundo López-Alarcón e Denicola (2013), a ação antioxidante engloba a regulação positiva de enzimas antioxidantes e desintoxicantes, modulação do redox celular e a expressão de genes, além de neutralizar os danos produzidos pela ação de radicais livres. Participam deste processo as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) (Barbosa *et al*., 2010; Reczek e Chandel, 2015), assim como as proteínas ligadas ao ferro, transferrina e ferritina, coenzima Q10, dentre outras, além dos antioxidantes adquiridos pela dieta, como as vitaminas C, E, A e os compostos flavonoides (Forbes-Hernández *et al*., 2014; Vasconcelos *et al*., 2014). Cabe ressaltar que, dependendo de sua concentração, muitos destes compostos podem agir como antioxidantes ou pró-oxidantes (Aust, 1995; Duarte e Lunec, 2005; Villanueva e Kross, 2012; Carocho e Ferreira, 2013).

A SOD é uma metaloproteina localizada na matriz mitocondrial (SOD-Mn), no espaço intermembrana mitocondrial e no citoplasma da célula (SOD-CuZn). Catalisa a dismutação do ânion superóxido em oxigênio molecular e peróxido de hidrogênio ($2O_2\bullet + 2H+ \rightarrow O_2 + H_2O_2$). Vários estudos

correlacionam alterações na SOD com doenças degenerativas (Buettner, 2011; Candas e Li, 2014). Outra enzima que catalisa o peróxido de hidrogênio em água é a CAT ($H_2O_2 \rightarrow 1/2\ O_2 + H_2O$). Esta enzima está presente em todos os tecidos e contém um átomo de ferro^{+3} em cada uma de suas quatro subunidades. A literatura associa doenças metabólicas, como o diabetes, com mutações no gene da CAT (Camara *et al*., 2010; Angeli, 2011). Da mesma forma que a CAT, a GPx transforma o peróxido de hidrogênio em água. Sua atividade depende da glutationa reduzida (GSH), por meio de NADPH, que é oxidada (GSSG), conforme a reação: $2GSH + H_2O_2 \rightarrow 3GSSG + 2H_2O$ ou $2GSH + ROOH \rightarrow GSSG + ROH + H_2O$ (Angeli, 2011). Baixos níveis de GPx podem estar correlacionados com doenças cardiovasculares (Arthur, 2000). Para a manutenção de um nível basal de GSH no organismo, a conversão de GSSG em GSH é catalisada pela enzima glutationa redutase ($GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$) (Arthur, 2000; Camara *et al*., 2010). Além disso, o tratamento com N-acetilcisteína (NAC), um aminoácido precursor da glutationa, poderia proteger as células de danos oxidativos por meio da manutenção dos níveis deste peptídeo (Raza *et al*., 2014). Outras proteínas antioxidantes são as tiorredoxinas (Trx), com ação sobre diferentes substratos, como o peróxido de hidrogênio, peroxinitrito e hidroperóxidos orgânicos. Sua atividade é regulada por fosforilação de um resíduo de cisteína, catalisando as reações: $H_2O_2 + Trx(SH)_2 \rightarrow 2H_2O + Trx(SS)$ e $R'\text{-}O\text{-}OH + 2\ RSH \rightarrow R'\text{-}OH + H_2O + RSSR$ (Camara *et al*., 2010).

Segundo Kannan *et al*. (2014), células com maior número de mitocôndrias podem apresentar maior absorção de oxigênio e suportar maior nível de ERO, bem como o acúmulo de mutações por oxidação do DNA.

Hipóteses como estas podem estar relacionadas com a adaptação celular ao estresse oxidativo e maior predisposição a doenças tumorais. Além do mais, falhas nos processos de reparo ou na indução de morte celular estão envolvidas com a etiopatogenia de diversas doenças hereditárias, degenerativas e tumorais (Barzilai *et al*., 2002; Barzilai e Yamamoto, 2004; David *et al*., 2007; Ziech *et al*., 2010; Kryston *et al*., 2011; Vurusaner *et al*., 2012; Federico *et al*., 2012; Wallace *et al*., 2012; Yan *et al*., 2013; Bogeski e Niemeyer, 2014; Mofluoglu *et al*., 2014; Pagano *et al*., 2014; Scott et al., 2014; Wang *et al*., 2014), como ocorre nas síndromes de Cockayne, Xeroderma Pigmentoso e Anemia de Fanconi, bem como nos defeitos de desenvolvimento, anomalias neurológicas e envelhecimento precoce (Da Silva e Ferrari, 2011; Berquist e Wilson, 2012; Dizdaroglu, 2012).

A ativação da resposta ao dano no DNA é mediada por proteínas cinases e poli (ADP-ribose) polimerase (PARP), comumente ativadas pela ruptura nas fitas de DNA (SSB ou DSB) e formação de sítios abásicos no DNA (Huber *et al*., 2004; Ciccia e Elledge, 2010; Ko e Ren, 2012; Gospodinov e Herceg, 2013). A poli (ADP-ribose) polimerase 1 (PARP-1) é a mais abundante de uma família de 18 proteínas que atuam em diversas vias, como na replicação do DNA e na regulação de processos celulares como transcrição, diferenciação celular, atividade da telomerase, organização do citoesqueleto e reparo do DNA (Damasceno, 2013). A PARP-1 atua como um sensor de danos no DNA e, quando ativada, pode contribuir para a sinalização de proteínas envolvidas no reparo do DNA, colaborando para a estabilidade genômica e a sobrevivência celular (Bouchard *et al*., 2003; Lilyestrom *et al*., 2010; Ko e Ren, 2012). PARP-1 media o acúmulo de proteínas cinases do complexo MRN

(Mre11, Rad 50 e Nbs1) que identificam quebras nas fitas do DNA e recrutam duas proteínas transdutoras, Ataxia Telangiectasia mutada (ATM) e Ataxia Telangiectasia relacionada (ATR), que promovem a fosforilação de outras proteínas efetoras no processo de reparo do DNA (Huber *et al*., 2004; Lisby *et al*., 2004; Guo *et al*., 2010; Polo e Jackson, 2011; Williams *et al*., 2011; Chen *et al*., 2012; Ditch e Paull, 2012; Hegde *et al*., 2012; Ray *et al*., 2012; Gobbini *et al*., 2013). Além disso, PARP-1 e PARP-2 também ativam modificações em histonas pela fosforilação de ɣH2AX, contribuindo para a expansão local da cromatina, dependente de ATP, a fim de possibilitar o acesso dos fatores envolvidos no reparo do DNA ao local da lesão (Huber *et al*., 2004; Yélamos *et al*., 2008; Ciccia e Elledge, 2010; Zhu e Wani, 2010; Ko e Ren, 2012). Outras alterações, como a acetilação e desacetilação, também desempenham papel fundamental no reparo de DSBs, promovendo o relaxamento de histonas pela diminuição da interação N-terminal destas proteínas com grupos fosfato do DNA (Tamburini e Tyler, 2005).

As proteínas sirtuínas, em especial a SIRT-1, também atuam na homeostase bioenergética celular e no controle da transcrição por meio de acetilação, agindo em diversos locais como núcleo, citoplasma e mitocôndria (Chang e Guarente, 2014). Sirtuínas são proteínas dependentes de NAD^+, e contribuem na regulação da resposta ao estresse oxidativo (Liu *et al*., 2009; Nakagawa e Guarente, 2011; Cho *et al*., 2013), além de atuar no reparo de DSBs (Rahman e Islan, 2011). Como a ativação de PARP-1 consome NAD^+, leva à diminuição da atividade de SIRT-1. Já a inibição de PARP-1 conserva os níveis de NAD^+, modulando a atividade de SIRT-1, importante no controle de doenças metabólicas e tumorais (Deng, 2009; Bai *et al*., 2011).

A PARP-1 desempenha papel chave ativando a via do BER (Sodhi *et al*., 2010), que atua primariamente no reparo da 8-oxoG. As proteínas DNA glicosilases OGG1 removem a 8-oxoG do par 8-oxoG•C e as MUTYH do par 8-oxoG•A, gerando um sítio apurínico (AP) no DNA (Lu *et al*., 2001; Paz-Elizur *et al*., 2008). Após a identificação e remoção da base alterada por endonucleases, a DNA polimerase e a DNA ligase restabelecem a integridade da fita do DNA concluindo o processo de reparo (David *et al*., 2007), conforme ilustrado na Figura 3. Sperotto *et al*. (2013) correlacionaram o dano oxidativo no DNA, induzido pelo óleo essencial de *P. gaudichaudianum*, em *Saccharomyces cerevisiae,* com o BER.

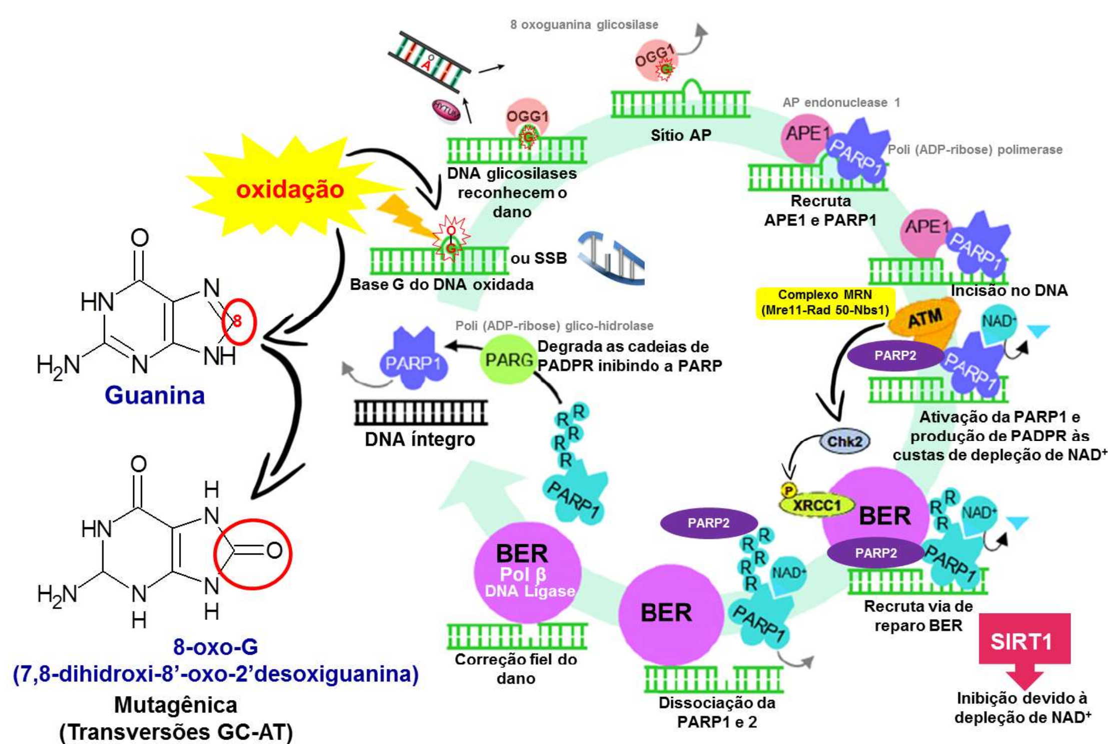

Figura 3: Mecanismo de reparo da via curta do BER na remoção da 8-oxoG: Após a identificação do dano (G-O), as DNA glicosilases OGG1 e MUTYH, em conjunto com endonucleases, removem a base alterada, gerando um sítio AP. A proteína PARP1 atua neste processo como um sensor de danos, atuando no reparo de quebras de fita simples (SSBs) no DNA. PARP1 inicia a síntese do

polímero de PADPR, o que promove sua ativação, e adiciona polímeros de ribose em outras proteínas, como a DNA polimerase β e a DNA ligase, recrutando-as para o reparo no DNA que é fielmente reconstituído. PARP1 e SIRT1 são dependentes de NAD^+ e a ativação de uma promove a inibição da outra (adaptado de David *et al*., 2007; Ko e Ren, 2012).

Todavia, dependendo da extensão do dano no DNA ou da inibição da atividade das proteínas envolvidas no processo de reparo, pode ocorrer a parada no ciclo celular e a ativação da morte celular por apoptose e/ou necrose (Boonstra e Post, 2004; Kroemer *et al*., 2007; Cairrão e Domingos, 2010). PARP-1 pode induzir a morte celular por apoptose ou necrose e a transcrição de genes pró-inflamatórios (Sodhi *et al*., 2010), pois toda a atividade da PARP ocorre com depleção energética celular e sua ativação consistente pode levar à morte celular por necrose, decorrente do colapso celular (Ying, 2008). Na morte celular por apoptose, a PARP-1 (113 kDa) sinaliza a liberação de AIF (fator indutor de apoptose) da mitocôndria para o núcleo, promovendo a clivagem da PARP-1 em 2 fragmentos, de 24 kDa e 89 kDa, pelas caspases 3 e 7. Pressupõe-se que isto ocorra, provavelmente, para preservar o ATP celular, inibindo o processo de reparo da célula, poupando a energia para os processos de morte por apoptose (Bouchard *et al*., 2003; Sodhi *et al*., 2010).

Na terapia contra o câncer, a inibição da PARP-1 pode aumentar a eficiência da terapia antitumoral, por impedir a inflamação por necrose induzida pela superexpressão de PARP-1, decorrente do dano no DNA (Giansanti *et al*., 2010). Javle e Curtin (2011) destacam o uso de inibidores da PARP (PARPi) como potencializadores no tratamento contra o câncer, pois estes PARPi atuariam em tecidos deficientes no mecanismo de reparo por recombinação homóloga (RH), sem danificar os tecidos normais.

Tendo em vista as diferentes propriedades biológicas atribuídas ao óleo essencial de *P. gaudichaudianum*, ainda há a necessidade de uma melhor compreensão sobre o seu mecanismo de ação. Neste sentido, este trabalho busca contribuir para o conhecimento dos processos celulares e moleculares envolvidos na citotoxicidade do óleo essencial de *P. gaudichaudianum*.

1.6. Referências Bibliográficas

Adams RP, Dev V. Synthesis and GC–MS analysis of angelates and tiglates as an aid to identification of these components in essential oils. Flavour Fragr J. 2010;25:71-4.

Afoulous S, Ferhout H, Raoelison EG, Valentin A, Moukarzel B, Couderc F *et al*. Chemical composition and anticancer, antiinflammatory, antioxidant and antimalarial activities of leaves essential oil of *Cedrelopsis grevei*. Food Chem Toxicol. 2013;56:352-62.

Agnoletto MH. Estudo de Lesões e Reparo de DNA em Pacientes com Câncer de Mama e em Fibroblastos Humanos [dissertação]. Porto Alegre (RS): Universidade Federal do Rio Grande do Sul; 2006.

Albiero ALM, Souza LA, Mourão KSM, Almeida OJG, Lopes WAL. Morfo-anatomia do caule e da folha de *Piper gaudichaudianum* Kuntze (*Piperaceae*). Acta Farm Bonaer. 2005;24(4):550-4.

Alexeyev M, Shokolenko I, Wilson G, LeDoux S. The Maintenance of Mitochondrial DNA Integrity—Critical Analysis and Update. Cold Spring Harb Perspect Biol. 2013;5:1-17.

Alfaro Y, Delgado G, Cárabez A, Anguiano B, Aceves C. Iodine and doxorubicin, a good combination for mammary cancer treatment: antineoplastic adjuvancy, chemoresistance inhibition, and cardioprotection. Mol Cancer. 2013;12(45):1-11.

Altenhöfer S, Radermacher KA, Kleikers PWM, Wingler K, Schmidt HHHW. Evolution of NADPH Oxidase Inhibitors: Selectivity and Mechanisms for Target Engagement. Antioxid Redox Signal. 2014;00:1-23.

Amiel E, Ofir R, Dudai N, Soloway E, Rabinsky T, Rachmilevitch S. β-Caryophyllene, a Compound Isolated from the Biblical Balm of Gilead (*Commiphora gileadensis*), Is a Selective Apoptosis Inducer for Tumor Cell Lines. Evid Based Complement Alternat Med. 2012;1-8.

Andrade EHA, Zoghbi MGB, Santos AS. Essential Oils of *Piper gaudichaudianun* Kunth and *P. regnellii* (Miq.). CDCJ Essent Oil Res. 1998;10:465-7.

Angeli JPF. Hidroperóxidos de lipídios como fontes de oxigênio molecular singlete (O_2 [$^1\Delta_g$]), detecção e danos em biomoléculas [tese]. São Paulo (SP): Universidade de São Paulo; 2011.

Ansari MA, Anurag A, Fatima Z, Hameed S. Natural Phenolic Compounds: A Potential Antifungal Agent. Formatex. 2013;2(4):1189-95.

Anthony KP, Deolu-Sobogun SA, Saleh MA. Comprehensive Assessment of Antioxidant Activity of Essential Oils. J Food Sci. 2012;77(8):C839-43.

Arruda DC, D'Alexandri FL, Katzin AM, Uliana SRB. Antileishmanial Activity of the Terpene Nerolidol. Antimicrob Agents Chemother. 2005;49(5):1679-87.

Arthur JR. The glutathione peroxidases. Cell Mol Life Sci. 2000;57:1825-35.

Aust SD. Ferritin as a source of iron and protection from iron-induced toxicities. Toxicol Lett. 1995;82/83:941-4.

Autran ES, Neves IA, da Silva CSB, Santos GKN, Câmara CAG, Navarro DMAF. Chemical composition, oviposition deterrent and larvicidal activities against *Aedes aegypti* of essential oils from *Piper marginatum* Jacq. (Piperaceae). Bioresour Technol. 2008;100:2284-8.

Azevedo SKS, Silva IM. Plantas medicinais e de uso religioso comercializadas em mercados e feiras livres no Rio de Janeiro, RJ, Brasil. Acta bot bras. 2006;20(1):185-94.

Bagheri H, Manap MYBA, Solati Z. Antioxidant activity of *Piper nigrum L*. essential oil extracted by supercritical CO_2 extraction and hydro-distillation. Talanta. 2014;121:220-8.

Bai P, Canto C, Oudart H, Brunyánszki A, Cen Y, Thomas C *et al*. PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. Cell Metab. 2011;13(4):461-8.

Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils – A review. Food Chem Toxicol. 2008;46:446-75.

Barbosa KBF, Costa NMB, Alfenas RCG, De Paula SO, Minim VPR, Bressan J. Estresse oxidativo: conceito, implicações e fatores modulatórios. Rev Nutr. 2010;23(4):629-43.

Bardelli KC, Kirizawa M, Sousa AVG. O gênero *Piper* L. (Piperaceae) da Mata Atlântica da Microbacia do Sítio Cabuçu-Proguaru, Guarulhos, SP, Brasil. Hoehnea. 2008;35(4):553-61.

Barreiros ALBS, David JM. Estresse Oxidativo: Relação entre Geração de Espécies Reativas e Defesa do Organismo. Quim Nova. 2006;29(1):113-23.

Bartosz G. Reactive oxygen species: Destroyers or messengers? Biochem Pharmacol. 2009;77:1303-15.

Barzilai A, Rotman G, Shiloh Y. ATM deficiency and oxidative stress: a new dimension of defective response to DNA damage. DNA Repair. 2002;1:3-25.

Barzilai A, Yamamoto K. DNA damage responses to oxidative stress. DNA Repair. 2004;3:1109-15.

Başer DHC, Buchbauer G. Handbook of essential oils: science, technology, and applications. CRC Press. 2010.

Batista Júnior JM, Lopes AA, Ambrósio DL, Regasini LO, Kato MJ, Bolzani VS *et al*. Natural Chromenes and Chromene Derivatives as Potential Anti-trypanosomal Agents. Biol Pharm Bull. 2008;31(3):538-40.

Batista Júnior JM, Batista ANL, Rinaldo D, Vilegas W, Ambrósio DL, Cicarelli RMB *et al*. Absolute Configuration and Selective Trypanocidal Activity of Gaudichaudianic Acid Enantiomers. J Nat Prod. 2011;74:1154-60.

Bereiter-Hahn J. Do we age because we have mitochondria? Protoplasma. 2014;251:3-23.

Berquist BR, Wilson DM. Pathways for repairing and tolerating the spectrum of oxidative DNA lesions. Cancer Lett. 2012;327:61-72.

Bezerra DP, Pessoa C, De Moraes MO, Saker-Neto N, Silveira ER, Costa-Lotufo LV. Overview of the therapeutic potential of piplartine (piperlongumine). Eur J Pharm Sci. 2013;48:453-63.

Bhattarai MD. Need of the Regulation for Profit Percentage Investment by Pharmaceutical Companies in New Drug Discovery Research from the Various Local Traditional Medicinal and Plant Systems. J Nepal Med Assoc. 2012;52(187):148-50.

Bogeski I, Niemeyer BA. Redox Regulation of Ion Channels. Antiox Redox Signal. 2014;21(6):859-62.

Bogner C, Leber B, Andrews DW. Apoptosis: embedded in membranes. Curr Opin Cell Biol. 2010;22:845-51.

Boire NA, Riedel S, Parrish NM. Essential Oils and Future Antibiotics: New Weapons against Emerging 'Superbugs'? J Anc Dis Prev Rem. 2013;1(2):1-5.

Boonstra J, Post, JA. Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. Gene. 2004;337:1-13.

Bouchard VJ, Rouleau M, Poirier G. PARP-1, a determinant of cell survival in response to DNA damage. Exp Hematol. 2003;31:446-54.

Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. Biochem J. 2011;435:297-312.

Brandão MGL, Pignal M, Romaniuc S, Grael CFF, Fagg CW. Useful Brazilian plants listed in the field books of the French naturalist Auguste deSaint-Hilaire(1779-1853). J Ethnopharmacol. 2012;143:488-500.

Brandão MGL, Cosenza GP, Pereira FL, Vasconcelos AS, Fagg CW. Changes in the trade in native medicinal plants in Brazilian public markets. Environ Monit Assess. 2013;185:7013-23.

Bratti C, Vieira MC, Zárate NAH, Oliveira APA, Marafiga BG, Fernandes SSL. Levantamento de Plantas Medicinais Nativas da Fazenda Azulão em Dourados-MS. Ver Bras Pl Med. 2013;15(4):675-83.

Brehm-Stecher BF, Johnson EA. Sensitization of *Staphylococcus aureus* and *Escherichia coli* to Antibiotics by the Sesquiterpenoids Nerolidol, Farnesol, Bisabolol, and Apritone. Antimicrob Agents Chemother. 2003;47(10):3357-60.

Buettner GR. Superoxide Dismutase in Redox Biology: The roles of superoxide and hydrogen peroxide. Anticancer Agents Med Chem. 2011;11(4):341-6.

Burton GJ, Jauniaux E. Oxidative stress. Best Pract Res Clin Obstet Gynaecol. 2011;25:287-99.

Cairrão F, Domingos PM. Apoptosis: Molecular Mechanisms. ESL. 2010:1-8.

Calderari MT. Estudo dos Óleos Essenciais de Piperaceae do Vale do Itajaí em Santa Catarina [dissertação]. Florianópolis (SC): Universidade Federal de Santa Catarina; 2002.

Callejas R. Studies in neotropical piperaceae I. a new species of *Piper* from Ecuador. Brittonia. 1989;41(3):325-7.

Callejas R. Studies in neotropical piperaceae. II. Four new species and a new combination from Colombia and Peru. Brittonia. 1990;42(1):70-82.

Callejas R, Jonhson DM. Piperaceae types from the t. G. Yuncker Herbarium (dpu) now filed in the New York Botanical Garden Herbarium (NY). Brittonia. 1989;41(3):297-324.

Camara AKS, Lesnefsky EJ, Stowe DF. Potential Therapeutic Benefits of Strategies Directed to Mitochondria. Antiox Redox Signal. 2010;13(3):279-350.

Campos FC, Victorino VJ, Martins-Pinge MC, Cecchini AL, Panis C, Cecchini R. Systemic toxicity induced by paclitaxel in vivo is associated with the solvent cremophor EL through oxidative stress-driven mechanisms. Food Chem Toxicol. 2014;68:78-86.

Candas D, Li JJ. MnSOD in Oxidative Stress Response-Potential Regulation via Mitochondrial Protein Influx. Antiox Redox Signal. 2014;20(10):1599-617.

Candé C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N *et al*. Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie. 2002;84:215-22.

Cao SS, Kaufman R. Endoplasmic Reticulum Stress and Oxidative Stress in Cell Fate Decision and Human Disease. Antiox Redox Signal. 2014;21(3):396-413.

Carocho M, Ferreira ICFR. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem Toxicol. 2013;51:15-25.

Carvalho-Okano RM, Alves SAM. Piperaceae C. Agardh da Estação Experimental Mata do Paraíso, Viçosa, MG. Acta bol bras. 1998;12(3):497-513.

Catanzaro I, Caradonna F, Barbata G, Saverini M, Mauro M, Sciandrello G. Genomic instability induced by α-pinene in Chinese hamster cell line. Mutagenesis. 2012;27(4):463-9.

Cavalheiro CN, Schneider F, Grillo HCZ, Corrêa NR, Almeida RVA, Spinelli R *et al*. Distribuição de *Piper Gaudichaudianum* Kuntze (Piperaceae) e Efeito de Borda em Fragmento Florestal do Jardim Botânico de Lajeado (JBL), Rio Grande Do Sul. Rev Dest Acad. 2013;5(3);131-9.

Cha JD, Kim JY. Essential Oil from *Cryptomeria japonica* Induces Apoptosis in Human Oral Epidermoid Carcinoma Cells via Mitochondrial Stress and Activation of Caspases. Molecules. 2012;17:3890-901.

Chandel NS. Mitochondria as signaling organelles. BMC Biol. 2014;12:34.

Chang H, Guarente L. SIRT1 and other sirtuins in metabolism. Trends Endocrinol Metab. 2014;25(3):138-45.

Chaudhari N, Talwar P, Parimisetty A, Lefebvre d'Hellencourt C, Ravanan P. A molecular web: endoplasmic reticulum stress, inflammation, and oxidative stress. Front Cell Neurosci. 2014;8(213):1-15.

Chen BPC, Li M, Asaithamby A. New insights into the roles of ATM and DNA-PKcs in the cellular response to oxidative stress. Cancer Lett. 2012;327:103-10.

Chen X, Lu R, Dan H, Liao G, Zhou M, Li XY *et al*. Honokiol: a promising small molecular weight natural agent for the growth inhibition of oral squamous cell carcinoma cells. Int J Oral Sci. 2011;3:34-42.

Chiu T, Chen C, Chien C, Li S, Tsai H, Chen Y. High ERCC1 expression predicts cisplatin-based chemotherapy resistance and poor outcome in

unresectable squamous cell carcinoma of head and neck in a betel-chewing area. J Transl Med. 2011;9(31):1-8.

Cho S, Cho M, Seo DB, Lee SJ, Suh Y. Identification of a small molecule activator of SIRT1 gene expression. AGING. 2013;5(3):174-82.

Ciccia A, Elledge SJ. The DNA Damage Response: Making it safe to play with knives. Mol Cell. 2010;40(2):179-204.

Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med. 2010;48:749-62.

Colvard MD, Cordell GA, Villalobos R, Sancho G, Soejarto DD, Pestle W *et al*. Survey of medical ethnobotanicals for dental and oral medicine conditions and pathologies. J Ethnopharmacol. 2006;107:134-42.

Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J. 2003;17:1195-214.

Cordell GA. Changing Strategies in Natural Products Chemistry. Phytochemistry. 1995;40(6):1585-612.

Cragg G M, Newman D J. Natural products: A continuing source of novel drug leads. Biochim Biophys Acta. 2013;1830:3670-95.

Crowell PL. Prevention and Therapy of Cancer by Dietary Monoterpenes. In: Symposium on Phytochemicals: Biochemistry and Physiology-Experimental Biology; 1996 Apr 14-18; Washington; 1996.

Da Silva JKR, Pinto LC, Burbano RMR, Montenegro RC, Guimarães EF, Andrade EHA *et al*. Essential oils of Amazon *Piper* species and their cytotoxic, antifungal, antioxidant and anti-cholinesterase activities. Ind Crops Prod. 2014; 58:55-60.

Da Silva WJM, Ferrari CKB. Metabolismo Mitocondrial, Radicais Livres e Envelhecimento. Rev Bras Geriatr Gerontol. 2011;14(3):441-51.

Damasceno JD. Caracterização molecular do envolvimento das proteínas LmHus1 e LmRad9 em mecanismos de reconhecimento e reparo de DNA no parasito *Leishmania major* [tese]. Ribeirão Preto (SP): Universidade de São Paulo; 2013.

David SS, O'Shea VL, Kundu S. Base-excision repair of oxidative DNA damage. Nature. 2007;447:941-50.

De Almeida RN, Agra MF, Maior FNS, De Sousa DP. Essential Oils and Their Constituents: Anticonvulsant Activity. Molecules. 2011;16(3):2726-42.

De Lima IP, Dos Reis NR. The availability of Piperaceae and the search for this resource by *Carollia perspicillata* (Linnaeus) (*Chiroptera, Phyllostomidae, Carolliinae*) in Parque Municipal Arthur Thomas, Londrina, Paraná, Brazil. Rev Bras Zool. 2004;21(2):371-7.

De Morais SM, Facundo VA, Bertini LM, Cavalcanti ESB, Dos Anjos Júnior JF, Ferreira SA *et al*. Chemical composition and larvicidal activity of essential oils from *Piper* species. Biochem Syst Ecol. 2007;35:670-5.

De Sousa DM. Analgesic-like Activity of Essential Oils Constituents. Molecules. 2011;16:2233-52.

Deng C. SIRT1, Is It a Tumor Promoter or Tumor Suppressor? Int J Biol Sci. 2009;5(2):147-52.

Di Stasi LC, Hiruma-Lima CA. Plantas medicinais na Amazônia e na Mata Atlântica. 2rd ed. São Paulo: UNESP; 2002.

Di Stasi LC, Oliveira GP, Carvalhaes MA, Queiroz-Juniora M, Tien OS, Kakinami SH *et al*. Medicinal plants popularly used in the Brazilian Tropical Atlantic Forest. Fitoterapia. 2002;73:69-91.

Dias CN, Moraes DFC. Essential oils and their compounds as *Aedes aegypti* L. (Diptera: Culicidae) larvicides: review. Parasitol Res. 2014;113:565-92.

Ditch S, Paull TT. The ATM protein kinase and cellular redox signaling: beyond the DNA damage response. Trends in Biochem Sci. 2012;37(1):15-22.

Dizdaroglu M. Oxidatively induced DNA damage: Mechanisms, repair and disease. Cancer Lett. 2012;327:26-47.

Do Carmo DF, Amaral ACF, Machado GMC, Leon LL, Silva JRA. Chemical and Biological Analyses of the Essential Oils and Main Constituents of *Piper* Species. Molecules. 2012;17:1819-29.

Dos Santos PRD, Moreira DL, Guimarães EF, Kaplan MAC. Essential oil analysis of 10 Piperaceae species from the Brazilian Atlantic forest. Phytochemistry. 2001;58:547-51.

Duarte TL, Lunec J. Review Part of the Series: From Dietary Antioxidants to Regulators in Cellular Signalling and Gene Expression Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free Radic Res. 2005;39(7):671-86.

El-Beltagi HS, Mohamed HI. Reactive Oxygen Species, Lipid Peroxidation and Antioxidative Defense Mechanism. Not Bot Horti Agrobo. 2013;41(1):44-57.

Elmann A, Mordechay S, Rindner M, Larkov O, Elkabetz M, Ravid U. Protective Effects of the Essential Oil of *Salvia fruticosa* and Its Constituents on Astrocytic Susceptibility to Hydrogen Peroxide-Induced Cell Death. J Agric Food Chem. 2009;57:6636-41.

European Pharmacopoeia. Sec ed. Maisonneuve, Sainte Ruffine, France; 1983.

Farrukh MR, Nissar UA, Afnan Q, Rafiq RA, Sharma L, Amin S *et al*. Oxidative stress mediated Ca^{2+} release manifests endoplasmic reticulum stress leading to unfolded protein response in UV-B irradiated human skin cells. J Dermatol Sci. 2014;75:24-35.

Federico A, Cardaioli E, Da Pozzo P, Formichi P, Gallus GN, Radi E. Mitochondria, oxidative stress and neurodegeneration. J Neurol Sci. 2012;322: 254-62.

Ferhat MA, Meklati BY, Smadja J, Chemat F. An improved microwave Clevenger apparatus for distillation of essential oils from orange peel. J Chromatogr A. 2006;1112:121-6.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C *et al*. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [periódico online]. Lyon, France: International Agency for Research on Cancer; 2013. Disponível em: http://globocan.iarc.fr, acesso em 29/11/2014.

Fernandes ES, Passos GF, Medeiros R, Da Cunha FM, Ferreira J, Campos MM *et al*. Anti-inflammatory effects of compounds alpha-humulene and (−)-trans-caryophyllene isolated from the essential oil of *Cordia verbenacea*. Eur J Pharmacol. 2007;569:228-36.

Ferraz ABF, Balbino JM, Zini CA, Ribeiro VLS, Bordignon SAL, Von Poser G. Acaricidal activity and chemical composition of the essential oil from three *Piper* species. Parasitol Res. 2010;107:243-8.

Ferreira FM, Palmeira CM, Oliveira MM, Santos D, Simões AM, Rocha SM *et al*. Nerolidol effects on mitochondrial and cellular energetics. Toxicol In Vitro. 2012;26:189-96.

Figueiredo RA, Sazima M. Pollination Biology of Piperaceae Species in Southeastern Brazil. Annals of Botany. 2000;85:455-60.

Forbes-Hernández TY, Giampieri F, Gasparrini M, Mazzoni L, Quiles JL, Alvarez-Suarez JM *et al*. The effects of bioactive compounds from plant foods on mitochondrial function: A focus on apoptotic mechanisms. Food Chem Toxicol. 2014;68:154-82.

Frutuoso AE, Nascimento NT, De Lemos TLG, Coelho EL, Teixeira DMA. Óleos Essenciais Aplicados em Alimentos: Uma Revisão. Rev Bras Pesquisa Alim. 2013;4(2):69-81.

Gautam N, Mantha AK, Mittal S. Essential Oils and Their Constituents as Anticancer Agents: A Mechanistic View. Biomed Res Int. 2014;1-23.

German P, Szaniszlo P, Hajas G, Radak Z, Bacsi A, Hazra TK *et al*. Activation of cellular signaling by 8-oxoguanine DNA glycosylase-1-initiated DNA base excision repair. DNA Repair. 2013;12:856-63.

Ghosh R, Darin K, Nath P, Deb P. An Overview of Various Piper Species for Their Biological Activities. IJPRR. 2014;3(1):67-75.

Giansanti V, Donà F, Tillhon M, Scovassi I. PARP inhibitors: New tools to protect from inflammation. Biochem Pharmacol. 2010;80:1869-77.

Gobbini E, Cesena D, Galbiati A, Lockhart A, Longhese MP. Interplays between ATM/Tel1 and ATR/Mec1 in sensing and signaling DNA double-strand breaks. DNA Repair. 2013;12:791-9.

Gobbo-Neto L, Lopes NP. Plantas Medicinais: Fatores de Influência no Conteúdo de Metabólitos Secundários. Quim Nova. 2007;30(2):374-81.

Gonçalves O, Pereira R, Gonçalves F, Mendo S, Coimbra MA, Rocha SM. Evaluation of the mutagenicity of sesquiterpenic compounds and their influence on the susceptibility towards antibiotics of two clinically relevant bacterial strains. Mutat Res. 2011;723:18-25.

Gospodinov A, Herceg Z. Chromatin structure in double strand break repair. DNA Repair. 2013;12:800-10.

Groussin AL, Antoniotti S. Valuable chemicals by the enzymatic modification of molecules of natural origin: Terpenoids, steroids, phenolics and related compounds. Bioresour Technol. 2012;115:237-43.

Guimarães AG, Quintans JSS, Quintans-Júnior LJ. Monoterpenes with Analgesic Activity-A Systematic Review. Phytother Res. 2013;27:1-15.

Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. ATM Activation by Oxidative Stress. Science. 2010;330:517-21.

Gurib-Fakim A. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Mol Aspects Med. 2006;27:1-93.

Gutierrez RMP, Gonzalez AMN, Hoyo-Vadillo C. Alkaloids from *Piper*: A Review of its Phytochemistry and Pharmacology. Mini Rev Med Chem. 2013;13:163-93.

HalliwelL B. Free Radicals and other reactive species in disease. ELS. 2001:1-7.

Halliwell B. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? Arch. Biochem. Biophys. 2008;476:107-12.

HalliwelL B. Free radicals and antioxidants: updating a personal view. Nutr Rev. 2012;70(5):257-65.

HalliwelL B, Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. Am J Clin Nutr. 1993;57:715S-25.

Halliwell B, Gutteridge JMC. The definition and measurement of antioxidants in Biological systems. Free Radic Biol Med. 1995;18(1):125-6.

Hegde ML, Mantha AK, Hazra TK, Bhakat KK, Mitra S, Szczesny B. Oxidative genome damage and its repair: Implications in aging and neurodegenerative diseases. Mech Ageing Dev. 2012;133:157-68.

Hemaiswarya S, Kruthiventi AK, Doble M. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine. 2008;15:639-52.

Hoeijmakers JHJ. Genome maintenance mechanisms for preventing cancer. Nature. 2001;411:366-74.

Holmgren PK, Kallunki JA, Thiers BM. A short description of the collections of The New York Botanical Garden Herbarium (NY). Brittonia. 1996;48(3):285-96.

Hu Z, Yang X, Ho PCL, Chen SY, Heng PWS, Chan E *et al*. Herb-Drug Interactions. A Literature Review. Drugs. 2005;65(9):1239-82.

Huber A, Bai P, De Murcia JM, De Murcia G. PARP-1, PARP-2 and ATM in the DNA damage response: functional synergy in mouse development. DNA Repair. 2004;3:1103-8.

Instituto Nacional de Câncer José Alencar Gomes da Silva. Estimativa 2014: Incidência de Câncer no Brasil. Rio de Janeiro: INCA; 2014.

Iwazaki MC, Souza LA, Oliveira JHG. Morfo-anatomia comparativa da flor de *Peperomia dahlstedtii* C. DC., *Ottonia martiana* Miq. e *Piper gaudichaudianum* Kunth (Piperaceae). Hoehnea. 2006;33(4):545-58.

Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009;461(7267):1071-8.

Javle M, Curtin NJ. The role of PARP in DNA repair and its therapeutic exploitation. Br J Cancer. 2011;105:1114-22.

Jo J, Park JS, Park YK, Chae YZ, Lee GH, Park GY *et al*. *Pinus densiflora* leaf essential oil induces apoptosis via ROS generation and activation of caspases in YD-8 human oral cancer cells. Int J Oncol. 2012;40:1238-45.

Jones DP. Disruption of mitochondrial redox circuitry in oxidative stress. Chem Biol Interact. 2006;163:38-53.

Kannan N, Nguyena LV, Makarema M, Donga Y, Shiha K, Eirewa P *et al*. Glutathione-dependent and -independent oxidative stress-control mechanisms distinguish normal human mammary epithelial cell subsets. PNAS. 2014;111(21):7789-94.

Kasymov RD, Grin I R, Endutkin AV, Smirnov SL, Ishchenko AA, Saparbaev MK *et al*. Excision of 8-oxoguanine from methylated CpG dinucleotides by human 8-oxoguanine DNA glycosylase. FEBS Lett. 2013;587:3129-34.

Ko HL, Ren EC. Functional Aspects of PARP1 in DNA Repair and Transcription. Biomolecules. 2012;2:524-48.

Koroch AR, Zygadlo JA, Juliani HR. Bioactivity of Essential oils and their components. In:Berger RG (ed.) Flavours-Chemistry, Technology and Resources. Springer-Verlag. Berlin. Heidelberg. 2007:87-116.

Kowaltowski AJ, Souza-Pinto NC, Castilho RF, Vercesi AE. Mitochondria and reactive oxygen species. Free Radic Biol Med. 2009;47:333-43.

Kroemer G, Galluzzi L, Brenner C. Mitochondrial Membrane Permeabilization in Cell Death. Physiol Rev. 2007;87:99-163.

Kryston TB, Georgieva AB, Pissis P, Georgakilasa AG. Role of oxidative stress and DNA damage in human carcinogenesis. Mutat Res. 2011;711:193-201.

Kubo I, Chaudhuri SK, Kubo Y, Sanchez Y, Ogura T, Saito T *et al*. Cytotoxic and antioxidative sesquiterpenoids from Heterotheca inuloides. Planta Med. 1996;62(5):427-30.

Lago JHG, Ramos CS, Casanova DCC, Morandim AA, Bergamo DCB, Cavalheiro AJ *et al*. Benzoic Acid Derivatives from *Piper* Species and Their Fungitoxic Activity against *Cladosporium cladosporioides* and *C. Sphaerospermum*. J Nat Prod. 2004;67:1783-8.

Lai L, Fu Q, Liu Y, Jiang K, Guo Q, Chen Q *et al*. Piperine suppresses tumor growth and metastasis in vitro and in vivo in a 4T1 murine breast cancer model. Acta Pharmacol Sin. 2012;33:523-30.

Lang G, Buchbauer G. A review on recent research results (2008–2010) on essential oils as antimicrobials and antifungals. A review. Flavour Fragr J. 2012;27:13-39.

Lee W, Kim K, Yu S, Kim S, Chun S, Ji J *et al*. Pipernonaline from *Piper longum* Linn. induces ROS-mediated apoptosis in human prostate cancer PC-3 cells. Biochem Biophys Res Communic. 2013;430:406-12.

Legault J, Pichette A. Potentiating effect of β-caryophyllene on anticancer activity of α-humulene, isocaryophyllene and paclitaxel. JPP. 2007;59:1643-7.

Lesgards JF, Gauthierc C, Iovannac J, Vidala N, Dollab A, Stockera P. Effect of reactive oxygen and carbonyl species on crucial cellular antioxidant enzymes. Chem Biol Interact. 2011;190:28-34.

Lesgards JF, Baldovini N, Vidal N, Pietri S. Anticancer Activities of Essential Oils Constituents and Synergy with Conventional Therapies: A Review. Phytother Res. 2014.

Lilyestrom W, Van Der Woerd MJ, Clark N, Luger K *et al*. Structural and Biophysical Studies of Human PARP-1 in Complex with Damaged DNA. J Mol Biol. 2010;395:983-94.

Lima ES, Abdalla DSP. Peroxidação lipídica: mecanismos e avaliação em amostras biológicas. Rev Bras Cienc Farm. 2001;37(3):293-303.

Lisby M, Barlow JH, Burgess RC, Rothstein R. Choreography of the DNA Damage Response: Spatiotemporal Relationships among Checkpoint and Repair Proteins. Cell. 2004;118:699-713.

Liu D, Gharavi R, Pitta M, Gleichmann M, Mattson MP. Nicotinamide Prevents NAD+ Depletion and Protects Neurons Against Excitotoxicity and Cerebral Ischemia: NAD+ Consumption by SIRT1 may Endanger Energetically Compromised Neurons. Neuromolecular Med. 2009;11(1):28-42.

Lobo SP. Características da indústria farmacêutica no Brasil durante o século XX: expansão e divisão internacional do trabalho. Rev PLURAIS Virtual. 2013;3(1):83-108.

Loizzo MR, Tundis R, Menichini F, Saab AM, Statti GA, Menichini F. Cytotoxic Activity of Essential Oils from *Labiatae* and *Lauraceae* Families Against In Vitro Human Tumor Models. Anticancer Res. 2007;27:3293-300.

Lopes AA, Baldoqui DC, López SN, Kato MJ, Bolzani VS, Furlan M. Biosynthetic origins of the isoprene units of gaudichaudianic acid in *Piper gaudichaudianum* (Piperaceae). Phytochemistry. 2007;68:2053-8.

López-Alarcón C, Denicola A. Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. Anal Chim Acta. 2013;763:1-10.

Lu A, Li X, Gu Y, Wright PM, Chang D. Repair of Oxidative DNA Damage. Cell Biochem Biophys. 2001;35:141-70.

Luziatelli G, Sorensen M, Theilade I, Molgaard P. Asháninka medicinal plants: a case study from the native community of Bajo Quimiriki, Junín, Peru. J Ethnobiol Ethnomed. 2010;6(21):1-23.

Ma Q. Transcriptional responses to oxidative stress: Pathological and toxicological implications. Pharmacol Ther. 2010;125:376-93.

Mansfield KD, Guzy RD, Pan Y, Young RM, Cash TP, Schumacker PT, Simon MC. Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-α activation. Cell Metab. 2005;1:393-9.

Marques AM, Barreto ALS, Curvelo JAR, Romanos MTV, Soares RMA, Kaplan MAC. Antileishmanial activity of nerolidol-rich essential oil from *Piper claussenianum*. Rev Bras Farmacogn. 2011;(5):908-14.

Martin-Ventura JL, Madrigal-Matute J, Martinez-Pinna R, Ramos-Mozo P, Blanco-Colio LM, Moreno JA *et al*. Erythrocytes, leukocytes and platelets as a source of oxidative stress in chronic vascular diseases: Detoxifying mechanisms and potential therapeutic options. Thromb Haemost. 2012;108(3):435-42.

Martins AP, Salgueiro L, Vila R, Tomi F, Cañigueral S, Casanova J *et al*. Essential Oils from Four *Piper* Species. Phytochemistry. 1998:49(7):2019-23.

McCullough AK, Dodson ML, Lloyd RS. Initiation of Base Excision Repair: Glycosylase Mechanisms and Structures. Annu Rev Biochem. 1999;68:255-85.

Medeiros ESS. Flora do Parque Estadual do Ibitipoca, MinasGerais, Brasil - Família Piperaceae [dissertação]. Rio de Janeiro (RJ): Instituto de Pesquisas Jardim Botânico do Rio de Janeiro/Escola Nacional de Botânica Tropical Rio de Janeiro; 2006.

Mendanha SA, Moura SS, Anjos JLV, Valadares MC, Alonso A. Toxicity of terpenes on fibroblast cells compared to their hemolytic potential and increase in erythrocyte membrane fluidity. Toxicol In Vitro. 2013;27:323-9.

Mesquita JMO, Cavaleiro C, Cunha AP, Lombardi JA, Oliveira AB. Estudo comparativo dos óleos voláteis de algumas espécies de Piperaceae. Rev Bras Farmacogn. 2005;15(1):6-12.

Meurer-Grimes B. Index to collectors of the piperaceae represented in the T. G. Yuncker Herbarium of Depauw University. Brittonia. 1989;41(3):285-96.

Miguel MG. Antioxidant and Anti-Inflammatory Activities of Essential Oils: A Short Review. Molecules. 2010;15:9252-87.

Mikich SB, Bianconi GV, Maia BHLNS, Teixeira SD. Attraction of the fruit-eating bat *Carollia perspicillata* to *Piper gaudichaudianum* essential oil. J Chem Ecol. 2003;29(10):2379-83.

Mishra BB, Tiwari VK. Natural products: An evolving role in future drug discovery. Eur J Med Chem. 2011;46:4769-807.

Mithöfer A, Boland W. Plant Defense Against Herbivores: Chemical Aspects. Annu Rev Plant Biol. 2012;63:431-50.

Monteiro LS, Bastos KX, Barbosa-Filho JM, Athayde-Filho PF, Diniz MFFM, Sobral MV. Medicinal Plants and Other Living Organisms with Antitumor Potential against Lung Cancer. eCAM. 2014;1-15.

Monzote L, García M, Montalvo AM, Scull R, Miranda M. Chemistry, cytotoxicity and antileishmanial activity of the essential oil from *Piper auritum*. Mem Inst Oswaldo Cruz. 2010;105(2):168-73.

Morandim AA, Pin AR, Pietro NAS, Alecio AC, Kato MJ, Young CM *et al*. Composition and screening of antifungal activity against *Cladosporium sphaerospermum* and *Cladosporium cladosporioides* of essential oils of leaves and fruits of *Piper* species. Afr J Biotechnol. 2010;9(37):6135-9.

Muftuoglu M, Mori MP, De Souza-Pinto NC. Formation and repair of oxidative damage in the mitochondrial DNA. Mitochondrion. 2014;17:164-81.

Müller M, Buchbauer G. Essential oil components as pheromones. A review. Flavour Fragr J. 2011;26:357-77.

Mundina M, Vila R, Tomi F, Gupta MP, Adzet T, Casanova J *et al*. Leaf Essential Oils of Three Panamanian *Piper* Species. Phytochemistry. 1998;47(7):1277-82.

Nakagawa T, Guarente L. Sirtuins at a glance. J Cell Sci. 2011;124(6):833-7.

Nerio LS, Olivero-Verbel J, Stashenko E. Repellent activity of essential oils: A review. Bioresour Technol. 2010;101:372-8.

Nogueira Neto JD, De Almeida AAC, Oliveira JS, Dos Santos OS, De Sousa P, De Freitas RM. Antioxidant Effects of Nerolidol in Mice Hippocampus After Open Field Test. Neurochem Res. 2013;38:1861-70.

Nuzhat T, Vidyasagar GM. Antifungal Investigations on Plant Essential Oils. A Review. Int J Pharm Pharm Sci. 2013;5(2):19-28.

Oh J, Bowling JJ, Carroll JF, Demirci B, Başer KHC, Leininger TD *et al*. Natural product studies of U.S. endangered plants: Volatile components of *Lindera*

melissifolia (Lauraceae) repel mosquitoes and ticks. Phytochemistry. 2012;80:28-36.

Oliveira FA, Andrade LN, De Sousa EBV, De Sousa DP. Anti-Ulcer Activity of Essential Oil Constituents. Molecules. 2014;19:5717-47.

Pagano G, Talamanca AA, Castello G, Cordero MD, D'Ischia M, Gadaleta MN et al. Oxidative Stress and Mitochondrial Dysfunction across Broad-Ranging Pathologies: Toward Mitochondria-Targeted Clinical Strategies. Oxid Med Cell Longev. 2014:1-27.

Park KR, Nam D, Yun HM, Lee SG, Jang HJ, Sethi G *et al*. β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. Cancer Lett. 2011;312:178-88.

Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jha A *et al*. Phytochemistry of the Genus *Piper*. Phytochemistry. 1997;46(4):591-673.

Paz-Elizur T, Sevilya Z, Leitner-Dagan Y, Elinger D, Roisman L, Livneh Z. DNA repair of oxidative DNA damage in human carcinogenesis: Potential application for cancer risk assessment and prevention. Cancer Lett. 2008;266(1):60-72.

Péres VF, Saffi J, Melecchi MIS, Abad FC, Jacques RA, Martinez MM *et al*. Comparison of soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acids and Vitamin E from *Piper gaudichaudianum* Kunth. J Chromatogr A. 2006a;1105:115-8.

Péres VF, Saffi J, Melecchi MIS, Abad FC, Martinez MM, Oliveira EC *et al*. Optimization of pressurized liquid extraction of *Piper gaudichaudianum* Kunth leaves. J Chromatogr A. 2006b;1105:148-53.

Péres VF, Moura DJ, Sperotto ARM, Damasceno FC, Caramão EB, Zini CA *et al*. Chemical composition and cytotoxic, mutagenic and genotoxic activities of the essential oil from *Piper gaudichaudianum* Kunth leaves. Food Chem Toxicol. 2009;47:2389-95.

Petrovska BB. Historical review of medicinal plants' usage. Pharmacogn Rev. 2012;6(11):1-5.

Phillipson JD. Phytochemistry and medicinal plants. Phytochemistry. 2001;56:237-43.

Phillipson JD. Phytochemistry and pharmacognosy. Phytochemistry. 2007;68:2960-72.

Picada JN, Kern AL, Ramos ALLP, Saffi J. O Estresse Oxidativo e as Defesas Antioxidantes. In: Da Silva J, Erdtmann, Henriques JAP. Genética Toxicológica. Porto Alegre: Alcance; 2003. p. 248-68.

Pículo F, Macedo CG, De Andrade SF, Maistro EL. In vivo genotoxicity assessment of nerolidol. J Appl Toxicol. 2011;31:633-9.

Polo SE, Jackson SP. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes Dev. 2011;25:409-33.

Pourmortazavi SM, Hajimirsadeghi SS. Supercritical fluid extraction in plant essential and volatile oil analysis. J Chromatogr A. 2007;1163:2-24.

Prakash B, Shukla R, Singh P, Kumar A, Mishra PK, Dubey NK. Efficacy of chemically characterized *Piper betle* L. essential oil against fungal and aflatoxin contamination of some edible commodities and its antioxidant activity. Int J Food Microbiol. 2010;142:114-9.

Prakash O, Kumar A, Kumar P, Ajeet. Anticancer Potential of Plants and Natural Products: A Review. Am J Pharm Sci. 2013;1(6):104-15.

Procházková D, Boušová I, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia. 2011;82:513-23.

Puhl MCMN, Cortez DAG, Ueda-Nakamura T, Nakamura CV, Dias Filho BP. Antimicrobial Activity of *Piper gaudichaudianum* Kuntze and its Synergism with Different Antibiotics. Molecules. 2011;16:9925-38.

Rahman S, Islam R. Mammalian Sirt1: insights on its biological functions. Cell Commun Signal. 2011;9(11):1-8.

Rai MP, Thilakchand KR, Palatty PL, Rao P, Rao S, Bhat HP *et al*. *Piper Betel* Linn (Betel Vine), the Maligned Southeast Asian Medicinal Plant Possesses Cancer Preventive Effects: Time to Reconsider the Wronged Opinion. Asian Pac J Cancer Prev. 2011;12:2149-56.

Ramos CS, Vanin SA; Kato MJ. Sequestration of prenylated benzoic acid and chromenes by *Naupactus bipes* (Coleoptera: Curculionidae) feeding on *Piper gaudichaudianum* (Piperaceae). Chemoecology. 2009;19:73-80.

Rates SMK. Plants as source of drugs. Toxicon. 2001;39:603-13.

Ravizza R, Gariboldi MB, Molteni R, Monti E. Linalool, a plant-derived monoterpene alcohol, reverses doxorubicin resistance in human breast adenocarcinoma cells. Oncol Rep. 2008;20:625-30.

Ray PD, Huang B, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 2012;24:981-90.

Raza H, John A, Shafarin J. NAC Attenuates LPS-Induced Toxicity in Aspirin-Sensitized Mouse Macrophages via Suppression of Oxidative Stress and Mitochondrial Dysfunction. PLOS ONE. 2014;9(7):1-10.

Reczek CR, Chandel NS. ROS-dependent signal transduction. Curr Opin Cell Biol. 2015;33:8-13.

Rodrigues E, Barnes J. Pharmacovigilance of Herbal Medicines: The Potential Contributions of Ethnobotanical and Ethnopharmacological Studies. Drug Saf. 2013;36:1-12.

Ruberto G; Baratta MT. Antioxidant activity of selected essential oil components in two lipid model systems. Food Chem. 2000;69:167-74.

Sancar A, Lindsey-Boltz LA, Ünsal-Kaçmaz K, Linn S. Molecular Mechanisms of Mammalian Dna Repair and The DNA Damage Checkpoints. Annu Rev Biochem. 2004;73:39-85.

Santos PG, Siani AC. A dinâmica Universidade-Empresa na área farmacêutica: Alguns indicadores gerais da Indústria, Academia Científica e Governo para o caso brasileiro. Rev Fitos. 2012;7(3):155-68.

Santos TG, Fukuda K, Katoc MJ, Sartorato A, Duarte MCT, Ruiz ALTG *et al*. Characterization of the essential oils of two species of Piperaceae by one- and two-dimensional chromatographic techniques with quadrupole mass spectrometric detection. Microchem J. 2014;115:113-20.

Sarnaglia Junior VB, Bermudez GMM, Guimarães EF. Diversidade de Piperaceae em um remanescente de Floresta Atlântica na região serrana do Espírito Santo, Brasil. Biotemas. 2014;27(1):49-57.

Schmit AP, Riffel C. Avaliação da atividade antifúngica de diferentes espécies do gênero *Piper* [monografia]. Itajaí (SC): Universidade do Vale do Itajaí; 2010.

Scott TL, Rangaswamy S, Wicker CA, Izumi T. Repair of Oxidative DNA Damage and Cancer: Recent Progress in DNA Base Excision Repair. Antiox Redox Signal. 2014;20(4):708-26.

Shen Z, Wu W, Hazen SL. Activated Leukocytes Oxidatively Damage DNA, RNA, and the Nucleotide Pool through Halide-Dependent Formation of Hydroxyl Radical. Biochemistry. 2000;39:5474-82.

Shu YZ. Recent Natural Products Based Drug Development: A Pharmaceutical Industry Perspective. J Nat Prod. 1998;6:1053-71.

Siegel R, Ma J, Zou Z, Jemal A. Cancer Statistics, 2014. CA Cancer J Clin. 2014;64:9-29.

Simões CMO, Schenkel EP, Gosmann G, De Mello JCP, Mentz LA, Petrovick PR. Farmacognosia, da Planta ao Medicamento. 6th ed. Porto Alegre: Editora da UFRGS; Florianópolis: Editora da UFSC; 2007.

Sireeratawong S, Khonsung P, Piyabhan P, Nanna U, Soonthornchareonnon N, Jaijoy K. Anti-inflammatory and anti-ulcerogenic activities of Chantaleela Recipe. Afr J Tradit Complement Altern Med. 2012a;9(4):485-94.

Sireeratawong S, Khonsung P, Nanna U, Vannasiri S, Lertprasertsuke N, Singhalak T *et al*. Anti-Diarrheal Activity and Toxicity of Learng Pid Samud Recipe. Afr J Tradit Complement Altern Med. 2012b;9(4):519-29.

Sisodiya PS. Plant Derived Anticancer Agents: A Review. Int J Res Dev Pharm L Sci. 2013;2(2):293-308.

Smelcerovic A, Djordjevic A, Lazarevic J, Stojanovic G. Recent Advances in Analysis of Essential Oils. Curr Anal Chem. 2013;9:61-70.

Sodhi RK, Singh N, Jaggi AS. Poly (ADP-ribose) polymerase-1 (PARP-1) and its therapeutic implications. Vascul Pharmacol. 2010;53:77-87.

Sperotto ARM, Moura DJ, Péres VF, Damasceno FC, Caramão EB, Henriques JAP *et al*. Cytotoxic mechanism of *Piper gaudichaudianum* Kunth essential oil and its major compound nerolidol. Food Chem Toxicol. 2013;57:57-68.

Sultana R, Mecocci P, Mangialasche F, Cecchetti R, Baglioni M, Butterfield DA. Increased Protein and Lipid Oxidative Damage in Mitochondria Isolated from Lymphocytes from Patients with Alzheimer's Disease: Insights into the Role of Oxidative Stress in Alzheimer's Disease and Initial Investigations into a Potential Biomarker for this Dementing Disorder. J Alzheimers Dis. 2011;24:77-84.

Sumathykutty MA, Rao JM, Padmakumari KP, Narayanan CS. Essential oil constituents of some *Piper* species. Flavour Fragr J. 1999;14:279-82.

Sylvestre M, Legault J, Dufour D, Pichette A. Chemical composition and anticancer activity of leaf essential oil of *Myrica gale* L. Phytomedicine. 2005;12:299-304.

Sylvestre M, Pichette A, Longtin A, Nagau F, Legault J. Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens* L. from Guadeloupe. J Ethnopharmacol. 2006;103:99-102.

Sylvestre M, Pichette A, Lavoie S, Longtin A, Legault J. Composition and Cytotoxic Activity of the Leaf Essential Oil of *Comptonia peregrina* (L.) Coulter Phytother Res. 2007;21:536-40.

Tak JK, Lee JH, Park JW. Resveratrol and piperine enhance radiosensitivity of tumor cells. BMB Rep. 2012;45(4):242-6.

Tamburini BA, Tyler JK. Localized Histone Acetylation and Deacetylation Triggered by the Homologous Recombination Pathway of Double-Strand DNA Repair. Mol Cell Biol. 2005;25(12):4903-13.

Teixeira SD. Estudo Fitoquímico de *Piper gaudichaudianum* e sua Interação com Morcegos Frugívoros [tese]. Curitiba (PR): Universidade Federal do Paraná; 2003.

Tisserand R, Young R. Essential Oil Safety. A Guide for Health Care Professionals. 2 th ed. Elsevier; 2014.

Thomas E, Semo L, Morales M, Noza Z, Nuñez H, Cayuba A *et al*. Ethnomedicinal practices and medicinal plant knowledge of the Yuracarés and Trinitarios from Indigenous Territory and National Park Isiboro-Sécure, Bolivian Amazon. J Ethnopharmacol. 2011;133:153-63.

Ueta E, Sasabe E, Yang Z, Osaki T, Yamamoto T. Enhancement of apoptotic damage of squamous cell carcinoma cells by inhibition of the mitochondrial DNA repairing system. Cancer Sci. 2008;99(11):2230-7.

Valko M, Leibfritz D, Moncola J, Cronin MTD, Mazura M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39:44-84.

Vasconcelos TB, Cardoso ARNR, Josinoc JB, Macenad RHM, Bastose VPD. Radicais Livres e Antioxidantes: Proteção ou Perigo? UNOPAR Cient Ciênc Biol Saúde. 2014;16(3):213-9.

Villanueva C, Kross RD. Antioxidant-Induced Stress. Int J Mol Sci. 2012;13:2091-109.

Von Poser GL, Rörig LR, Henriques AT, Lamaty G, Menut C, Bessière JM. Aromatic Plants from Brazil. III. The Chemical Composition of *Piper gaudichaudianum* Kunth and *P. mikanianum* (Kunth) Steudel Essential Oils. J Essent Oil Res. 1994;6(4):337-40.

Vurusaner B, Poli G, Basaga H. Tumor suppressor genes and ROS: complex networks of interactions. Free Radic Biol Med. 2012;52:7-18.

Wallace SS, Murphy DL, Sweasy JB. Base Excision Repair and Cancer. Cancer Lett. 2012;327(1-2):73-89.

Wang Y. Morris‑Natschke SL, Yang J, Niu H, Long C, Lee K. Anticancer Principles from Medicinal *Piper* (*胡椒Hú Jiāo*) Plants. J Tradit Complement Med. 2014;4(1):8‑16.

Williams GJ, Williams RS, Williams JS, Moncalian, Arvai AS, Limbo O. ABC ATPase signature helices in Rad50 link nucleotide state to Mre11 interface for DNA repair. Nat Struct Mol Biol. 2011;18(4):423-34.

Yamaguchi LF, Freitas GC, Yoshida NC, Silva RA, Gaia AM, Silva AM *et al*. Chemometric Analysis of ESIMS and NMR Data from *Piper* Species. J Braz Chem Soc. 2011;22(12):2371-82.

Yan MH, Wang X, Zhu X. Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. Free Radic Biol Med. 2013;62:90-101.

Yélamos J, Schreiber V, Dantzer F. Toward specific functions of poly (ADP-ribose) polymerase-2. Trends Mol Med. 2008;14(4):169-78.

Ying W. NAD^+/NADH and $NADP^+$/NADPH in Cellular Functions and Cell Death: Regulation and Biological Consequences. Antiox Redox Signal. 2008;10(2):179-215.

Yousef MI, Hussien HM. Cisplatin-induced renal toxicity via tumor necrosis factor-α, interleukin 6, tumor suppressor P53, DNA damage, xanthine oxidase, histological changes, oxidative stress and nitric oxide in rats: Protective effect of ginseng. Food Chem Toxicol. 2015;78:17–25.

Yuncker TG. The Piperaceae-a family profile. Brittonia. 1958;10:1-7.

Yuncker TG. New taxa of peruvian Piperaceae. Brittonia. 1961;13:58-63.

Zhu Q, Wani AA. Histone Modifications: Crucial Elements for Damage Response and Chromatin Restoration. J Cell Physiol. 2010;223(2):283-8.

Ziech D, Francob R, Georgakilasc AG, Georgakilac S, Malamou-Mitsid V, Schonevelde O *et al*. The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development. Chem Biol Interact. 2010;188:334-9.

2. Objetivos

2.1. Objetivo Geral

A proposta deste trabalho é ampliar o conhecimento sobre os mecanismos envolvidos na citotoxicidade do óleo essencial das folhas de *P. gaudichaudianum* em células de fibroblastos de pulmão de hamster chinês (V79).

2.2. Objetivos específicos

a) Identificar a composição química do óleo essencial de *P. gaudichaudianum*, utilizando as técnicas de cromatografia gasosa com ionização de chama (GC-FID) e cromatografia gasosa acoplada à espectrometria de massa (GC-MS).

b) Avaliar a viabilidade celular após o tratamento com diferentes concentrações do óleo essencial de *P. gaudichaudianum* utilizando o ensaio de azul de trypan.

c) Identificar o tipo de morte celular induzida pelo óleo essencial de *P. gaudichaudianum* utilizando marcação com anexina V-PE e 7-AAD.

d) Avaliar a interferência do óleo essencial de *P. gaudichaudianum* no potencial mitocondrial através de marcação com rodamina 123.

e) Verificar a formação de espécies reativas induzidas pelo óleo essencial de *P. gaudichaudianum* utilizando a marcação com DCFH.

f) Analisar a atividade das enzimas envolvidas na resposta ao estresse oxidativo, superoxido dismutase e catalase, após o tratamento com o óleo essencial de *P. gaudichaudianum*.

g) Verificar a ação da N-acetilcisteína na citotoxicidade induzida pelo óleo essencial de *P. gaudichaudianum*.

h) Verificar o nível de expressão das proteínas SIRT-1 e PADPR após tratamento com o óleo essencial de *P. gaudichaudianum*.

3. Artigo científico

"*Piper gaudichaudianum* essential oil triggers cell death by necrosis through mitochondrial dysfunction and ROS-dependent mechanisms involving PARP activation"

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Para facilitar a leitura do artigo, as figuras foram introduzidas ao longo do texto.

Piper gaudichaudianum essential oil triggers cell death by necrosis through mitochondrial dysfunction and ROS-dependent mechanisms involving PARP activation

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Abstract

The *Piper gaudichaudianum* Kunth essential oil (EO) has pharmacological properties such as anti-inflammatory, antimicrobial and antitumor activities as a result of the synergistic action of its major components. In order to improve the understanding of the cytotoxicity and the mechanism of action of the EO in mammalian cells, our study aims to evaluate the chemical composition of the EO, as well as cell viability, mitochondrial function, formation of free radicals, proteins involved in cellular response and induction of cell death after 3 h and 24 h of treatment with different concentrations of EO. 93.32% of EO components were identified by GC-FID and GC-MS. The major components were (E)-nerolidol (31.13%) and α-humulene (14.83%). The EO decreased the percentage of viable V79 cells, exerting dose-dependent cytotoxic effect, with IC50 4.15 μg/ml and 4.49 μg/mL, after 3 h and 24 h treatment, respectively; increased mitochondrial depolarization, indicating that mitochondrial membranes are damaged by permeabilization; increased ROS production and reduced SOD activity, resulting in a pro-oxidant state; increased PADPR and decreased SIRT1 levels, acting in the cell death induction, by necrosis and late apoptosis. Furthermore, pretreatment of cells with N-acetyl cysteine (2 mM) restored cell viability, confirming the involvement of ROS in mediating the induced effects. Therefore, this work is an important contribution to the understanding of the cytotoxicity and the mechanism of action of the EO in mammalian cells.

Keywords: *Piper gaudichaudianum* Kunth, essential oil, citotoxity.

1. Introduction

Medicinal plants have been used over time, always with growing interest in the research and development of new drugs containing their active principles (Chen et al., 2011; Gurib-Fakim, 2006; Monteiro et al., 2014; Rai et al., 2011; Shu, 1998). Essential oils are natural and volatile aromatic compounds, consisting of a variety of terpenes and terpenoids (Bakkali et al., 2008). They can be extracted from different parts of the plant by different methods, among them hydrodistillation (Ferhat et al., 2006), and their composition can be analyzed through gas chromatography coupled to mass spectrometry (GC/MS) (Bakkali et al., 2008; Başer and Buchbauer, 2010; Smelcerovic et al., 2013). Essential oils have biological properties such as analgesic, anti-inflammatory (Fernandes et al., 2007), bactericidal, virucidal, fungicidal (Boire et al., 2013; Schmit and Riffel, 2010), antiparasitic (Ferraz et al., 2010; Marques, et al., 2011), insecticidal and larvicidal (De Morais et al., 2007; Dias and Moraes, 2014; Ferraz et al., 2010; Mithöfer and Boland, 2012), with applications in medicine, agriculture and food industry (Frutuoso et al., 2013; Gutierrez et al., 2013). Some studies characterized some essential oils as antioxidants (Anthony et al., 2012; Ruberto and Baratta, 2000) and antitumoral agent (Legault et al., 2003; Legault and Pichette, 2007; Lesgards et al., 2011; Lesgards et al., 2014; Monteiro et al., 2014). On the other hand, other studies show that essential oils may have pro-oxidant effects by acting on cell membranes and organelles such as mitochondria (Forbes-Hernández et al., 2014). These effects are dependent on the concentration used and the synergism of the major compounds of the EO (Bakkali et al., 2008). They can induce cytotoxicity, genotoxicity and mutagenicity (Bakkali et al., 2008; Tisserand and Young, 2014.). Cytotoxicity appears to be related to the formation of reactive oxygen species (ROS) and induction of cell death by apoptosis and/or necrosis (Gautam et al., 2014; Jo et al., 2012).

The family Piperaceae comprises about three thousand species distributed in eight genera, of which *Piper* and *Peperomia* are the largest (Callejas, 1989, 1990; Callejas and Johnson, 1989; Meurer-Grimes, 1989; Luziatelli et al., 2010; Yuncker, 1958, 1961), with 265 and 166 described species in Brazil, respectively, which were found particularly in the Atlantic Forest (Bardelli et al., 2008; Carvalho-Okano and Alves, 1998; Cavalheiro et al., 2013; Figueiredo and Sazima, 2000; Medeiros, 2006; Sarnaglia Junior et al., 2014). The species *Piper gaudichaudianum* Kunth belongs to the Piperaceae family and is popularly known as jaborandi or iaborandi, or as murta and pariparoba (Di Stasi and Hiruma-Lima, 2002). In folk medicine, the leaves of *P. gaudichaudianum* are used to relieve toothache, and several studies have been conducted to identify analgesic, anti-inflammatory and antifungal activities (Di Stasi et al., 2002; Di Stasi and Hiruma-Lima, 2002; Péres et al., 2009).

The first published work on *P. gaudichaudianum* species deals with the analysis of the essential oil and phytochemical study of the leaves, reporting as major compounds flavonoids, triterpenes and alkaloids (Gutierrez et al., 2013; Parmar, 1997). Our group started the study on the essential oil of *P. gaudichaudianum* (EO) with Péres et al. (2006a, 2006b, 2009) by investigating the chemical composition, as well as cytotoxicity, mutagenicity and genotoxicity of the EO in Chinese hamster fibroblasts lung cells (V79 cells). Oxidative damage was probably the cause of genotoxic damage; however, we have suggested the need for further testing to validate this hypothesis. Continuing, Sperotto et al. (2013) suggested that neither the essential oil nor one of its major compounds, nerolidol, were mutagenic. The authors also claimed that the cytotoxicity induced by the EO was related to the formation of oxidative lesions, nerolidol being primarily responsible for biological effects induced by the EO.

Some of the components present in the EO, such as nerolidol, caryophyllene and α-humulene, have cytotoxic activity against tumor cells (Legault et al., 2003; Loizzo et al., 2007; Sylvestre et al., 2007). There is evidence that the lipophilic character of the essential oil may affect the structure of cell walls and membranes, particularly mitochondrial membranes, which may interfere with the cellular redox state as a result of a pro-oxidant effect. This might cause damage to lipids, proteins and DNA, inducing apoptosis and/or necrosis (Bakkali et al., 2008; Gautam et al., 2014; Lesgards et al., 2014; Ueta et al., 2008). In order to improve the understanding of the cytotoxicity and the mechanism of action of the EO in mammalian cells, our study aims to evaluate the chemical composition of the essential oil, as well as cell viability, mitochondrial function, free radicals formation, proteins involved in the oxidative stress response and cell death induction in V79 cells after treatment with *P. gaudichaudianum* essential oil.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin–ethylenediaminetetraacetic acid (EDTA), L-glutamine, antibiotics (penicillin/streptomycin) were purchased from Gibco BRL (Grand Island, NY, USA). Annexin V-Phycoerythrin (PE) and 7-Amino-Actinomycin (7-AAD) were purchased from BD Biosciences (San Diego, CA). Trypan blue, hydrogen peroxide (H_2O_2), 2',7'-dichlorofluorescein diacetate (DCFH-DA), dimethylsulfoxide (DMSO) and rhodamine 123 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-PADPR antibody and horseradish peroxidase-conjugated anti-mouse IgGs were supplied by Abcam (Cambridge, UK). Anti-SIRT1 and anti-actin specific antibodies, horseradish

peroxidase-conjugated anti-rabbit and anti-mouse IgGs were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All others reagents were analytical grade.

2.2. Sample

The leaves of *P. gaudichaudianum* were acquired from Riozinho (Rio Grande do Sul) in January, 2013. The leaves were dried outdoors in the shade. The essential oil was obtained by hydrodistillation process for 4 h, using a Clevenger type apparatus, in accordance with the method recommended by the British Pharmacopoeia Commission (2013), producing 0.5% (w/v) of essential oil. The oil was dried over anhydrous sodium sulfate and stored in a closed dark vial at 4 °C until use. The essential oil was diluted (1:100 v/v) in hexane prior to GC-FID and GC-MS.

2.3. Analysis by Gas Chromatography-Flame Ionization (GC-FID) and Gas Chromatography-Mass Spectrometry (GC-MS)

Analyses were performed with a Shimadzu GC-FID (Model G17A) and two capillary columns: OV-05 and Supelcowax 10 (60 m x 0.25 mm i.d., film thickness 0.25 μm). A Shimadzu GC-MS (Model QP5050-A) was also employed using 70 eV as the ionization energy in the electron impact mode. The carrier gas was helium with a flow rate of 1 mL/min. Initial column temperature for the OV-05 was 60 °C and it was programmed to reach 250 °C at a rate increase of 3 °C/min. For the Supelcowax the temperature program was set to start at 40 °C with a rate increase of 3 °C/min to a final temperature of 220 °C. Triplicate analyses were performed in both chromatographic systems with 1 μL of a 1% (v/v) hexanic solution of the essential oil. Compounds were identified by (I) comparison of their retention indexes (RI) relative to C9-C22 n-alkanes obtained on OV-05 columns, with those provided in the literature and (II) comparison of their mass spectra with those recorded in NIST 08 (National Institute of Standards

and Technology) and Wiley 275 libraries or with mass spectra from the literature (Adams and Dev, 2010). The percentage composition of the essential oil was computed by the normalization method from the GC peak areas, assuming identical mass response factor for all compounds. Results were calculated as mean values of two injections from essential oil, without using correction factors. All determinations were performed in triplicate and averaged.

2.4. Cell culture and treatments

V79 cells were cultured under standard conditions in DMEM supplemented with 10% heat-inactivated FBS, 0.2 mg/mL L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were kept in tissue-culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2 and harvested by treatment with 0.15% trypsin-0.08% EDTA in PBS. The EO, dissolved in DMSO, was added to FBS-free media to achieve the different designed concentrations (1, 2.5, 5 and 10 μg/mL). The final DMSO concentration in the media never exceeded 0.2%, and the negative control was exposed to an equivalent concentration of solvent. Hydrogen peroxide (H_2O_2) and doxorubicin were used as a positive controls. Cells (5 x 10^5 cells) were seeded in complete media and grown for one day prior to treatment with the essential oil. The cells were treated for 3 h and 24 h under standard conditions. After treatment, cells were analyzed with trypan blue (survival), DCFH-DA (reactive species formation), rhodamine (mitochondrial function), Annexin V-PE and 7-AAD (cell death). To measure SOD and CAT activity, 1 x 10^6 cells were grown overnight before treatment, as described below.

2.5. Trypan blue exclusion assay (TB)

Cell viability was measured by TB exclusion, as previously described by Uliasz and Hewett (2000). Briefly, after treatment cells were detached using trypsin, resuspended in PBS, stained with trypan blue, and counted using a Neubauer chamber, and then their viability was calculated. The survival was calculated as a relative percentage to the survival of the negative control (considered 100% viability value).

2.6. Assessment of cell death by flow cytometric analysis

Annexin V-PE was used in conjunction with a vital dye, 7-AAD, to distinguish apoptotic (Annexin V-PE positive, 7-AAD negative) from necrotic (Annexin V-PE positive, 7-AAD positive) cells. After treatment, cells were trypsinized, collected and resuspended in 40 µL of binding buffer with 2 µL Annexin V-PE. Cells were incubated for 15 min in the dark at room temperature. After incubation, 160 µL of binding buffer and 2 µL of 7-AAD were added. Cells were incubated for 5 min and additional 200 µL of binding buffer were added. Before analysis, cells were filtered through a cell strainer cap fitted to a polystyrene round bottom flow cytometric tube. Data were collected and analyzed by a FACS Calibur flow cytometer with CellQuest software, in a total of 10,000 events per sample; fluorescence was quantified and the percentage of viable, early apoptotic, late apoptotic and necrotic cells was determined.

2.7. Rhodamine assay to determination of mitochondrial depolarization

Transmembrane mitochondrial potential was evaluated using Rho-123 incorporation according to the method described by Marinho-Filho et al. (2010). Rho-123 is a cell-permeable, cationic, fluorescent dye that is readily sequestered by active mitochondria without inducing cytotoxic effects. After treatment cells were washed and

incubated with Rhodamine 123 for 15 min in the dark at 37 °C. After incubation, cells were washed and incubated with PBS for 30 min in the dark. Rho-123 fluorescence was measured using 488 nm excitation and 530/30 nm band pass emission filters using a FACS Calibur flow cytometer with CellQuest software. A total of 10,000 events were quantified per sample and the percentage of mitochondrial depolarization was determined.

2.8. DCFH-DA assay for the determination of ROS generation

Levels of intracellular ROS were estimated after different treatments using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as a fluorescent probe. The dye is a non-polar compound that readily penetrates into cells. Intracellular peroxides oxidize DCFH-DA to a highly fluorescent compound 2’-7’-dichlorofluorescein (DCF) (Kumar et al., 2014). After treatment cells were washed and incubated with 10 µM DCFH-DA for 30 min at 37 °C in the dark. After incubation, cells were washed with PBS and analyzed using FACS Calibur (Becton Dickinson, San Jose, CA, USA). The DCFH-oxidation was measured using 488 nm excitation and 530/30 nm band pass emission filters. As a rule, 10,000 cells were counted in each experiment. The Cell Quest software (Becton Dickinson) was used to calculate the mean fluorescence. Data were expressed as percentages of control values.

2.9. SOD and CAT activity determination

SOD activity was evaluated by quantifying the inhibition of superoxide-dependent autoxidation of epinephrine, verifying the absorbance of the samples at 480 nm (Misra and Fridovich, 1972). Briefly, to each sample aliquots were added a mixture containing 50 mM Glycine buffer pH 10.2 and 10 mM catalase. After that, epinephrine was added and the absorbance was immediately recorded each 30 s for 12 min at

480 nm in SpectraMax M^{2e} Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The inhibition of autoxidation of epinephrine occurs in the presence of SOD, whose activity can then be indirectly assayed spectrophotometrically. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of epinephrine autoxidation and the specific activity is reported as SOD Units/mg protein. CAT activity was assayed according to the method described by Aebi (1984), based on the disappearance of H_2O_2 at 240 nm. Briefly, each sample aliquots were added to 20 mM potassium phosphate buffer pH 7.2. Subsequently, 10 mM H_2O_2 were added and the absorbance was immediately recorded each 30 s for 10 min at 240 nm using SpectraMax M^{2e} Microplate Reader. One CAT unit is defined as one µmol of H_2O_2 consumed per minute and the specific activity is calculated as CAT Units/mg protein.

2.10. Effect of N-acetyl cysteine on P. gaudichaudianum *essential oil-induced V79 cell death*

To investigate the mechanism underlying the EO effect, V79 cells were initially pretreated for 2 h with NAC (2 mM), cells were then washed and treated with EO at 2.5, 5 and 10 µg/mL. Cell viability, using trypan blue assay, was then evaluated after 3 h, and compared to cells treated only with EO.

2.11. Protein expression analyses

After treatment, cells were lysed in lysis buffer [50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1% DMSO, 1 mM PMSF, plus protease inhibitors EDTA-free tablets (Roche)]. Proteins were first separated by SDS-PAGE (10%) and then transferred to a nitrocellulose membrane. Nitrocellulose sheets were then blocked by incubation with blocking buffer (3% BSA and 0.05% Tween 20 in PBS) for 2 h at room temperature. Afterwards, membranes were incubated with a monoclonal anti-PADPR

antibody (1:1000) or polyclonal anti-SIRT1 antibody (1:1000) in blocking buffer for 18 h. The monoclonal anti-actin antibody was used as a constitutive control. Assays were performed in triplicate. Secondary antibodies peroxidase-conjugated (1:3000) were then used and visualized using an ECL advanced western blotting detection kit. Photos of protein bands were taken by Molecular Imager Gel Logic 2200 (Carestream, Rochester, NY, USA).

2.12. Statistical analysis

All experiments were independently repeated at least three times, with triplicate samples for each treatment. Results are expressed as means ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using the Dunnets test, with $P < 0.05$ considered as statistically significant.

3. Results

3.1. Essential oil analysis

Composition of the essential oil of the *P. gaudichaudianum* leaves was established by GC-FID and GC-MS and 53 components in total were identified representing 93.32 % of essential oil. Table 1 shows the identified compounds and their percentages obtained by GC-FID as well as the retention times listed in order of their elution from the capillary column used.

Table 1. Composition of the *P. gaudichaudianum* essential oil.

	Compounds	%	RI[a]	Identification
1	n.i.	0,11	-	-
2	Tricyclene	0,09	935	MS, RI
3	α-Pinene	0,38	936	MS, RI
4	n.i.	0,09	940	-
5	β-Pinene	0,28	983	MS, RI
6	Myrcene	0,23	987	MS, RI
7	Linalool	0,21	1099	MS, RI
8	Δ-Elemene	0,56	1338	MS, RI
9	α-Copaene	0,41	1383	MS, RI
10	β-Bourbonene	0,09	1393	MS, RI
11	β-Elemene	0,82	1395	MS, RI
12	α-Gurjunene	0,11	1417	MS, RI
13	n.i.	0,25	1428	-
14	*(E)*-Caryophyllene	6,79	1431	MS, RI
15	Elemene gamma	0,21	1435	MS, RI
16	n.i.	0,12	1440	-
17	α-Guaiene	0,47	1443	MS, RI
18	Aromadendrene	1,90	1450	MS, RI
21	Muurola-3,5-diene trans	0,10	1456	MS, RI
22	α-humulene	14,83	1469	MS, RI
23	Allo-aromadendrene	2,48	1472	MS, RI
24	Gurjunene gamma	0,61	1483	MS, RI
25	Muurolene gamma	1,68	1486	MS, RI
26	Germacrene D	0,81	1491	MS, RI
29	Germacrene A	2,55	1501	MS, RI
30	Butylated hydroxytoluene	5,66	1506	MS, RI
31	α-Bulnesene	0,84	1511	MS, RI
32	n.i.	0,08	1513	-
33	Cadinene gamma	0,80	1522	MS, RI
34	Cadinene delta	0,90	1525	MS, RI
35	Calamenene trans	0,26	1529	MS, RI
36	n.i.	0,13	1531	-
37	Nerolidol Z	0,10	1534	MS, RI
38	Cubebol 10-epi	0,14	1539	MS, RI
39	Cadinene alpha	0,09	1545	MS, RI
40	Calacorene alpha	0,05	1551	MS, RI
41	Elemol	0,29	1556	MS, RI
42	n.i.	0,17	1559	-
43	*(E)*-Nerolidol	31,13	1566	MS, RI
44	Longipinanol	0,29	1574	MS, RI
45	n.i.	0,24	1584	-
46	Spathulenol	2,80	1590	MS, RI
47	Caryophyllene oxide	3,33	1597	MS, RI
48	Globulol	0,79	1600	MS, RI
49	viridiflorol	0,18	1609	MS, RI

50	Bisabolol-11-ol Z	0,63	1613	MS, RI
51	Eudesmol 10-epi-gamma	0,73	1620	MS, RI
52	Citronellyl pentanoate	5,55	1626	MS, RI
53	Alloaromadendrene epoxy-allo	0,31	1631	MS, RI
54	n.i.	1,40	1638	MS, RI
55	ni	0,22	1644	MS, RI
56	n.i.	1,43	1647	MS, RI
57	Caryophylla-4(12),8(13)-dien-5.alpha-ol	0,41	1651	MS, RI
58	Muurolol alpha	0,46	1652	MS, RI
59	Cadinol alpha	0,24	1654	MS, RI
60	n.i.	0,19	1654	MS, RI
61	n.i.	0,45	1667	MS, RI
62	n.i.	0,70	1667	MS, RI
63	Valerianol	0,55	1669	MS, RI
64	Bulnesol	0,71	1672	MS, RI
65	n.i.	0,71	1672	MS, RI
66	Caryophyllene 14-hydroxy-9-epi-(E)	0,41	1681	MS, RI
67	abaixo do S/N	0,00	1684	MS, RI
68	n.i.	0,10	1688	MS, RI
69	Bisabolol alpha	0,08	1693	MS, RI
70	n.i.	0,04	1697	MS, RI
71	n.i.	0,06	1704	MS, RI
72	n.i.	0,04	1742	MS, RI
73	n.i.	0,05	1754	MS, RI
74	n.i.	0,08	1824	MS, RI
	Total identified	93,32		

a = The retention index on the OV-05 column relative to C8 to C22 n-alkanes.

3.2. Cytotoxicity in V79 cells

Dose-dependent changes in the viability of EO-treated cells were evaluated based on their effects on cell growth. As previously demonstrated by our group (Péres et al., 2009), results indicated that the essential oil exerts cytotoxic effect in a dose-responsive manner in both treatment times used (3 h and 24 h) (Figure 1). The response parameter (IC_{50}) was calculated and shows similar values regardless the treatment time, with values of 4.15 µg/mL and 4.49 µg/mL after 3 h and 24 h treatments, respectively. Since the cell death profile was very similar in both times of treatment used, the investigation of the cell death mechanism induced by EO was then conducted after 3 h of exposure.

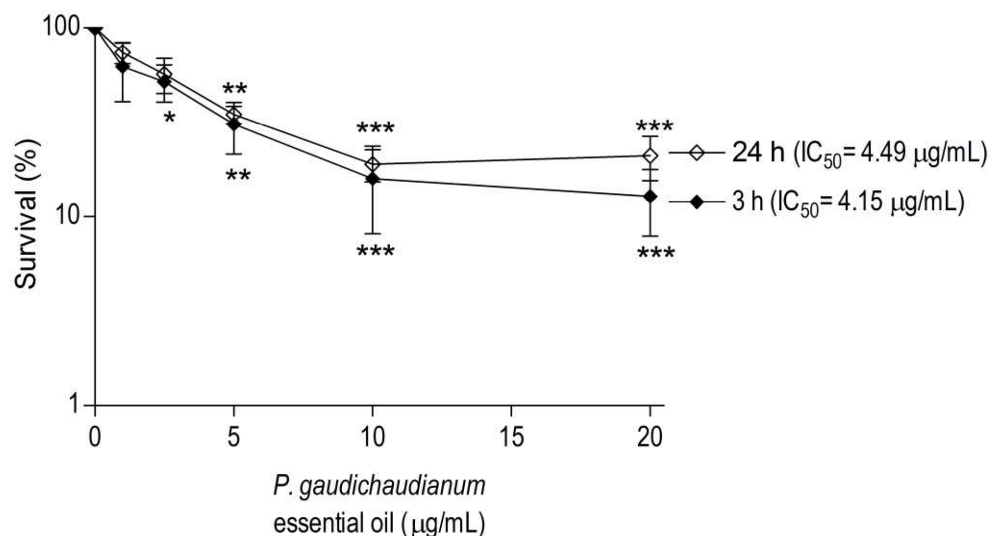

Figure 1. Cytotoxic effects of EO oil upon V79 cells, after 3 h and 24 h treatment, by trypan blue exclusion assay. Results are expressed as mean percentage in treated cells compared to control (solvent) ± standard deviation of three independent experiments. In detail the IC_{50} value for each cell line. *Significant difference as compared to negative control treatment at P<0.05; **P<0.01; ***P<0.001/One-way ANOVA/Dunnett's Multiple Comparison Test.

3.3. Cell death type determination

To verify the type of cell death induced by treatment of V79 cells with EO, flow cytometry analysis was conducted using dual staining with annexin V-PE and 7-AAD, which were used to distinguish viable, early apoptotic, late apoptotic or necrotic cells. As expected, there is a decrease in viable cells (unlabeled with either annexin-V or 7-AAD) treated with increasing concentrations of EO (Figure 2). After 3 h of treatment with EO at the highest tested concentration (10 µg/mL), approximately 24% of the V79 cells were in necrosis (labeled with 7-AAD), 25% of cells were in late apoptosis (labeled with both annexin V-PE and 7-AAD), and 12% in apoptosis (Figure 2). A similar profile of cell death was observed at 5 µg/mL of EO.

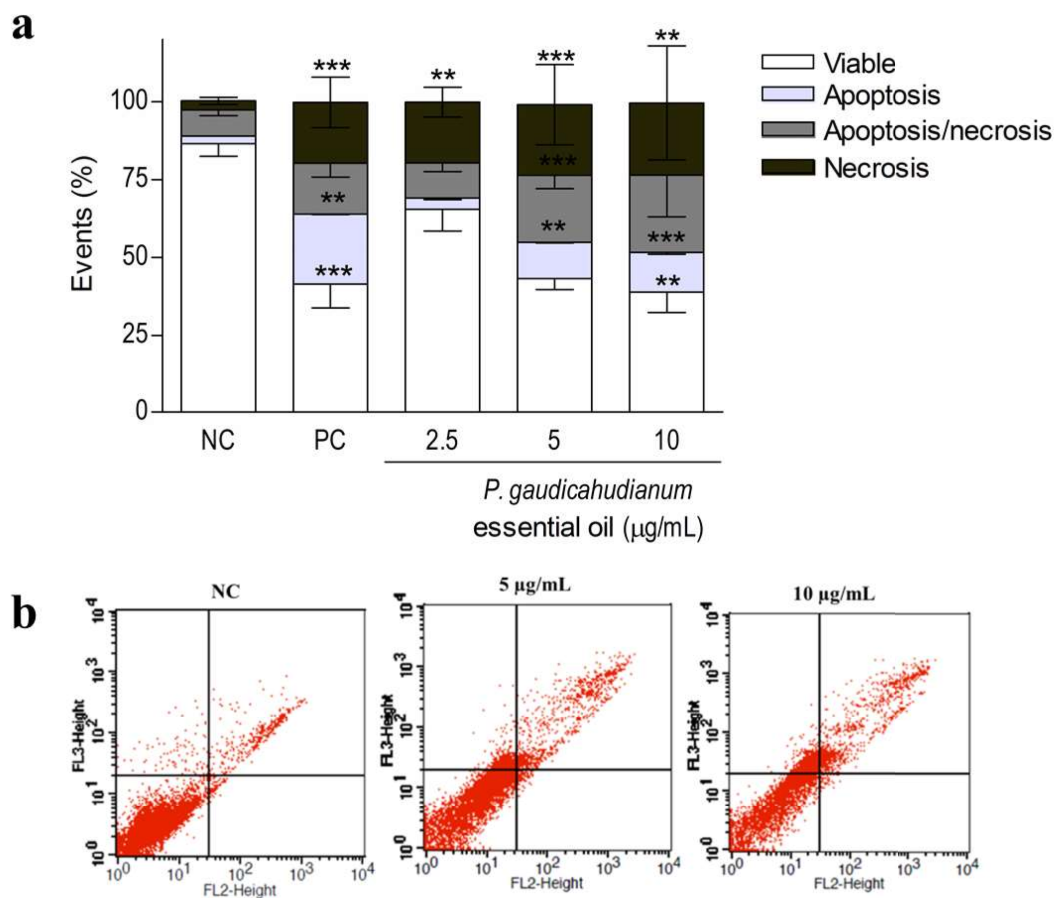

Figure 2. Cell death induction in V79 cells after *P. gaudichaudianum* essential oil treatment. Quantification of the viable cells (unlabeled with either annexin-V or 7-AAD), early apoptosis (labeled with annexin V-PE), late apoptosis (labeled with both annexin V-PE and 7-AAD) and necrosis (labeled with 7-AAD). a) Bars represent the mean ± standard deviation of three independent experiments in triplicate. b) Representative dot blot of the cell death profile and the quadrants represent, LL: Viable cells, LR: early apoptotic cells, UL: necrotic cells and UR: late apoptotic. *Significant difference as compared to negative control (NC) treatment, in each type of labeling, at $P<0.05$; **$P<0.01$; ***$P<0.001$/One-way ANOVA/Dunnett's Multiple Comparison Test. The negative control (NC) was treated with the vehicle (DMSO) used to dilute the essential oil. Doxorubicin was used as positive control (PC). The percentage of annexin-V-positive cells was determined in the whole-cell population (10,000 cells) by FACS Calibur flow cytometry and Cell Quest software.

3.4. Alteration in mitochondrial potential

To examine the role of mitochondria in the EO-induced effect on V79 cells, the effect of varying essential oil concentration was determined by rhodamine cell incorporation. Our flow cytometry results with V79 cells showed that, after treatment with EO, there is an alteration in mitochondrial function resulting in depolarization of this organelle (Figure 3).

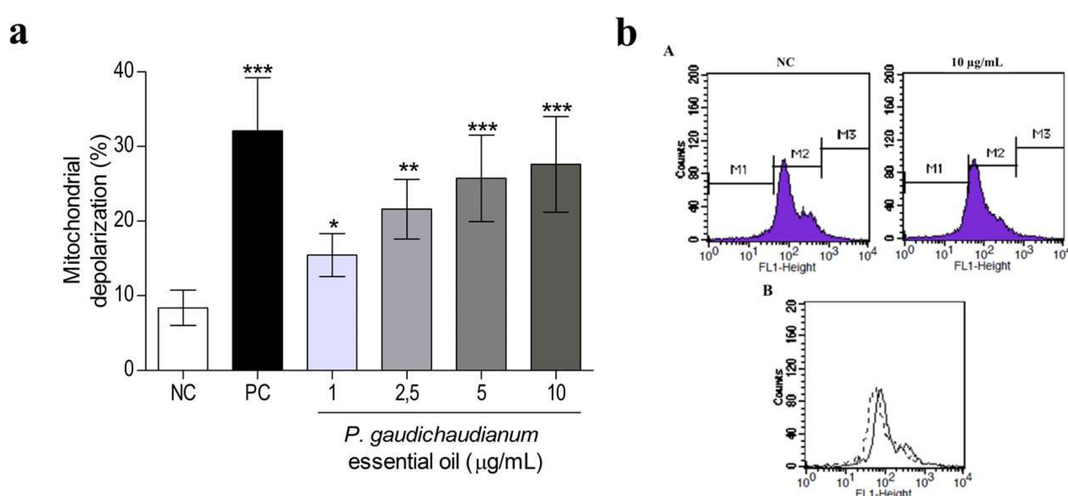

Figure 3. Evaluation of *P. gaudichaudianum* essential oil on V79 cells mitochondrial depolarization at the indicated concentrations for 3 h. a) Bars represent the mean ± standard deviation of three independent experiments in triplicate. b) Representative histogram as measured of rhodamine cell incorporation. In detail the overlay of negative control (NC) and 10 µg/mL treatments. The NC was treated with the vehicle (DMSO) used to dilute the essential oil. Hydrogen peroxide was used as positive control (PC). *Significant difference as compared to negative control treatment at $P<0.05$; **$P<0.01$; ***$P<0.001$/One-way ANOVA/Dunnett's Multiple Comparison Test.

3.5. ROS generation

In previous studies, our research group described EO as having cytotoxic activity in yeast and mammalian cells and that the mechanism of action is related to induction of reactive oxygen species generation (ROS) (Péres et al., 2009; Sperotto et

al., 2013). Continuing the study we analyzed the possible involvement of the EO in the generation of ROS using DCFH-DA assay. Our results confirm that EO caused an increase in the intracellular levels of ROS (Figure 4).

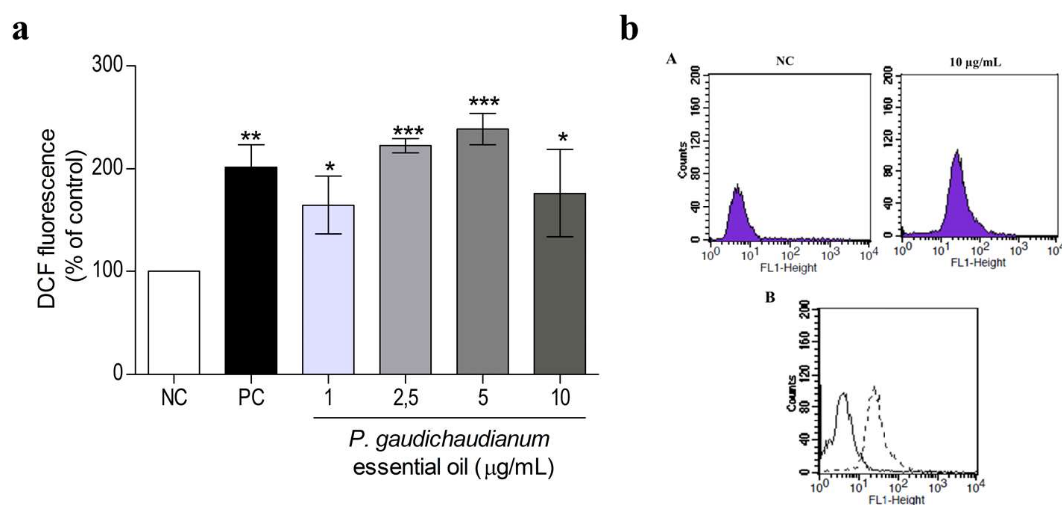

Figure 4. Evaluation of *P. gaudichaudianum* essential oil on V79 cells inducing ROS formation at the indicated concentrations for 3 h. a) Bars represent the mean ± standard deviation of three independent experiments in triplicate. b) Representative by DCF oxidation as measured of the ROS formation. In detail the percent increase of fluorescence intensity of dye fluorescent compound DCF after 10 µg/mL essential oil treatment. The negative control (NC) was treated with the vehicle (DMSO) used to dilute the essential oil. Hydrogen peroxide was used as positive control (PC). *Significant difference as compared to negative control treatment at $P<0.05$; **$P<0.01$; ***$P<0.001$/One-way ANOVA/Dunnett's Multiple Comparison Test.

3.6. *Effect of* P. gaudichaudianum *on SOD and CAT activity*

Our results showed that, in V79 cells exposed to EO, the SOD activity was significantly reduced in all concentrations tested after 3 h of treatment when compared to the unexposed cells (negative control), while no significant changes in CAT activity was observed after treatment with EO (Figure 5).

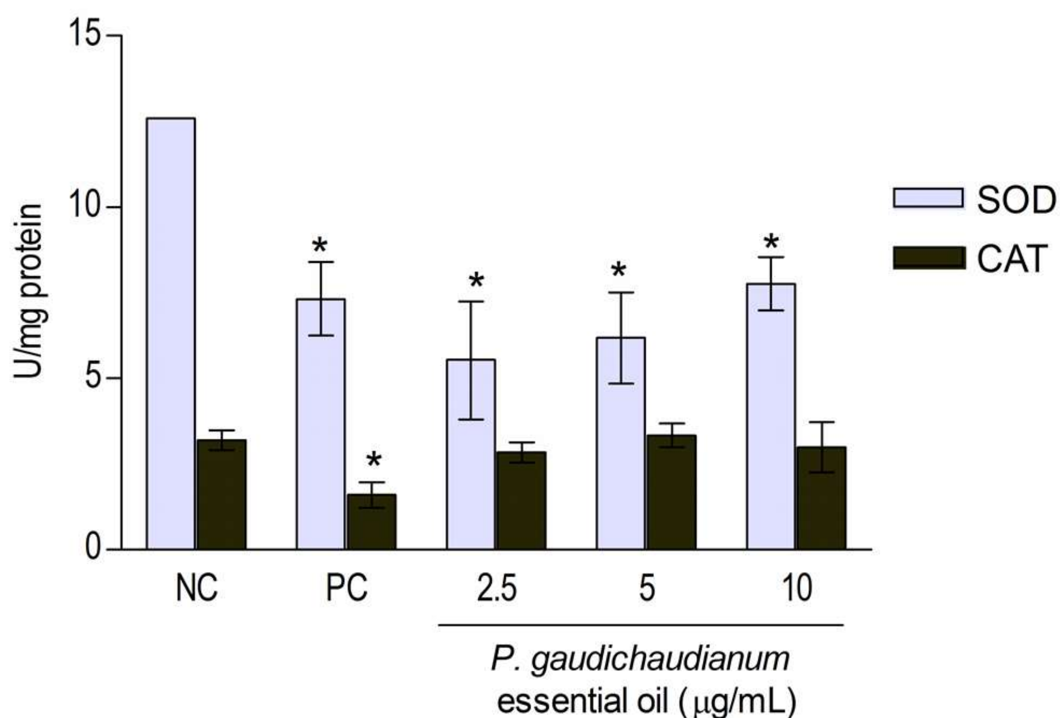

Figure 5. SOD and CAT level in V79 cells treated with EO. The negative control (NC) was treated with the vehicle (DMSO) used to dilute the essential oil. Doxorubicin was used as positive control (PC). Bars represent the mean ± standard deviation of three independent experiments in triplicate. *Significant difference as compared to negative control treatment at $P<0.05$; **$P<0.01$; ***$P<0.001$/One-way ANOVA/Dunnett's Multiple Comparison Test.

3.7. Antioxidant NAC suppresses the cytotoxic effects of P. gaudichaudianum *essential oil*

Results showed that the pretreatment with NAC (2 mM), a glutathione precursor, protected V79 cells from the cytotoxic effect of EO as estimated by trypan blue exclusion assay (Figure 6).

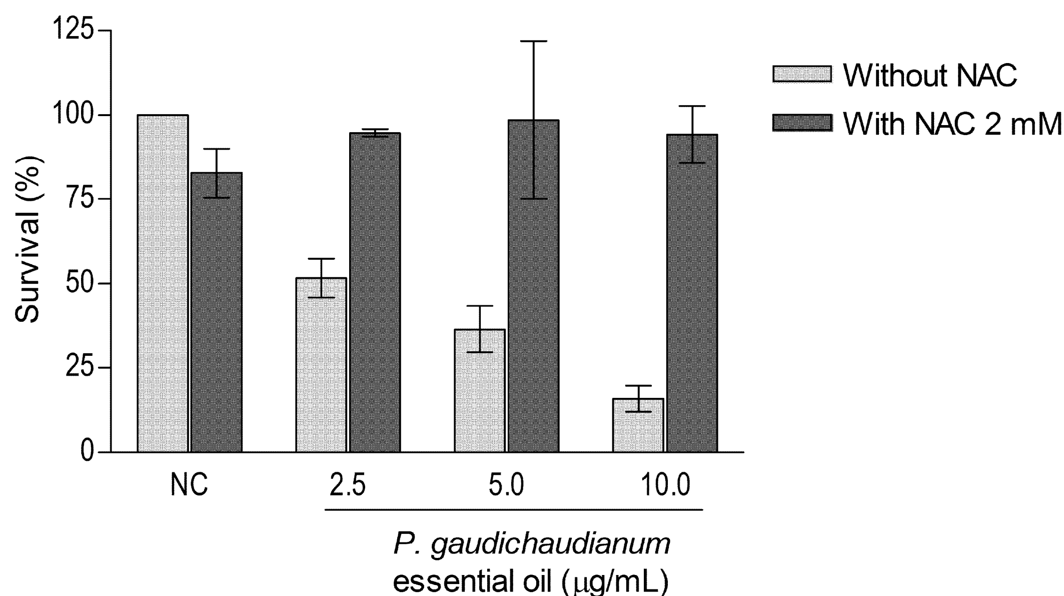

Figure 6. Effect of treatment with NAC (2 mM) for 2 h prior to EO on cell viability assessed by trypan blue exclusion assay. The negative control (NC) was treated with the vehicle (DMSO) used to dilute the essential oil. Bars represent the mean ± standard deviation of three independent experiments in triplicate. *Significant difference as compared to negative control treatment at $P<0.05$; **$P<0.01$; ***$P<0.001$/One-way ANOVA/Dunnett's Multiple Comparison Test.

3.8. P. gaudichaudianum *essential oil upregulated Sirt1 and PADPR expression*

Various enzyme systems are on duty to cope with free radical-induced damage and hence to protect against free radical-provoked damage, among them, PARPs and SIRTs play prominent roles. Therefore, we examined whether EO could affect SIRT1 and PADPR in V79 cells. As shown in Figure 7, treatment with 5 μg/mL EO for 3 h induces a slight decreased in SIRT1 expression. On the other hand, treatment with the EO induces an increase in PADPR expression (Figure 7). These results indicated that EO may exert its effects by modulating SIRT1 and PADPR expression in V79 cells.

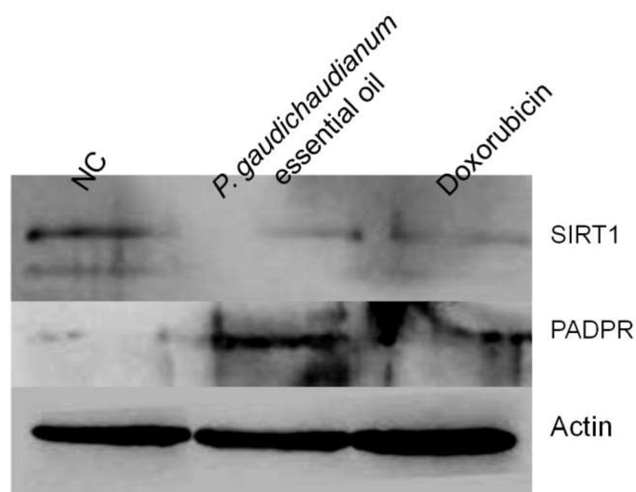

Figure 7. *P. gaudichaudianum* essential oil (EO) affected the protein levels of PADPR and SIRT1 in V79 116 cells. Cells were treated with 5 µg/mL of EO for 3 h. The total proteins were harvested and the levels of PADPR and SIRT1 were analyzed by Western Blotting. Actin was used as a loading control.

4. Discussion

The essential oil of *P. gaudichaudianum* analyzed in the present study showed similar composition of the EO described by Péres et al. (2009). The amount of components identified (93.32%) was also similar to the amounts identified by Péres et al. (2009) (92.3%) and Sperotto et al., (2013) (93.86%). However, we identified compounds that were not reported in the previous studies. We have unpublished data demonstrating that the EO is more efficient in the induction of cytotoxic activity than its isolated major compounds (*E*)-nerolidol, α-humulene and β-caryophyllene. Therefore, we conducted the further experiments on the cytotoxic mechanisms using the essential oil.

Previously, we have shown that EO presents strong cytotoxic, genotoxic and mutagenic effects in V79 cells, which can be related to its oxidative potential (Péres et al., 2009). In addition, it was shown that this essential oil induces significant cytotoxic

effects on *Saccharomyces cerevisiae* that are related to ROS generation and the formation of single-strand breaks (Sperotto et al., 2013). Now, we deepen our understanding of the oil toxicity mechanisms using V79 cells. The EO cytotoxicity was confirmed using trypan blue exclusion assay after 3 h (IC_{50} = 4.15 μg/mL) and 24 h (4.49 μg/mL) of cell exposure to the EO (Figure 1). To further investigate the means by which EO kills V79 cells, anexin V-PE/7-AAD double staining and flow cytometry analyses were performed, and showed that the cells mostly die by necrosis and late apoptosis (Figure 2). This could be the consequence of mitochondrial dysfunction and extensive DNA damage (previously demonstrated by Péres et al., 2009), which in turn leads to ATP depletion. Because apoptosis requires ATP, the necrotic pathway will be activated. The late apoptosis may be a result of cell membrane disruption; as typical lipophiles, the essential oil compounds pass through the cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids, thus permeabilize them (Bakkali et al., 2008).

Some works suggested that the essential oils, by penetrating through the cell wall and cytoplasmic membrane, disrupt and permeabilize them and especially damage mitochondrial membranes (Azmi et al., 2006; Bakkali et al., 2008; Park et al., 2011; Lesgards et al., 2014). The mitochondria, by changes in electron flow through the electron transport chain, produces free radicals which oxidize and damage lipids, proteins and DNA. Our results confirmed that mitochondrial dysfunction and oxidative stress are involved in essential oil-induced toxicity. Cells treated with EO showed significant increase in the mitochondrial depolarization (Figure 3) that probably results in ROS formation increase (Figure 4), indicating that the mitochondrial membranes are first damaged by permeabilization and this results in a prooxidant status thereafter. The

relationship between EO cytotoxicity and mitochondrial function (R^2= 0.964) or ROS production (R^2= 0.528) confirms this hypothesis.

The production of free radicals has been found in a diverse range of essential oil, which is one of the mechanisms of toxicity (Rathore et al., 2008; Ferreira et al., 2014; Sinha et al., 2014). It may result in oxidative stress, inflammation, and consequent damage to proteins, membranes and DNA (Schieber et al., 2014; Zorov et al., 2014). Thus, in our study we investigated the SOD and CAT antioxidant enzymes levels in the cells exposed to EO. Depletions in the SOD level were found after 3 h exposure, however no significant changes were observed in the CAT levels (Figure 5). The SOD decrease indicates a condition of oxidative stress in the cells, which may arise due to imbalance in the reactive oxygen species (ROS) formation and antioxidant defense system of the cells (Halliwell et al., 2012).

Interestingly, pretreatment of cells with an antioxidant prior to EO treatment exerted a protective effect. A restoration in V79 cells viability was obtained when cells were pretreated with NAC (Figure 6). Besides being used as a drug, NAC is a well-established antioxidant that acts by increasing intracellular GSH and by scavenging ROS directly (Raza et al., 2014). NAC protection against the decrease in cell viability confirms the involvement of ROS in mediating the observed induced effects.

Bioenergetic regulators such as SIRT1 and PARP1 are nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes that play a vital role in many cellular events; in particular, they act as decision makers in oxidative stress-induced cell death (Liu et al., 2009). In this work we have measured the PADPR [Poly (ADP-ribose)] level, a polymer synthesized by PARP that becomes attached to nuclear proteins, that under normal conditions has low basal level, but can dramatically increase in cells exposed to

DNA damaging agents. Our results showed a decrease in SIRT1 with concomitant increase in PADPR-1, suggesting that bioenergetic regulators have the same kind of involvement in the death induced by EO (Figure 7). Apparently, PARP and SIRT-1, under oxidative conditions, regulate the activity of each other through various mechanisms. SIRT-1 induction leads to protection against oxidative damage, while PARP-1 activation is a detrimental consequence of oxidative stress (Cantó et al., 2013). Early observations indicated that the decline of NAD^+ and the rise of NAM promoted by enhanced PARP activity correlates with a downregulation of sirtuin activity (Bai et al., 2011). PARP synthesis reflects an early cellular reaction especially to DNA single strand break, which reinforces our previous results showing that the increase in ROS, which oxidize and damage DNA, induces formation of single-strand breaks and activation of base excision repair (BER) machinery (Péres et al., 2009; Sperotto et al., 2013). Furthermore, this increase in PARP activity results in the massive synthesis of poly (ADP-ribose) (PADPR) from NAD+ and, in consequence, intracellular NAD+ and ATP are rapidly depleted (Xu et al., 2006). The lack of energy prevents the progression of the apoptotic program and turns cell death into necrosis (Leist et al., 1997; Leist et al., 1999).

In conclusion, the molecular cell death mechanism induced by EO is summarized in Figure 8. The present study revealed that EO (I) decreased the percentage of viable cells; (II) increased the mitochondrial depolarization; (III) increased the ROS production; (IV) reduced SOD activity; (V) upregulated the protein levels of PADPR and dowregulated the protein levels of SIRT1; and (VI) induced cell death by necrosis and late apoptosis. Moreover, our previous results showed the DNA damage and increased lipid peroxidation. In general terms, this work is an important contribution for understanding the *P. gaudichaudianum* essential oil cytotoxicity in

mammalian cells. Further studies are required to determine its potential use as an antimicrobial or antitumor, alone or in combination with other conventional drugs. In this sense, *P. gaudichaudianum* essential oil-activated cell death in tumoral cell lines is under intensive investigation in our lab.

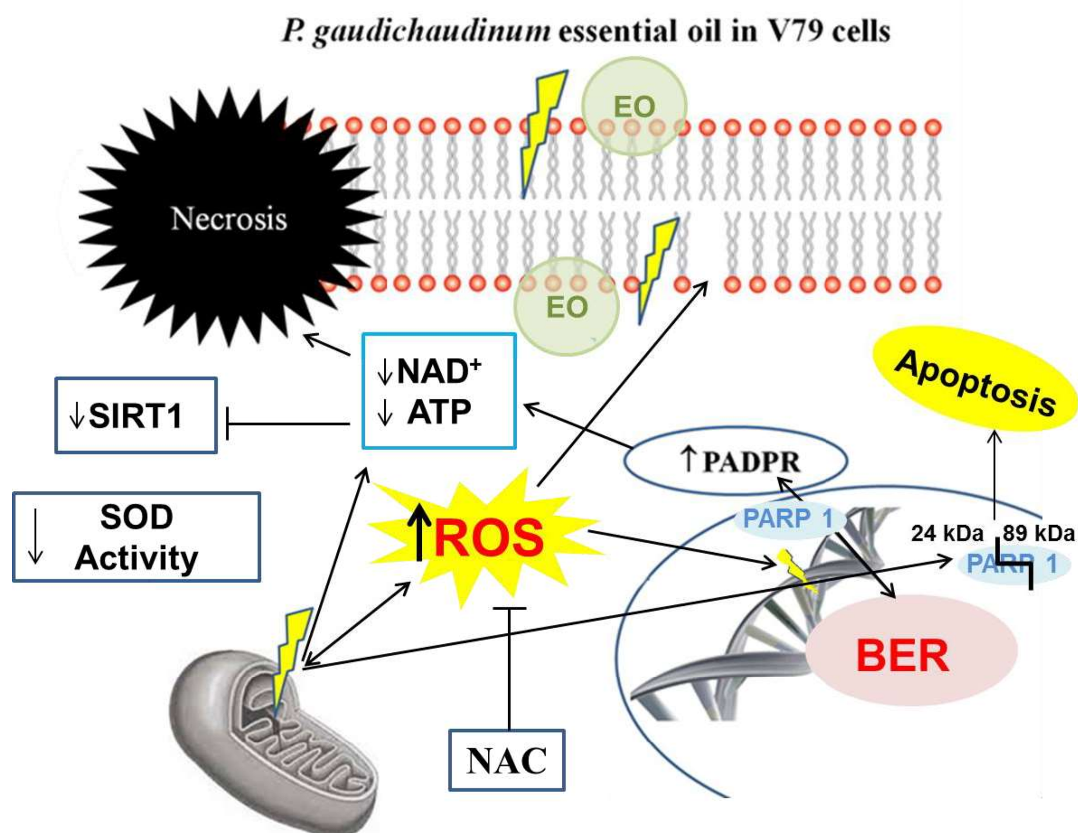

Figure 8. A proposed model for the action of *P. gaudichaudianum* essential oil (EO) on V79 cells. EO induces necrosis by decreasing in NAD+ and ATP, which results from mitochondrial dysfunction, and PARP activation as consequence of ROS increase (see text for details).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

Adams, R.P., Dev, V., 2010. Synthesis and GC–MS analysis of angelates and tiglates as an aid to identification of these components in essential oils. Flavour Fragr. J. 25, 71-74. DOI: 10.1002/ffj .1968.

Aebi, H., 1984. Catalase in vitro. Meth. Enzymol. 105, 121-126.

Anthony, K.P., Deolu-Sobogun, S.A., Saleh, M.A., 2012. Comprehensive Assessment of Antioxidant Activity of Essential Oils. J. Food Sci. 77, C839-C843. DOI: 10.1111/j.1750-3841.2012.02795.x

Azmi, A.S., Bhat, S.H., Hanif, S., Hadi, S.M., 2006. Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: A putative mechanism for anticancer properties. FEBS Lett. 580, 533-538. DOI: 10.1016/j.febslet.2005.12.059

Bai, P., Canto, C., Oudart, H., Brunyánszki, A., Cen, Y., Thomas, C., et al., 2011. PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. Cell Metab. 13, 461-468. DOI: 10.1016/j.cmet.2011.03.004

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils – A review. Food Chem. Toxicol. 46, 446-475. DOI: 10.1016/j.fct.2007.09.106

Bardelli, K.C., Kirizawa, M., Sousa, A.V.G., 2008. O gênero Piper L. (Piperaceae) da Mata Atlântica da Microbacia do Sítio Cabuçu-Proguaru, Guarulhos, SP, Brasil. Hoehnea. 35, 553-561.

Başer, D.H.C., Buchbauer, G., 2010. Handbook of essential oils: science, technology, and applications, CRC Press, New York.

Boire, N.A., Riedel, S., Parrish, N.M., 2013. Essential Oils and Future Antibiotics: New Weapons against Emerging 'Superbugs'? J. Anc. Dis. Prev. Rem. 1, 1-5. DOI: 10.4172/2329-8731.1000105

British Pharmacopoeia Commission, 2013. British Pharmacopoeia 2013. Stationery Office, London.

Callejas, R., 1989. Studies in neotropical piperaceae I. a new species of Piper from Ecuador. Brittonia. 41, 325-327.

Callejas, R., 1990. Studies in neotropical piperaceae. II. Four new species and a new combination from Colombia and Peru. Brittonia. 42, 70-82.

Callejas, R., Jonhson, D.M., 1989. Piperaceae types from the t. G. Yuncker Herbarium (dpu) now filed in the New York Botanical Garden Herbarium (NY). Brittonia. 41, 297-324.

Cantó, C., Sauve, A.A., Bai, P., 2013. Crosstalk between poly(ADP-ribose) polymerase and sirtuin enzymes. Mol. Aspects Med. 34, 1-62. DOI: 10.1016/j.mam.2013.01.004

Carvalho-Okano, R.M., Alves, S.A.M., 1998. Piperaceae C. Agardh da Estação Experimental Mata do Paraíso, Viçosa, MG. Acta bot. bras. 12, 497-513.

Cavalheiro, C.N., Schneider, F., Grillo, H.C.Z., Corrêa, N.R., Almeida, R.V.A., Spinelli, R., et al., 2013. Distribuição de *Piper Gaudichaudianum* Kuntze

(Piperaceae) e Efeito de Borda em Fragmento Florestal do Jardim Botânico de Lajeado (JBL), Rio Grande Do Sul. Rev. Dest. Acad. 5, 131-139.

Chen, X., Lu, R., Dan, H., Liao, G., Zhou, M., Li, X.Y., et al., 2011. Honokiol: a promising small molecular weight natural agent for the growth inhibition of oral squamous cell carcinoma cells. Int. J. Oral Sci. 3, 34-42. DOI: 10.4248/IJOS11014

De Morais, S.M., Facundo, V.A., Bertini, L.M., Cavalcanti, E.S.B., Dos Anjos Júnior, J.F., Ferreira, S.A. et al., 2007. Chemical composition and larvicidal activity of essential oils from Piper species. Biochem. Syst. Ecol. 35, 670-675. DOI: 10.1016/j.bse.2007.05.002

Di Stasi, L.C., Hiruma-Lima, C.A., 2002. Plantas medicinais na Amazônia e na Mata Atlântica, second ed. UNESP, São Paulo.

Di Stasi, L.C., Oliveira, G.P., Carvalhaes, M.A., Queiroz-Juniora, M., Tien, O.S., Kakinami, S.H. et al., 2002. Medicinal plants popularly used in the Brazilian Tropical Atlantic Forest. Fitoterapia. 73, 69-91.

Dias, C.N., Moraes, D.F.C., 2014. Essential oils and their compounds as Aedes aegypti L. (Diptera: Culicidae) larvicides: review. Parasitol. Res. 113, 565-592. DOI: 10.1007/s00436-013-3687-6

Ferhat, M.A., Meklati, B.Y., Smadja, J., Chemat, F., 2005. An improved microwave Clevenger apparatus for distillation of essential oils from orange peel. J. Chromatogr. A. 1112, 121-126. DOI: 10.1016/j.chroma.2005.12.030

Fernandes, E.S., Passos, G.F., Medeiros, R., Da Cunha, F.M., Ferreira, J., Campos, M.M. et al., 2007. Anti-inflammatory effects of compounds alpha-humulene and (−)-trans-caryophyllene isolated from the essential oil of Cordia verbenacea. Eur. J. Pharmacol. 569, 228-236. DOI: 10.1016/j.ejphar.2007.04.059

Ferraz, A.B.F., Balbino, J.M., Zini, C.A., Ribeiro, V.L.S., Bordignon, S.A.L., Von Poser, G., 2010. Acaricidal activity and chemical composition of the essential oil from three Piper species. Parasitol. Res. 107, 243-248. DOI: 10.1007/s00436-010-1878-y

Ferreira, P., Cardoso, T., Ferreira, F., Fernandes-Ferreira, M., Piper, P., Sousa, M.J., 2014. *Mentha piperita* essential oil induces apoptosis in yeast associated with both cytosolic and mitochondrial ROS-mediated damage. FEMS Yeast Res. 14, 1006-1014. DOI: 10.1111/1567-1364.12189

Figueiredo, R.A., Sazima, M., 2000. Pollination Biology of Piperaceae Species in Southeastern Brazil. Ann. Bot. 85, 455-460. DOI: 10.1006/anbo.1999.1087

Forbes-Hernández, T.Y., Giampieri, F., Gasparrini, M., Mazzoni, L., Quiles, J.L., Alvarez-Suarez, J.M. et al., 2014. The effects of bioactive compounds from plant foods on mitochondrial function: A focus on apoptotic mechanisms. Food Chem. Toxicol. 68, 154-182. DOI: 10.1016/j.fct.2014.03.017

Frutuoso, A.E., Nascimento, N.T., De Lemos, T.L.G., Coelho, E.L., Teixeira, D.M.A., 2013. Óleos Essenciais Aplicados em Alimentos: Uma Revisão. REBRAPA. 4, 69-81. DOI: 10.14685/rebrapa.v4i2.134

Gautam, N., Mantha, A.K., Mittal, S., 2014. Essential Oils and Their Constituents as Anticancer Agents: A Mechanistic View. Biomed. Res. Int. 1-23. DOI: 10.1155/2014/154106

Gurib-Fakim, A., 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Mol. Aspects Med. 27, 1-93. DOI: 10.1016/j.mam.2005.07.008

Gutierrez, R.M.P., Gonzalez, A.M.N., Hoyo-Vadillo, C., 2013. Alkaloids from *Piper*: A Review of its Phytochemistry and Pharmacology. Mini Rev. Med. Chem. 13, 163-193. DOI: 1875-5607/13

Halliwel, L. B., 2012. Free radicals and antioxidants: updating a personal view. Nutr. Rev. 70, 257-265. DOI: 10.1111/j.1753-4887.2012.00476.x

Jo, J., Park, J.S., Park, Y.K., Chae, Y.Z., Lee, G.H., Park, G.Y. et al., 2012. *Pinus densiflora* leaf essential oil induces apoptosis via ROS generation and activation of caspases in YD-8 human oral cancer cells. Int. J. Oncol. 40, 1238-1245. DOI: 10.3892/ijo.2011.1263

Kumar, D., Kumari, J., Pakrashi, S., Dalai, S., Raichur, A.M., Sastry, T.P. et al., 2014. Qualitative toxicity assessment of AgNPs on the fresh water bacterial isolates and consortium at low level of exposure concentration. Ecotoxicol. Environ. Saf. 108, 152-160. DOI: 10.1016/j.ecoenv.2014.05.033

Legault, J., Dahl, W., Debiton, E., Pichette, A., Madelmont, J., 2003. Antitumor Activity of Balsam Fir Oil: Production of Reactive Oxygen Species Induced by α-Humulene as Possible Mechanism of Action. Planta Med. 69, 402-407. DOI: 10.1055/s-2003-39695

Legault, J., Pichette, A., 2007. Potentiating effect of β-caryophyllene on anticancer activity of α-humulene, isocaryophyllene and paclitaxel. JPP. 59, 1643-1647. DOI: 10.1211/jpp.59.12.0005

Leist, M., Single, B., Castoldi, A.F., Kühnle, S., Nicotera, P., 1997. Intracellular Adenosine Triphosphate (ATP) Concentration: A Switch in the Decision Between Apoptosis and Necrosis. J. Exp. Med. 185, 1481-1486. DOI: 0022-1007/97/04/1481/06

Leist, M., Single, B., Naumann, H., Fava, E., Simon, B., Kühnle, S., Nicotera, P., 1999. Inhibition of Mitochondrial ATP Generation by Nitric Oxide Switches Apoptosis to Necrosis. Exp. Cell Res. 249, 396-403.

Lesgards, J.F., Gauthierc, C., Iovannac, J., Vidala, N., Dollab, A., Stockera, P., 2011. Effect of reactive oxygen and carbonyl species on crucial cellular antioxidant enzymes. Chem. Biol. Interact. 190, 28-34. DOI: 10.1016/j.cbi.2010.12.028

Lesgards, J.F., Baldovini, N., Vidal, N., Pietri, S., 2014. Anticancer Activities of Essential Oils Constituents and Synergy with Conventional Therapies: A Review. Phytother. Res. DOI: 10.1002/ptr.5165

Liu, D., Gharavi, R., Pitta, M., Gleichmann, M., Mattson, M.P., 2009. Nicotinamide Prevents NAD+ Depletion and Protects Neurons Against Excitotoxicity and Cerebral Ischemia: NAD+ Consumption by SIRT1 may Endanger Energetically Compromised Neurons. Neuromolecular Med. 11, 28-42. DOI: 10.1007/s12017-009-8058-1

Loizzo, M.R., Tundis, R., Menichini, F., Saab, A.M., Statti, G.A., Menichini, F., 2007. Cytotoxic Activity of Essential Oils from Labiatae and Lauraceae Families Against *In Vitro* Human Tumor Models. Anticancer Res. 27, 3293-3300.

Luziatelli, G., Sorensen, M., Theilade, I., Molgaard, P., 2010. Asháninka medicinal plants: a case study from the native community of Bajo Quimiriki, Junín, Peru. J. Ethnobiol. Ethnomed. 6, 1-23. DOI: 10.1186/1746-4269-6-21

Marinho-Filho, J.D.B., Bezerra, D.P., Araújo, A.J., Montenegro, R.C., Pessoa, C., Diniz, J.C. et al., 2010. Oxidative stress induction by (+)-cordiaquinone J triggers both mitochondria-dependent apoptosis and necrosis in leukemia cells. Chem. Biol. Interac. 183, 369-379. DOI: 10.1016/j.cbi.2009.11.030

Marques, A.M., Barreto, A.L.S., Curvelo, J.A.R., Romanos, M.T.V., Soares, R.M.A., Kaplan, M.A.C., 2011. Antileishmanial activity of nerolidol-rich essential oil from *Piper claussenianum*. Rev. Bras. Farmacogn. 21, 908-914. DOI: 10.1590/S0102-695X2011005000157

Medeiros, E.S.S., 2006. Flora do Parque Estadual do Ibitipoca, MinasGerais, Brasil - Família Piperaceae [dissertação]. Rio de Janeiro (RJ): Instituto de Pesquisas Jardim Botânico do Rio de Janeiro/Escola Nacional de Botânica Tropical Rio de Janeiro.

Meurer-Grimes, B., 1989. Index to collectors of the piperaceae represented in the T. G. Yuncker Herbarium of Depauw University. Brittonia. 41, 285-96.

Misra, H.P., Fridovich, I., 1972. The Role of Superoxide Anion in the Autoxidation of Epinephrine and a Simple Assay for Superoxide Dismutase. J. Biol. Chem. 247, 3170-3175.

Mithöfer, A., Boland, W., 2012. Plant Defense Against Herbivores: Chemical Aspects. Annu. Rev. Plant. Biol. 63, 431-450. DOI: 1543-5008/12/0602-0431

Monteiro, L.S., Bastos, K.X., Barbosa-Filho, J.M., Athayde-Filho, P.F., Diniz, M.F.F.M., Sobral, M.V., 2014. Medicinal Plants and Other Living Organisms with Antitumor Potential against Lung Cancer. eCAM. 1-15. DOI: 10.1155/2014/604152

Park, K.R., Nam, D., Yun, H.M., Lee, S.G., Jang, H.J., Sethi, G. et al., 2011. β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. Cancer Lett. 312, 178-188. DOI: 10.1016/j.canlet.2011.08.001

Parmar, V.S., Jain, S.C., Bisht, K.S., Jain, R., Taneja, P., Jha, A., et al., 1997. Phytochemistry of the Genus *Piper*. Phytochemistry. 46, 591-673.

Péres, V.F., Saffi, J., Melecchi, M.I.S., Abad, F.C., Jacques, R.A., Martinez, M.M. et al., 2006a. Comparison of soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acids and Vitamin E from *Piper gaudichaudianum* Kunth. J. Chromatogr. A. 1105, 115-118. DOI: 10.1016/j.chroma.2005.07.113

Péres, V.F., Saffi, J., Melecchi, M.I.S., Abad, F.C., Martinez, M.M., Oliveira, E.C. et al., 2006b. Optimization of pressurized liquid extraction of *Piper gaudichaudianum* Kunth leaves. J. Chromatogr. A. 1105, 148-153. DOI: 10.1016/j.chroma.2005.08.036

Péres, V.F., Moura, D.J., Sperotto, A.R.M., Damasceno, F.C., Caramão, E.B., Zini, C.A. et al., 2009. Chemical composition and cytotoxic, mutagenic and genotoxic activities of the essential oil from *Piper gaudichaudianum* Kunth leaves. Food Chem. Toxicol. 47, 2389-2395. DOI: 10.1016/j.fct.2009.06.035

Rai, M.P., Thilakchand, K.R., Palatty, P.L., Rao, P., Rao, S., Bhat, H.P. et al., 2011. Piper Betel Linn (Betel Vine), the Maligned Southeast Asian Medicinal Plant Possesses Cancer Preventive Effects: Time to Reconsider the Wronged Opinion. Asian Pac. J. Cancer Prev. 12, 2149-2156.

Rathore, P., Dohare, P., Varma, S., Ray, A., Sharma, U., Jaganathanan, N.R. et al., 2008. Curcuma Oil: Reduces Early Accumulation of Oxidative Product and is Anti-apoptogenic in Transient Focal Ischemia in Rat Brain. Neurochem. Res. 33, 1672-1682. DOI: 10.1007/s11064-007-9515-6

Raza, H., John, A., Shafarin, J., 2014. NAC Attenuates LPS-Induced Toxicity in Aspirin Sensitized Mouse Macrophages via Suppression of Oxidative Stress and Mitochondrial Dysfunction. PLOS ONE. 9, 1-10.

Ruberto, G., Baratta, M.T., 2000. Antioxidant activity of selected essential oil components in two lipid model systems. Food Chem. 69, 167-174. DOI: 10.1016/S0308-8146(99)00247-2

Sarnaglia Junior, V.B., Bermudez, G.M.M., Guimarães, E.F., 2014. Diversidade de Piperaceae em um remanescente de Floresta Atlântica na região serrana do

Espírito Santo, Brasil. Biotemas. 27, 49-57. DOI: 10.5007/2175-7925.2014v27n1p49

Schieber, M., Chandel, N.S., 2014. ROS Function in Redox Signaling and Oxidative Stress. Curr. Biol. 24, R453-R462. DOI: 10.1016/j.cub.2014.03.034

Schmit, A.P., Riffel, C., 2010. Avaliação da atividade antifúngica de diferentes espécies do gênero Piper [monografia]. Itajaí (SC): Universidade do Vale do Itajaí.

Shu, Y.Z., 1998. Recent Natural Products Based Drug Development: A Pharmaceutical Industry Perspective. J. Nat. Prod. 61, 1053-1071. DOI: S0163-3864(98)00010-X

Sinha, S., Jothiramajayam, M., Ghosh, M., Mukherjee, A., 2014. Evaluation of toxicity of essential oils palmarosa, citronella, lemongrass and vetiver in human lymphocytes. Food Chem. Toxicol. 68, 71-77. DOI: 10.1016/j.fct.2014.02.036

Smelcerovic, A., Djordjevic, A., Lazarevic, J., Stojanovic, G., 2013. Recent Advances in Analysis of Essential Oils. Curr. Anal Chem. 9, 61-70.

Sperotto, A.R.M., Moura, D.J., Péres, V.F., Damasceno, F.C., Caramão, E.B., Henriques, J.A.P., et al., 2013. Cytotoxic mechanism of *Piper gaudichaudianum* Kunth essential oil and its major compound nerolidol. Food Chem. Toxicol. 57, 57-68. DOI: 10.1016/j.fct.2013.03.013

Sylvestre, M., Pichette, A., Lavoie, S., Longtin, A., Legault, J., 2007. Composition and Cytotoxic Activity of the Leaf Essential Oil of *Comptonia peregrina* (L.) Coulter Phytother. Res. 21, 536-540. DOI: 10.1002/ptr.2095

Tisserand, R., Young, R., 2014. Essential Oil Safety. A Guide for Health Care Professionals. second ed. Elsevier, New York.

Ueta, E., Sasabe, E., Yang, Z., Osaki, T., Yamamoto, T., 2008. Enhancement of apoptotic damage of squamous cell carcinoma cells by inhibition of the

mitochondrial DNA repairing system. Cancer Sci. 99, 2230-2237. DOI: 10.1111/j.1349-7006.2008.00918.x

Uliasz, T.F., Hewett, S.J., 2000. A microtiter trypan blue absorbance assay for the quantitative determination of excitotoxic neuronal injury in cell culture. J. Neurosci. Methods. 100, 157-163.

Xu, Y., Huang, S., Liu, Z., Han, J., 2006. Poly (ADP-ribose) Polymerase-1 Signaling to Mitochondria in Necrotic Cell Death Requires RIP1/TRAF2-mediated JNK1 Activation. J. Biol. Chem. 281, 8788-8795. DOI: 10.1074/jbc.M508135200

Yuncker, T.G., 1958. The Piperaceae-a family profile. Brittonia. 10, 1-7.

Yuncker, T.G., 1961. New taxa of peruvian Piperaceae. Brittonia. 13, 58-63.

Zorov, D.B., Juhaszova, M., Sollott, S.J., 2014. Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. Physiol. Rev. 94, 909-950. DOI: 10.1152/physrev.00026.2013

4. Conclusões e Considerações Finais

O estudo sobre a análise química de *Piper gaudichaudianum* Kunth teve início no nosso grupo de pesquisa em 2005, gerando a publicação dos artigos "Comparison of soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acids and Vitamin E from *Piper gaudichaudianum* Kunth" (Péres et al., 2006a) e "Optimization of pressurized liquid extraction of *Piper gaudichaudianum* Kunth leaves" (Péres et al., 2006b). Em 2009, foi realizada a análise citotóxica, genotóxica e mutagênica deste espécime vegetal, com a publicação do artigo "Chemical composition and cytotoxic, mutagenic and genotoxic activities of the essential oil from *Piper gaudichaudianum* Kunth leaves" (Péres et al., 2009). Em 2013, o grupo avaliou as propriedades dos compostos majoritários contidos no óleo essencial de *P. gaudichaudianum* com a realização do trabalho intitulado "Cytotoxic mechanism of *Piper gaudichaudianum* Kunth essential oil and its major compound nerolidol" (Sperotto et al., 2013).

Neste trabalho, o óleo essencial das folhas de *P. gaudichaudianum* foi extraído por hidrodestilação e 93.32% dos seus componentes foram identificados por GC-FID e GC-MS. Os componentes majoritários encontrados foram (E)-nerolidol (31,13%), α-humuleno (14,83%) e (E)-cariofileno (6,79%). Portanto, observa-se que a composição deste óleo manteve-se estável durante os diferentes períodos em que foi realizada a sua análise química, mostrando o predomínio de sesquiterpenos.

O tratamento com o óleo essencial, durante os períodos de 3h e de 24h, diminuiu a viabilidade celular, com IC50 de 4,15 µg/mL e 4,49 µg/mL,

respectivamente. O pré-tratamento com N-acetilcisteína restabeleceu a viabilidade celular, mostrando efeito protetor contra a citotoxicidade induzida pelo óleo essencial de *P. gaudichaudianum*. Observou-se um aumento da despolarização mitocondrial, da produção de espécies reativas e a redução da atividade da superóxido dismutase. Verificou-se também o aumento da expressão de PADPR e a diminuição da expressão de SIRT1. O tratamento com o óleo essencial de *P. gaudichaudianum* induziu a morte celular por necrose e apoptose tardia, exercendo efeito citotóxico, concentração-dependente, pela indução de estresse oxidativo celular com envolvimento de dano mitocondrial e modulação da resposta antioxidante e de reparo no DNA.

O conjunto destes resultados, somados aos trabalhos anteriores, fornece uma contribuição importante para a compreensão da citotoxicidade do óleo essencial de *P. gaudichaudianum*. Nosso objetivo principal é contribuir na identificação de novos princípios ativos celulares e no desenvolvimento de novas drogas com potencial antimicrobiano e antitumoral, com efetividade superior às existentes, ou que potencialize o efeito de outras drogas atuais, promovendo uma menor toxicidade para o paciente e contribuindo para uma melhora na sua qualidade de vida.

5. Perspectivas

Os resultados obtidos neste estudo revelam o mecanismo de citotoxicidade do óleo essencial de *P. gaudichaudianum* em células de mamífero, com envolvimento de danos na mitocôndria e no DNA, com indução de estresse oxidativo e de morte celular por apoptose e/ou necrose, bem como a modulação da atividade de proteínas dos sistemas antioxidante e de reparo do DNA. Estudos adicionais são necessários para identificar a capacidade antimicrobiana ou antitumoral do óleo essencial de *P. gaudichaudianum* e de seus componentes majoritários. Para tal, podem ser realizados ensaios como a quantificação dos níveis de ATP, ensaios de viabilidade combinando diferentes concentrações do óleo essencial ou de seus componentes majoritários com antimicrobianos e quimioterápicos conhecidos, além de novas abordagens em células tumorais.

6. Anexos

6.1. Normas para publicação da revista *Food and Chemical Toxicology*

FOOD AND CHEMICAL TOXICOLOGY

AUTHOR INFORMATION PACK

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