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ATIVIDADE ANTIPROLIFERATIVA DE EXTRATO DE *ARAUCARIA ANGUSTIFOLIA* EM CÉLULAS TUMORAIS DE LARINGE HEp-2

CÁTIA DOS SANTOS BRANCO

Caxias do Sul

2016

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Atividade antiproliferativa de extrato de *Araucaria angustifolia* em células tumorais de laringe HEp-2

Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, visando à obtenção do grau de Doutora em Biotecnologia.

Orientadora: Profª. Drª. Mirian Salvador

Co-orientadora: Profª. Drª. Valeria Weiss Angeli

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TESE APROVADA EM 02 DE DEZEMBRO DE 2016.

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LISTA DE ABREVIATURAS

C I	Complexo I
C II	Complexo II
C III	Complexo III
C IV	Complexo IV
C V	Complexo V
CAT	Catalase
CTE	Cadeia de transporte de elétrons
DNA	Ácido desoxirribonucléico
DNMT	DNA metiltransferases
DNPH	Dinitrofenilhidrazina
DTNB	5,5´-ditiobis- ácido 2-nitrobenzóico
EAA	Extrato de *Araucaria angustifolia*
ERO	Espécies reativas de oxigênio
HIF-1α	Fator indutor de hipóxia-1α
IARC	Agência Internacional de Pesquisa em Câncer
INCA	Instituto Nacional do Câncer
LPO	Lipoperoxidação
mtDNA	DNA mitocondrial
NE	Nanoesferas
NO	Nitric oxide
NP	Nanopartículas

NPCL	Nanoesferas de policaprolactona / nanospheres of polycaprolactone
ON	Óxido nítrico
8-OHdG	8-hidroxi-2-deoxiguanosina
PDH	Piruvato desidrogenase
PDK1	Piruvato desidrogenase kinase 1
PKM2	Isoenzima piruvato kinase M2
ROS	Reactive oxygen species
SIR 3	Sirtuína 3
SOD	Superóxido dismutase
TBARS	Produtos reativos ao ácido tiobarbitúrico
TET	Proteínas de translocação (*ten- eleven- translocation*)
WHO	Organização Mundial da Saúde

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ESTRUTURA DA TESE

A presente tese está estruturada da seguinte forma: introdução geral, objetivos do trabalho (geral e específicos), capítulos e discussão geral. As conclusões obtidas são apresentadas, seguida das perspectivas, referências bibliográficas e os anexos.

A introdução da tese apresenta generalidades sobre a planta *A. angustifolia*, suas caratrerísticas e propriedades biológicas, identificação de compostos químicos com propriedades antitumorais, aspectos sobre o câncer e tecnologias inovadoras de encapsulação de ativos para o tratamento do câncer.

O Capítulo I apresenta o trabalho publicado na revista "Journal of Organic and Inorganic Chemistry", o qual abordou uma revisão completa da espécie *A. angustifolia* e suas atividades biológicas, bem como seus constituintes químicos e as técnicas utilizadas para a identificação das principais classes de compostos presentes em diferentes partes da planta. Este trabalho contribuiu significativamente para o conhecimento da planta escolhida e seu potencial terapêutico.

O Capítulo II se refere ao artigo publicado na revista "Chemico-Biological Interactions", que abordou o efeito antitumoral seletivo e apoptótico do EAA e seus principais alvos moleculares.

O Capítulo III apresenta um artigo no formato *highlight* publicado à convite na revista "Cancer Cell & Microenvironment", abordando os aspectos antitumorais seletivos do extrato evidenciados no capítulo anterior, incluindo as principais hipóteses para corroborar os achados experimentais iniciais.

No Capítulo IV encontra-se o recente trabalho realizado no Canadá durante o período sanduíche, o qual será submetido na revista "British Journal of Cancer (Nature)",

abordando os mecanismos de ação antitumoral do extrato em nível mitocondrial e epigenético.

O Capítulo V, por fim, aborda o artigo a ser submetido na revista "Química Nova", contemplando a preparação e caracterização de um sistema nanovetorizado e a análise deste sistema em células humanas *in vitro* a fim de conhecer sua possível toxicidade visando a incorporação do EAA para a potencialização do seu efeito antitumoral.

Os anexos I e II contém publicações desenvolvidas durante o doutorado, que não estão diretamente relacionadas ao presente trabalho, mas que foram utilizadas na discussão deste. O Anexo I contém o artigo científico intitulado "*Araucaria angustifolia* (Bert.) O. Kuntze induces oxidative and genotoxic damage in *Anticarsia gemmatalis* larvae Hübner (Lepidoptera: Erebidae)", publicado na revista "International Journal of Pest Management", no qual foram avaliados os efeitos pró-oxidativos e genotóxicos do EAA em insetos em estágio larval. O Anexo II compreende o artigo publicado na revista "Cogent Food and Agriculture", intitulado "Redox imbalance mediates entomotoxic effects of the conifer *Araucaria angustifolia* in *Anticarsia gemmatalis* velvetbean caterpillar" e propôs aprofundar os mecanismos de ação do EAA sobre o metabolismo redox dos insetos.

A discussão geral aborda os resultados dos capítulos apresentados, a relação entre os mesmos e a importância desse estudo como contribuição científica. Finalmente é apresentada a conclusão final do trabalho desenvolvido e as perspectivas.

RESUMO

Os produtos naturais constituem uma das fontes mais promissoras para o descobrimento de novos ativos na terapêutica do câncer. O carcinoma de laringe é um dos mais comuns tipos de câncer envolvendo as áreas de cabeça e pescoço, e apresenta elevada taxa de morbidade e mortalidade em pacientes com estágio avançado. Terapias alternativas e/ou adjuvantes para o tratamento deste tipo de câncer representam uma necessidade emergente. Uma das alternativas mais promissoras é o desenvolvimento de nanocarreadores contendo ativos antitumorais. *Araucaria angustifolia* (Bert. O Kuntze) pertence à família Araucariaceae e é uma planta reconhecidamente medicinal. Seus estróbilos femininos dão origem a pinha, constituída por pinhões (sementes verdadeiras) e brácteas (sementes não desenvolvidas). O objetivo do presente estudo foi avaliar o efeito antiproliferativo do extrato aquoso de brácteas de *A. angustifolia* (EAA) em células tumorais de laringe HEp-2 e seus mecanismos de ação. Além disso, a possibilidade de associar o EAA a nanoesferas (NE) a fim de potencializar seu efeito antitumoral também foi investigado. A análise química por meio de Espectrometria de Massas de Alta Resolução evidenciou a presença majoritária de polifenóis no EAA. Os resultados mostraram que o EAA induziu citotoxicidade nas células tumorais HEp-2 através de dois diferentes ensaios de viabilidade celular (ensaio de MTT e de exclusão do corante *Trypan blue*). No entanto, o mesmo não foi capaz de induzir citotoxicidade significativa em células normais HEK-293, utilizadas como controle, indicando um efeito diferencial seletivo do EAA sobre as células tumorais. As células HEp-2 tratadas com EAA apresentaram níveis aumentados de peroxidação lipídica, danos oxidativos a proteínas e

aumento da produção de ON, juntamente com depleção das defesas antioxidantes superóxido dismutase (Sod) e catalase (Cat). Além disso, o EAA induziu danos ao DNA, juntamente com fragmentação nuclear e condensação da cromatina nestas células. Alterações nos marcadores epigenéticos, como hipometilação do DNA e redução da atividade de DNMT1 foram também observadas. A exposição das células tumorais ao extrato aumentou a expressão de proteínas apoptóticas de via intrínseca mitocondrial, mediada pela ativação da proteína Bax, liberação de AIF e foi independente da ativação de p53. O EAA modificou o metabolismo energético das células tumorais, elevando os níveis de piruvato desidrogenase (PDH) e estimulando a fosforilação oxidativa mitocondrial. Embora tenha ativado a mitocôndria destas células, o mesmo causou falhas no potencial de membrana mitocondrial ($\Delta\Psi m$), juntamente com diminuição dos níveis de proteínas dos complexos I e III da CTE, inibição da atividade do complexo I, produção de ERO e depleção de ATP. A associação do EAA à NE permitiu a obtenção de sistemas com tamanho de partícula inferior a 200 nm, índice de polidispersibilidade abaixo de 1, potencial zeta negativo e pH estável pelo período de 30 dias, na condição testada. No entanto, a taxa de associação obtida foi baixa (19%), indicando a necessidade de futuros estudos a fim de aumentar a eficiência de incorporação do extrato. As NE *per se* demonstraram capacidade de reduzir a viabilidade de células tumorais e induzir alterações redox, sensibilizando estas células, demonstrando ser um possível carreador para a vetorização de ativos antitumorais. O conjunto de dados deste estudo demonstra a potencialidade dos compostos presentes nas brácteas de *A. angustifolia* para o desenvolvimento de novas estratégicas terapêuticas para o câncer.

Palavras-chave: carcinoma de laringe, pinheiro-brasileiro, polifenóis, mitocôndria, alterações redox, nanocarreadores

ABSTRACT

Natural products are among one of the most promising fields in finding new active substances in cancer therapy. Laryngeal carcinoma is one of the most common cancers affecting the head and neck regions, and is associated with high morbidity and mortality in patients with the advanced stage. Alternative and/or complementary therapies for treating this cancer represents an emerging need. One of the most promising alternatives is the development of nanocarriers containing antitumor substances. *Araucaria angustifolia* (Bert. O Kuntze) belongs to Araucariaceae family and it is recognized as medicinal plant. Female strobilus originates the pinecone, which contains seeds and undeveloped seeds, commonly known as bracts. The aim of this study was to evaluate the antiproliferative effects of *A. angustifolia* bracts aqueous extract (AAE) in HEp-2 cancer cells and its action mechanisms. Moreover, the possibility to associate AAE in nanospheres (NS) to improve its antitumor effect was also investigated. Chemical analysis using High Resolution Mass Spectrometry (HRMS) revealed the major presence of polyphenols in AAE. The results showed that AAE induced cytotoxicity in HEp-2 cells, by using two differents approaches (MTT and Trypan blue assays). However, the extract was not able to induce significant cytotoxicity in HEK-293 normal cells used as control, indicating a selective differential effect of AEE in tumor cells. HEp-2 treated cells presented high levels of lipid peroxidation, oxidative damage to proteins and increment on NO production, along with depletion on antioxidante defenses superoxide dismutase (Sod) and catalase (Cat). In addition, AAE induced DNA damage, nuclear fragmentation and chromatin condensation in these cells. Epigenetic alterations, such as

DNA hypomethylation and DNMT1 activity were also observed. Cell exposition to the AAE increased expression of proteins of the mitochondrial intrinsic pathway, via Bax-triggered, along with AIF release, and it is independent of p53 incitement. AAE changed energetic metabolism of cancer cells, increasing levels of pyruvate dehydrogenase (PDH) and stimulating mitochondrial oxidative phosphorylation. Although it enabled the mitochondria of these cells, the extract caused loss of mitochondrial membrane potential ($\Delta\Psi m$), reduction on protein expression levels of complex I and III, inhibition of complex I activity, ROS generation and ATP depletion. The association of EAA to NS allowed obtaining systems with particle size lower than 200 nm, polydispersity index less than 1, negative zeta potential and pH stable for the period of 30 days, under tested condition. However, the association rate obtained was low (19%); therefore, further studies are needed to improve AAE encapsulation. The NS *per se* demonstrated ability to reduce the viability of tumor cells and induce redox stress, sensitizing these cells, proving to be a possible carrier for delivery of antitumor substances. The data set of this study demonstrates the potentiality of the compounds present in the *A. angustifolia* bracts for the development of new therapeutic strategies for cancer.

Keywords: laryngeal carcinoma, Brazilian pine, polyphenols, mitochondria, redox alterations, nanocarriers

1 INTRODUÇÃO

1.1 *Araucaria angustifolia*: aspectos etnobotânicos e fitoquímicos

A *Araucaria angustifolia* (Bert.) O. Kuntze, pertencente à família Araucariaceae, é uma conífera nativa brasileira, concentrando-se principalmente nos estados de Paraná, Santa Catarina e Rio Grande do Sul, ocorrendo também nos estados de São Paulo, Minas Gerais e Rio de Janeiro, como populações esparsas. Conhecida como "pinheiro-do-paraná" ou "pinheiro-brasileiro", a *A. angustifolia* é uma espécie de grande importância econômica e ecológica para o país. Trata-se de uma árvore de grande porte (Figura 1 A), alcançando, quando adulta, 20 a 50 metros de altura (Klein 1960). Sua área de ocorrência abrange altitudes que vão de 500 metros a 1.500 metros (Carvalho 1994). Existem aproximadamente 1,5 mil quilômetros quadrados de áreas de pinheiros nativos no Rio Grande do Sul, sendo que cerca de 3% encontram-se na reserva original da Mata Atlântica (BRDE 2005). Devido à intensa atividade madeireira no passado, sua área de concentração foi bastante reduzida e, atualmente, a espécie está classificada como vulnerável na Lista Vermelha de espécies ameaçadas de extinção (Thomas 2015).

A araucária é uma espécie predominantemente dióica (Danner et al. 2014), possuindo flores femininas e masculinas em árvores distintas. As flores femininas apresentam-se em estróbilo globoso, protegido por várias folhas bem aglomeradas (Figura 1 B), enquanto que as flores masculinas são cilíndricas e alongadas (Figura 1 C), tendo cerca de 10 a 15 cm de comprimento e 2 a 4 cm de diâmetro (Dallimore & Jackson 1948). O processo reprodutivo é longo. Nas populações naturais, a produção de sementes (pinhão) normalmente ocorre após 15 a 20 anos de idade e quando plantadas, as árvores isoladas iniciam a produção de sementes entre 10 e 15 anos.

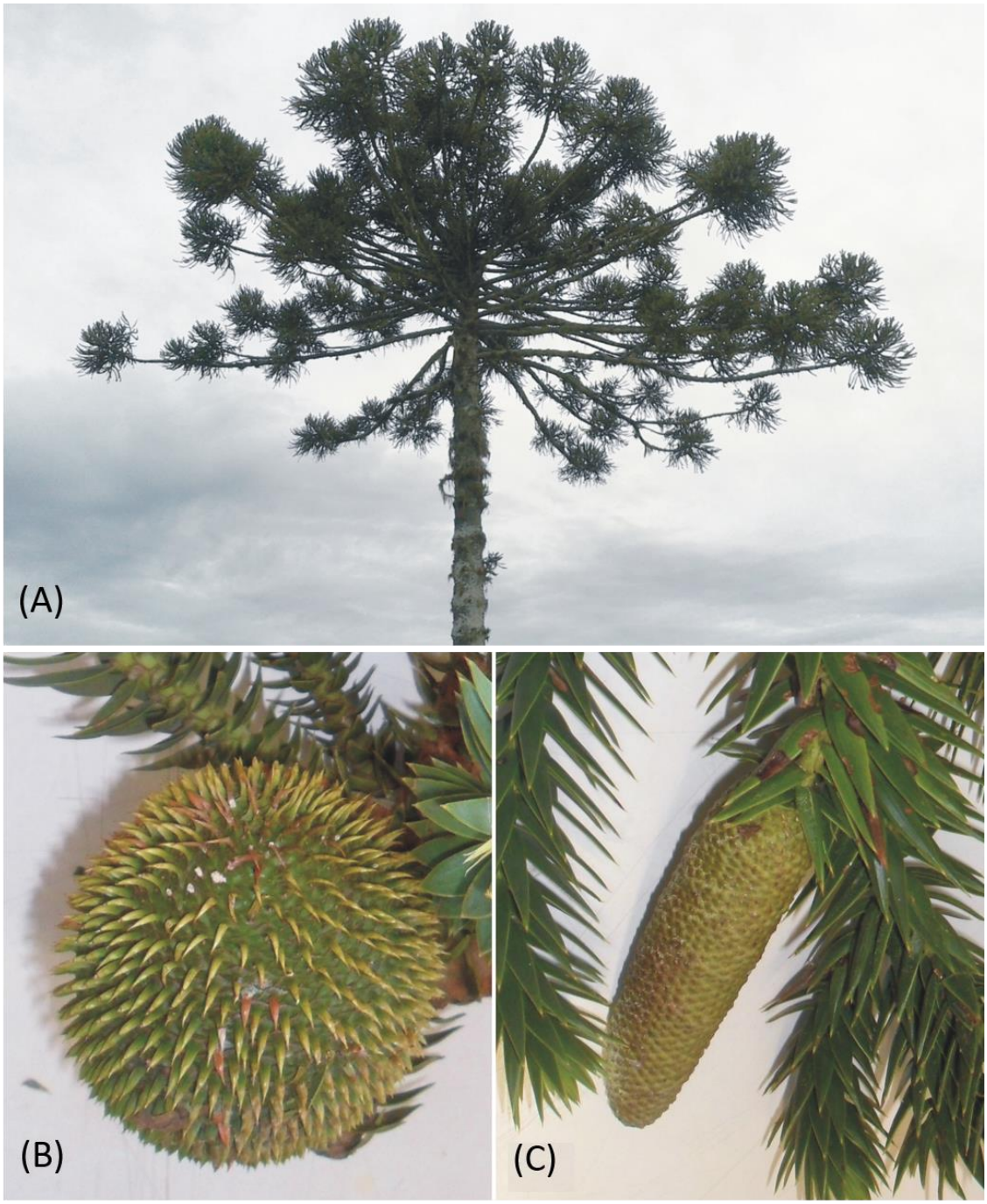


Figura 1. Fotografia do pinheiro *Araucaria angustifolia*, mostrando um aspecto umbeliforme peculiar na parte superior e um tronco retilíneo e cilíndrico (A). Estróbilo feminino (B) e masculino (C) da planta. Adaptado de (Branco et al. 2016).

Nos primeiros anos, a produção de pinhão é pequena e, mesmo quando atinge a plena produção, as safras são cíclicas. Durante 2 ou 3 anos, produz abundantemente, reduzindo a produção posterior, gradativamente, nos 2 ou 3 anos seguintes (Carvalho 1994; Mantovani et al. 2004; Martins-Ramos et al. 2008). A polinização ocorre durante os meses de setembro e outubro e, uma vez fertilizada, as pinhas amadurecem em 2 a 3 anos (Mantovani et al. 2004; Bittencourt 2007). Após a polinização, as brácteas férteis desenvolvem somente um óvulo fecundado (o pinhão), unindo seus bordos com os da bráctea acima e comprimindo as brácteas estéreis (falhas) conforme o pinhão vai se desenvolvendo (Bittencourt 2007). A pinha, então fecundada, é constituída por pinhões e brácteas (Figura 2).

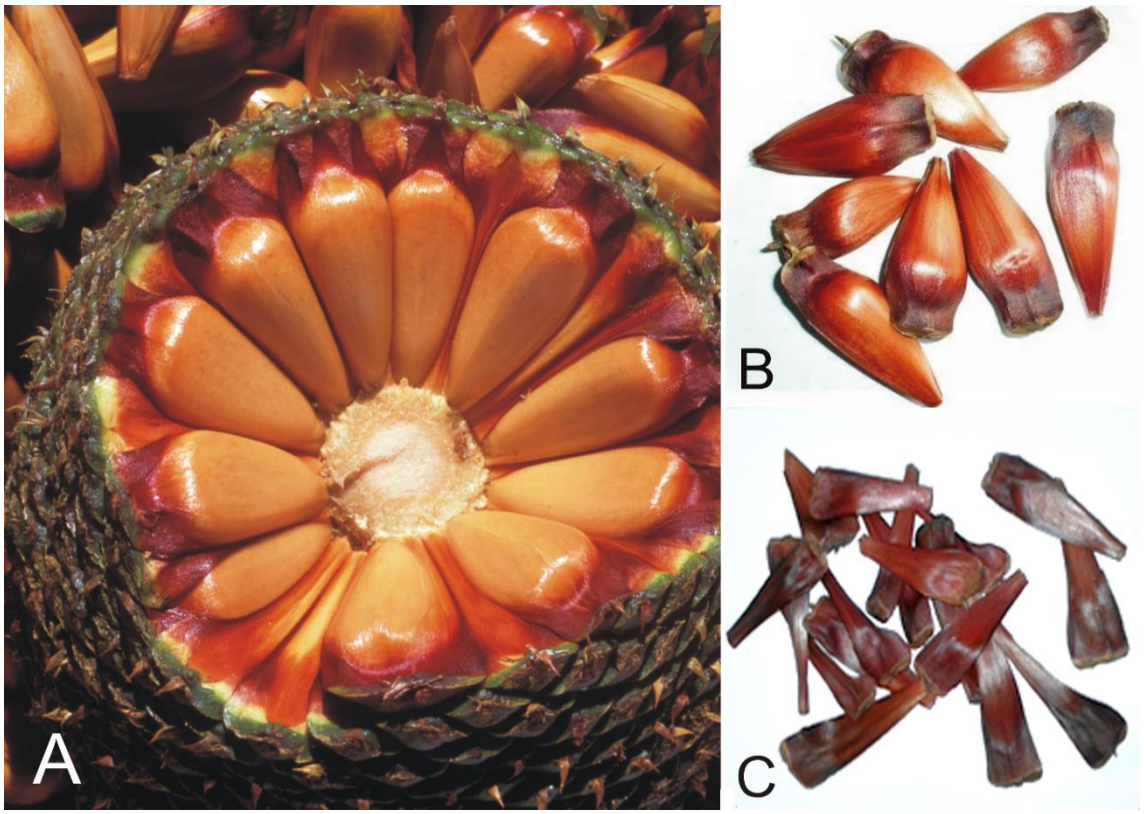


Figura 2. Pinha madura de *A. angustifolia* mostrando o eixo cônico central (A), pinhões (B) e brácteas (C). Adaptado de (Branco et al. 2016).

Os pinhões são encontrados em maior quantidade nos meses de abril a junho, porém o maior volume de comercialização ocorre nos meses de junho e julho. Embora sua comercialização seja disciplinada pela Portaria Normativa DC nº20, grande parte da venda ainda é clandestina (sem emissão de notas fiscais), o que dificulta a mensuração da magnitude deste mercado (BRDE 2005). Segundo os dados mais recentes do Instituto Brasileiro de Geografia e Estatística, a quantidade de pinhão produzido no Rio Grande do Sul, em 2013, foi de 881 toneladas (IBGE 2015) sendo a região dos Campos de Cima da Serra, a maior produtora. O pinhão *in natura* exibe uma coloração amarelo-amarronzada, sendo constituído por uma membrana externa muito resistente e uma membrana interna fina, aderente à semente (Figura 3).

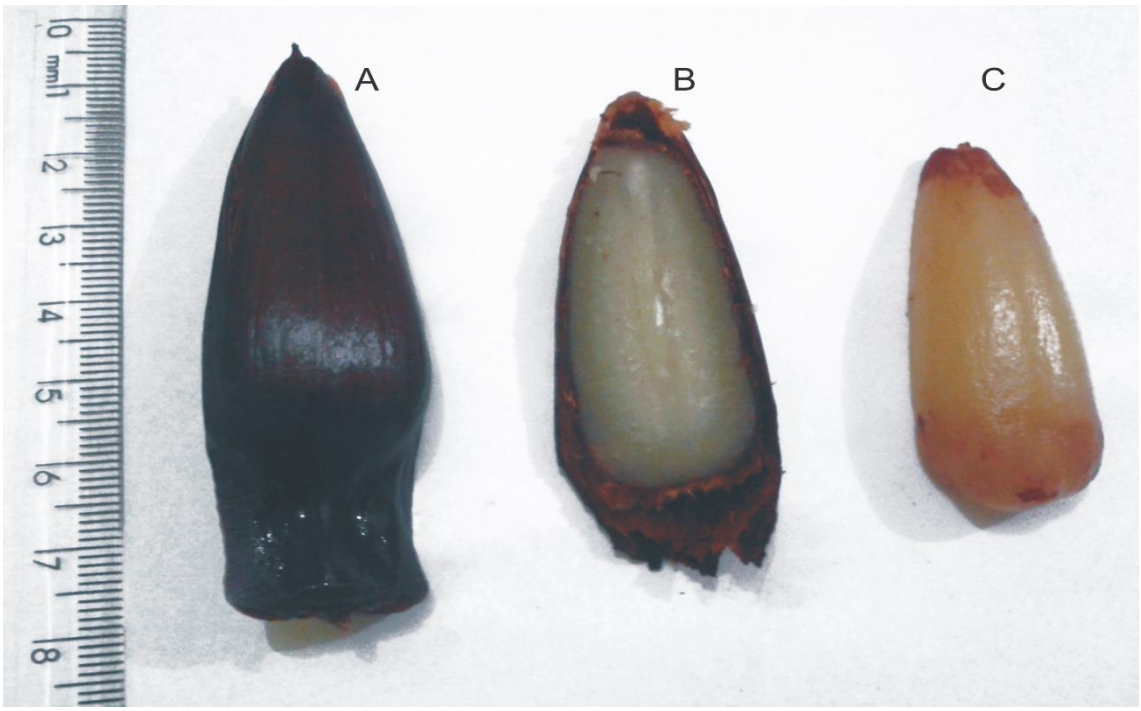


Figura 3. Sementes cozidas (pinhão) da *A. angustifolia* mostrando o revestimento externo (A), corte longitudinal evidenciando o interior da semente (B) e porção interna comestível (C). Adaptado de (Branco et al. 2016).

Possuem formato oblongo, alcançando 3 a 8 cm de comprimento e pesando aproximadamente 8 g (Cordenunsi et al. 2004; Cladera-Olivera et al. 2008). O pinhão é

muito apreciado como alimento pela população e é considerado uma excelente fonte de energia e nutrientes, possuindo proteínas e carboidratos de alto valor nutritivo, além da presença de minerais e ferro (Cordenunsi et al. 2004; Pires et al. 2006; Brandelli et al. 2008; Brazil 2011; Koehnlein et al. 2012).

Além da importância nutricional da *A. angustifolia*, a planta também se destaca do ponto de vista terapêutico. Inicialmente, o seu uso empírico foi explorado pelas populações indígenas, as quais conheciam o seu potencial medicinal contra enfermidades. Posteriormente, o conhecimento tradicional foi ganhando destaque em outras partes da América do Sul e desde então, diferentes partes de *A. angustifolia* (casca do tronco, resina, nós, folhas e sementes) vêm sendo empregadas, empiricamente, no tratamento de algumas doenças (Franco & Fontana 2001). Tinturas de nós vêm sendo usadas oralmente e/ou topicamente para o tratamento do reumatismo. Infusões de nós são ingeridas para o tratamento de doenças renais, enquanto que a infusão da casca vem sendo utilizada topicamente para tratar tensões musculares e varizes. O xarope produzido com a resina é utilizado para o tratamento de infecções do trato respiratório, e as folhas da árvore são utilizadas tanto para o preparo de infusões, quanto para o preparo de tinturas, sendo empregadas no tratamento da fadiga, infecções respiratórias, gastrite e anemia (Marquesini 1995; Carvalho 2003; Martins-Ramos et al. 2008).

Apesar de seu uso tradicional, são poucos os estudos visando elucidar o seu potencial farmacológico. No entanto, estudos sobre a composição fitoquímica desta planta têm relatado a presença de metabólitos primários e secundários de importância biológica. Em relação aos metabólitos secundários, estudos identificaram majoritariamente compostos fenólicos, além de outras substâncias bioativas, como esteróis e terpenos. Em estudo prévio foi mostrado que a casca do tronco da *A.*

angustifolia é rica em compostos fenólicos, principalmente flavanois e proantocianidinas poliméricas (Seccon et al. 2010). Estudos utilizando as folhas da árvore identificaram frações contendo altos níveis de biflavonóides com atividade biológica (Yamaguchi et al. 2005; Yamaguchi et al. 2009). Outras partes da *A. angustifolia* também apresentam polifenóis em sua composição. Tem sido mostrado que extratos obtidos a partir da semente da Araucária contêm quantidades significativas de compostos fenólicos, incluindo catequina, quercetina e ácido gálico (Cordenunsi et al. 2004; Koehnlein et al. 2012). Além da semente, as brácteas também apresentam compostos fenólicos com importância farmacológica, incluindo catequina, epicatequina, quercetina, rutina e apigenina (Michelon et al. 2012; Souza et al. 2014), flavonoides com importante potencial farmacológico.

1.2 Polifenóis: classificação química e propriedades anticarcinogênicas

Os polifenóis, ou compostos fenólicos, são moléculas fitoquímicas complexas com estruturas químicas diferenciadas. Estes compostos estão presentes nos vegetais em sua forma livre, como também conjugados a açúcares (glicosídios) e proteínas, representando os mais abundantes antioxidantes da dieta humana. Possuem pelo menos um anel aromático no qual ao menos um hidrogênio é substituído por um grupamento hidroxila, e são classificados segundo o tipo de esqueleto principal, dividindo-se em flavonóides e não-flavonóides (Del Rio et al. 2012). Os compostos não-flavonóides compreendem os ácidos fenólicos, benzóicos e cinâmicos, e outros derivados fenólicos como os estilbenos. Já os flavonóides representam um dos grupos fenólicos mais importantes, sendo classificados em várias subclasses que se distinguem pelo grau de oxidação de seu núcleo pirano. São metabólitos secundários das plantas apresentando uma estrutura hidrocarbonada comum (Figura 1) do tipo C6-C3-C6 (difenilpropano), e são divididos

em diversas classes (flavan-3-ol, flavona, flavonol, flavanona, flavononol, antocianidina, chalcona, aurona).

Os flavonóides têm sido descritos como agentes neutralizadores de radicais livres e quelantes de íons metálicos, principalmente ferro e cobre, os quais são os de maior relevância para as reações iniciais de formação de radicais. Além disso, estes compostos podem inibir algumas enzimas envolvidas na geração de espécies reativas, dessa forma protegendo as células contra os danos oxidativos (Halliwell 2007; Halliwell 2008).

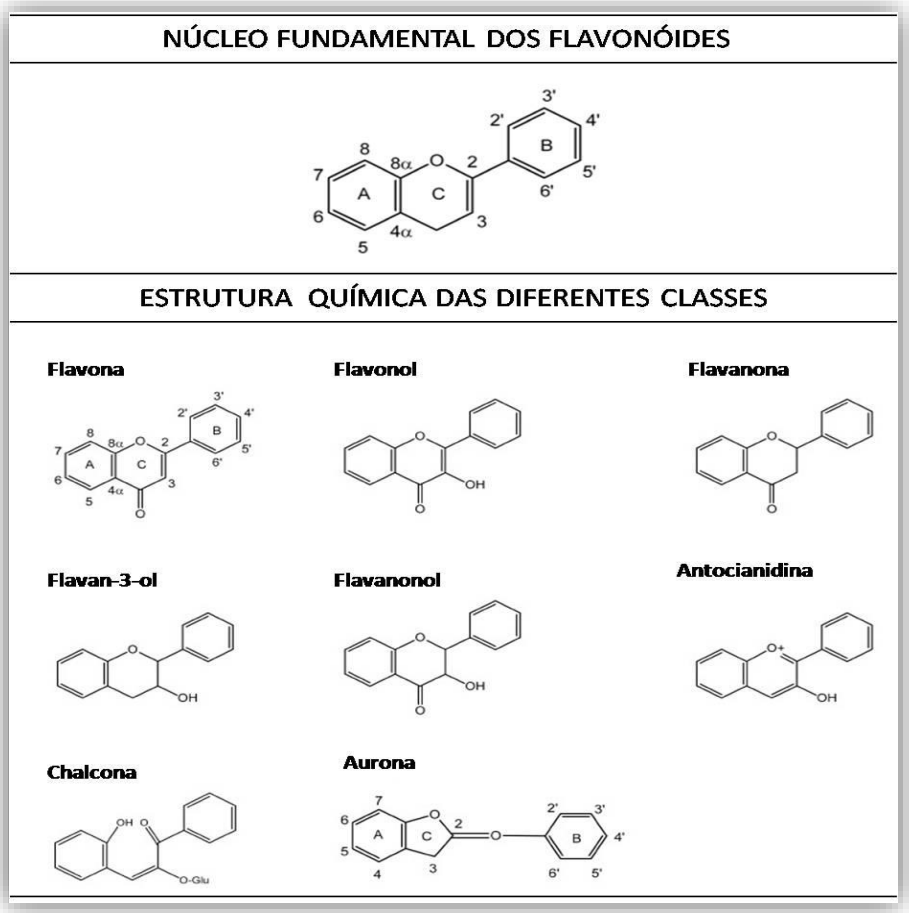


Figura 4. Núcleo fundamental e classificação dos flavonoides. Adaptado de (Aron & Kennedy 2008).

A literatura científica tem mostrado que os compostos fenólicos possuem diversas propriedades biológicas, incluindo antioxidante e anticarcinogênica (Khan et al. 2006;

Corcoran et al. 2012; Khan et al. 2012; Del Rio et al. 2012; Khan et al. 2014). Além disso, tem sido demonstrado que os polifenóis são importantes agentes profiláticos para a minimização da incidência do câncer, atuando também como co-adjuvantes, aumentando a efetividade dos antineoplásicos (Khan et al. 2006; Lambert & Elias 2010; González-Vallinas et al. 2013). Estudos mostram que os polifenóis possuem propriedades antitumorais e indutoras de apoptose em diferentes linhagens tumorais de câncer (Nair et al. 2007; Hadi et al. 2007; Dai & Mumper 2010; Asensi et al. 2011; Khan et al. 2014; Braconi et al. 2014). Os principais mecanismos de ação destes compostos frente à inibição do processo carcinogênico incluem a inativação de moléculas genotóxicas exógenas ou endógenas, modulação das vias de expressão de enzimas antioxidantes (Zinov & Spasov 2011), sinalização de rotas apoptóticas (Khan et al. 2012) e imunomodulação (Zhao et al. 2007).

1.3 Carcinogênese e produtos naturais

O câncer é um grupo de doenças caracterizadas por modificações genéticas que levam ao desequilíbrio entre sobrevivência e morte celular (Khan et al. 2007; Fulda 2014). A palavra câncer é utilizada para designar um conjunto de mais de 200 doenças, que inclui tumores malignos de diferentes localizações. Embora de origens e causas ainda não muito esclarecidas, as neoplasias surgem devido à mutações genéticas, espontâneas ou induzidas por agentes citotóxicos, incluindo espécies reativas, radiação ionizante, xenobióticos, entre outros. A exposição a estes agentes promove desordens no ciclo celular, ocorrendo excesso nas taxas de proliferação e deficiência nas taxas de apoptose, culminando com a formação de agrupamentos de clones de células neoplásicas, os tumores (Jones & Thompson 2009). Evidências demonstram que a resistência à apoptose é uma das características mais marcantes da maioria dos tumores malignos. Estes são

responsáveis por um número expressivo e crescente de pacientes em todo o mundo, e representam a segunda causa de morte da população mundial, totalizando aproximadamente 13% dos óbitos de causa conhecida (GLOBOCAN 2012; WHO 2016).

Segundo a Agência Internacional para a Pesquisa em Câncer (IARC) e a Organização Mundial de Saúde (WHO 2016) o impacto global do câncer dobrou nos últimos 30 anos, devendo-se principalmente ao crescimento populacional e ao envelhecimento da população, sendo que o maior número de novos casos e óbitos estão previstos principalmente para os países em desenvolvimento.

Dos mais de 100 tipos de carcinomas existentes (WHO 2016), o de laringe é um dos mais comuns, representando 25% dos tumores malignos que acometem a área de cabeça e pescoço (INCA 2016). O tipo histológico mais prevalente é o carcinoma epidermóide, estando associado a mais de 90% dos pacientes, em sua maioria do sexo masculino. De acordo com a localização e a extensão do câncer, ele pode ser tratado com cirurgia e/ou radioterapia e com quimioterapia associada à radioterapia (WHO 2016; INCA 2016). No entanto, existe uma alta taxa de morbidade e mortalidade associada a este tipo de câncer em pacientes em estágio avançado.

Apesar da introdução de novos medicamentos no arsenal terapêutico contra o câncer, vários tumores ainda não dispõem de tratamento adequado, e estes medicamentos ainda apresentam sérios efeitos colaterais (Costa-Lotufo et al. 2010; INCA 2016). Atualmente, há um crescente interesse nos produtos naturais, os quais demonstram forte potencial biológico para o desenvolvimento de novos fármacos, principalmente na terapêutica antineoplásica. De fato, mais de 60% dos medicamentos utilizados no tratamento do câncer tem, em alguma instância, sua origem relacionada a uma fonte natural (Costa-Lotufo et al. 2010). Tradicionalmente, as plantas superiores, assim como

os micro-organismos, revelaram-se fontes ricas em moléculas naturais indispensáveis para o tratamento de doenças como o câncer. Exemplos bem conhecidos são o taxol (Paclitaxel®), de *Taxus brevifolia*; o etoposide (Vesepid®), derivado da síntese parcial da podofilotoxina isolada de *Podophyllum peltatum*; a Rapamicina® derivada do *Streptomyces hygroscopicus;* alcalóides da Vinca (*Catharanthus roseus)* Vinblastina®, Vincristina®, entre outros. Considerando a necessidade crescente de buscar novas formas alternativas para a prevenção e tratamento do câncer, torna-se importante a caracterização e estudo dos ativos (a substância ou conjunto delas que é responsável pelos efeitos terapêuticos) existentes nas plantas, as quais representam um importante reservatório de moléculas com potencial farmacológico.

1.4 Avaliação do efeito antiproliferativo e mecanismos de citotoxicidade

A cultura de células de mamíferos constitui-se num importante instrumento para o estudo das atividades biológicas de diferentes compostos, bem como para um *screening* de novas moléculas e identificação de novos alvos terapêuticos, facilitando a análise de propriedades e processos que não seriam facilmente realizados em nível de organismo intacto. A manutenção de células requer conhecimento e prática de algumas poucas técnicas essenciais, sendo então, um procedimento relativamente simples. Devido a essa praticidade, segurança e efetividade dos ensaios, diversos trabalhos utilizam esses sistemas experimentais (Kim et al. 2012; Matthews et al. 2012; Fauser et al. 2013; Muntané et al. 2013; Link et al. 2013).

A linhagem HEp-2 de carcinoma de laringe humana é considerada um bom modelo de estudo celular, podendo ser utilizada para ensaios de atividade citotóxica, genotóxica e avaliação do ciclo celular, principalmente por ser de fácil cultivo e manutenção, crescimento rápido e curto período de adaptação, tempo de geração entre 12 e 24 horas e

capacidade de iniciar um rápido crescimento exponencial a partir de um pequeno inóculo (ATCC 2015).

O cultivo de células possibilita a avaliação dos efeitos da administração de diferentes compostos sobre a viabilidade celular, a qual pode ser mensurada por diferentes técnicas, incluindo a redução metabólica do 3-(4,5-dimetiltiazol 2-il)-2,5 difenil brometo de tetrazolina – MTT (Denizot & Lang 1986) e a capacidade de metabolização do corante *Trypan blue* (Strober 2001). Estes ensaios colorimétricos servem de parâmetro para a descoberta de novas substâncias com potencial de interferir na biologia celular. A análise morfológica das células também serve de critério para avaliação das alterações provocadas por diferentes tratamentos.

O mecanismo de citotoxicidade parece estar relacionado a diferentes rotas do metabolismo celular, entre elas a apoptose. Muitas vezes a morte celular programada é induzida frente a exposições moderadas de estresse oxidativo, com processos irreversíveis de oxidação que inibem proteases específicas, as caspases. As caspases, em especial a caspase 3, participa ativamente do processo de iniciação e execução da apoptose (Salvesen & Dixit 1997; Cohen 1997; Hangen et al. 2010). A proteína p53, por exemplo, tem sua expressão acumulada quando o DNA encontra-se danificado e previne a replicação celular, interrompendo assim o ciclo durante a interfase, dando tempo para que a célula se repare (Spurgers et al. 2006; Teoh & Chng 2014; Zhang et al. 2016). Essas proteínas são consideradas importantes marcadores apoptóticos e seu nível global de expressão pode ser quantificado por meio de *immunoblotting*, bem como o nível de expressão gênica pode ser avaliado por meio de qRT-PCR. Como a expressão destas proteínas está associada a lesões genômicas, estas podem ser avaliadas através de diferentes técnicas, tais como o ensaio Cometa e a quantificação dos níveis de expressão

de 8-hidroxideoxiguanosina (8-OHdG), um marcador de lesões oxidativas na base nitrogenada guanina do DNA. Lesões genômicas podem ainda ser avaliadas por meio da análise da morfologia nuclear empregando imunocitoquímica.

As conseqüências do aumento nos níveis de espécies reativas de oxigênio (ERO) já são conhecidas e levam, entre outros fatores, à oxidação de lipídios, proteínas e ácidos nucléicos, potencializando o colapso das membranas celulares (Halliwell 2007; Halliwell 2008). A integridade dos lipídios é de vital importância, uma vez que os ácidos graxos poli-insaturados são os maiores constituintes da membrana das células e são alvos preferenciais do processo peroxidativo, juntamente com as proteínas, cujo dano pode ser avaliado pelo aumento dos níveis de proteínas carboniladas (Wills 1966; Levine et al. 1990). A quantificação dos níveis de óxido nítrico (ON) também é um parâmetro importante, uma vez que esta molécula reage rapidamente com outras espécies radicalares levando à geração de mais espécies reativas e causando a oxidação de diversas classes de biomoléculas (Wink DA, Mitchell 1998). Os níveis de estresse oxidativo podem ser controlados pela ação das enzimas antioxidantes, dentre as quais destacam-se a superóxido dismutase (Sod) e a catalase (Cat), consideradas como a primeira linha de defesa enzimática contra ERO (Halliwell 2007). Nos sistemas biológicos, a mitocôndria é a principal fonte endógena de ERO, especialmente em nível dos complexos da cadeia de transporte de elétrons (CTE) (Grivennikova & Vinogradov 2006; Vartak et al. 2014). O complexo I, também conhecido como NADH-ubiquinona oxidoredutase, é composto por 45 subunidades, sendo 7 oriundas do DNA mitocondrial (mtDNA) e 38 oriundas de genoma nuclear. Além do complexo I, os complexos III (ubiquinona citocromo c oxidoredutase), IV (citocromo c oxidase) e V (ATP sintase) também apresentam subunidades originadas tanto do DNA nuclear quanto mitocondrial. O complexo II

(succinato ubiquinona oxidoredutase), por sua vez, apresenta somente subunidades de origem mtDNA (El-Hattab & Scaglia 2016). A ação conjunta destes 5 complexos é responsável pela eficiência da fosforilação oxidativa, a qual é permanentemente regulada. No entanto, quando há falhas no controle global da respiração mitocondrial, em função da ineficiência de atividade dos complexos I-V ocorre a disfunção mitocondrial, em parte pela perda de energia requerida para o metabolismo celular, mas também pela superprodução de ERO. Estes fenômenos podem afetar diversas vias de transdução de sinais responsáveis pela transformação e progressão tumoral (Hanahan & Weinberg 2011), dessa forma exercendo importante papel na biologia do câncer.

Sabe-se que o câncer é uma doença eminentemente genômica e dessa forma as alterações genéticas, incluindo mutações, rearranjos, deleções e inserções são consideradas como eventos importantes para sua etiologia. Além disso, existem alterações genômicas potencialmente reversíveis que, embora herdáveis, não alteram a sequência de bases do DNA. Estas características são singulares dos fenômenos epigenéticos (Feinberg & Tycko 2004). Alterações epigenéticas são modificações do DNA e histonas transmitidas durante as divisões celulares que são capazes de modificar a expressão gênica e são consideradas a chave para o entendimento das diferenças entre crescimento e senescência celular de células tumorais e não tumorais (Feinberg & Tycko 2004; Oronsky et al. 2014). Existem três mecanismos principais de alterações epigenéticas: metilação do DNA, modificações de histonas e *imprinting* genômico (Feinberg & Tycko 2004). Entre as modificações que as histonas podem sofrer, estão: metilação, fosforilação e acetilação, no entanto, na molécula de DNA ocorre apenas metilação. A metilação consiste em uma modificação covalente do DNA na qual um grupamento metil (CH3) é transferido da S-adenosilmetionina para o carbono 5 de uma

citosina (5-MeC) que geralmente precede a uma guanina (dinucleotídeo CpG), pela ação de uma família de enzimas denominada de DNA metiltransferase (DNMT) (Szyf 2007). As DNA metiltransferases estão divididas em duas classes, sendo as envolvidas na metilação de fitas hemimetiladas do DNA (fitas de DNA em processo de replicação), conhecidas como metilases de manutenção, tais como a DNMT1; e a outra classe responsável pela maioria dos processos de metilação *de novo*, que ocorre em sítios com nenhum tipo de indicação de metilação, ou seja, sem a presença de metilação prévia, incluindo as DNMT2, DNMT3A e DNMT3B1, as quais realizam a transferência do grupo metil da S-adenosil-L-metionina (SAM) para a citosina (Szyf 2007). Além das DNA metiltransferases, as metilcitosina dioxigenases da família das proteínas conhecidas como *ten-eleven translocation* (TET), também exercem importante papel na regulação das modificações epigenéticas, catalisando a demetilação do DNA (Hill et al. 2014). A família de proteínas reguladoras TET é composta de três membros, quais sejam TET1, TET2 e TET3, cada qual com abundância variável em diferentes tipos celulares. A metilação do DNA controla várias funções do genoma, sendo essencial durante a morfogênese para que ocorra desenvolvimento normal. Alterações nos padrões de metilação tais como aberrações, podem promover instabilidade genômica e carcinogênese (Ushijima & Asada 2010).

Considerando a complexidade do câncer, o desenvolvimento de drogas com diferentes alvos moleculares de ação é um fator primordial para o desenho de novos fármacos com maior especificidade e efetividade.

1.5 Tecnologias inovadoras na terapia antineoplásica: nanotecnologia

Diversos estudos vêm sendo realizados com o objetivo de aperfeiçoar o processo de liberação dos fármacos no organismo, principalmente no que se refere à toxicidade dos

mesmos. Nesse sentido, os sistemas nanoparticulados constituem uma importante alternativa de vetorização, sendo capazes de reduzir os efeitos adversos das substâncias neles associadas, assim como protegê-las de reações de oxidação e reações enzimáticas (Weiss 2001; Cancino et al. 2014). Do ponto de vista da estabilidade química, alguns estudos destacam a utilização destes sistemas para a incorporação de substâncias quimicamente lábeis, como por exemplo o ácido lipóico (Külkamp et al. 2009) e os flavonoides quercetina (Weiss-Angeli et al. 2008; Pool et al. 2012) e catequina (Pool et al. 2012). Outra vantagem da utilização destes sistemas está relacionada à otimização da resposta farmacológica exibida pelo fármaco, já que a sua distribuição no organismo se baseia nas suas propriedades físico-químicas, o que muitas vezes não permite que este atinja o local de ação. Neste contexto, a associação de ativos a um sistema que permita a adequação de suas propriedades físico-químicas sem alterar seu mecanismo de ação representa uma alternativa valiosa para o tratamento clínico. Algumas destas novas formas de administração incluem sistemas dispersos coloidais constituídos por polímeros naturais, sintéticos ou semissintéticos desenvolvidos à escala nanométrica (abaixo de 1μm), tais como as nanopartículas (NP) (Cancino et al. 2014). As NP possuem a capacidade de incorporar tanto substâncias hidrofílicas quanto lipofílicas, dessa forma aumentando a biodisponibilidade de substâncias pouco solúveis (Rawat et al. 2006). Dentre as NP, destacam-se as nanoesferas.

As nanoesferas (NE) são definidas como estruturas matriciais esféricas, constituídas por um núcleo sólido polimérico e com um diâmetro médio de 200 nm. Estas estruturas não apresentam óleo em sua composição. Neste caso a substância ativa pode encontrar-se dissolvida no núcleo e/ou incluída ou adsorvida na parede polimérica (Sinha et al. 2004; Guterres et al. 2007).

A utilização destes sistemas nanovetorizados na terapia do câncer baseia-se no fato de que as partículas possuem afinidade a certos tumores primários ocasionando um aumento da eficiência e redução da toxicidade de alguns agentes quimioterápicos e imunomoduladores (Kedar et al. 2010). Embora o FDA/USA já tenha aprovado diversos nanofármacos, no Brasil este número ainda é pequeno. Atualmente, estão liberados pela ANVISA nanofármacos à base de doxorrubicina e gentuzumab para o tratamento de câncer. Estima-se que nos próximos anos cerca de 50% dos novos medicamentos sejam de base nanotecnológica (Cancino et al. 2014; Xie et al. 2016). No entanto, estudos detalhados acerca dos aspectos farmacológicos e toxicológicos dos ativos, das estruturas carreadoras e de sua combinação devem ser exaustivamente examinados.

Diante dos aspectos mencionados sobre a necessidade de novas terapias para o tratamento do câncer, destaca-se a importância da identificação e do estudo de novos ativos. Em vista disso, neste trabalho estudou-se o efeito antiproliferativo do extrato de *A. angustifolia*, bem como seus constituintes químicos e alvos moleculares mitocondriais e epigenéticos em linhagem tumoral humana de laringe HEp-2. Além disso, considerando o interessante potencial do extrato de *A. angustifolia* frente ao câncer, foi escolhido um sistema nanovetorizado baseado em NE para encapsulação do mesmo, a fim de potencializar seu efeito antitumoral.

2 OBJETIVOS

2.1 Objetivo Geral

Este trabalho teve como objetivo avaliar o efeito antiproliferativo e os mecanismos de ação do extrato de brácteas de *Araucaria angustifolia* em linhagem tumoral humana HEp-2, bem como incorporar o extrato à nanoesferas (NE).

2.2 Objetivos Específicos

- ✓ Caracterizar quimicamente o extrato de brácteas de *A. angustifolia*.
- ✓ Avaliar o possível efeito antiproliferativo e alterações morfológicas induzidas pelo tratamento com o extrato em linhagem tumoral HEp-2.
- ✓ Analisar as características nucleares das células HEp-2 (marcada com 4'6'-diamidino-2-fenilindol) após o tratamento com o extrato de *A. angustifolia*.
- ✓ Avaliar a possível genotoxicidade do extrato através da quantificação dos níveis de danos ao DNA (ensaio Cometa) em células da linhagem tumoral HEp-2.
- ✓ Quantificar os danos oxidativos a lipídios, proteínas, atividade de enzimas antioxidantes e níveis de óxido nítrico, nas células HEp-2 tratadas com o extrato de *A. angustifolia*.
- ✓ Avaliar a expressão dos marcadores de apoptose, incluindo p53 e caspase-3, nas células HEp-2 tratadas com o extrato.
- ✓ Avaliar os possíveis alvos mitocondriais de ação do extrato de *A. angustifolia* em células tumorais HEp-2.
- ✓ Investigar, em nível epigenético, os padrões globais de metilação e hidroximetilação de células HEp-2 tratadas com o extrato de *A. angustifolia*.

- ✓ Avaliar a factibilidade de preparação de sistemas carreadores do tipo NE contendo o extrato de *A. angustifolia*.
- ✓ Avaliar as características físico-químicas das NE contendo o extrato e das NE sem o extrato (*per se*).
- ✓ Investigar a possível toxicidade das formulações de NE sobre a viabilidade celular e parâmetros de estresse oxidativo em células HEp-2.
- ✓ Correlacionar os compostos majoritários do extrato de *A. angustifolia* com os efeitos biológicos observados.

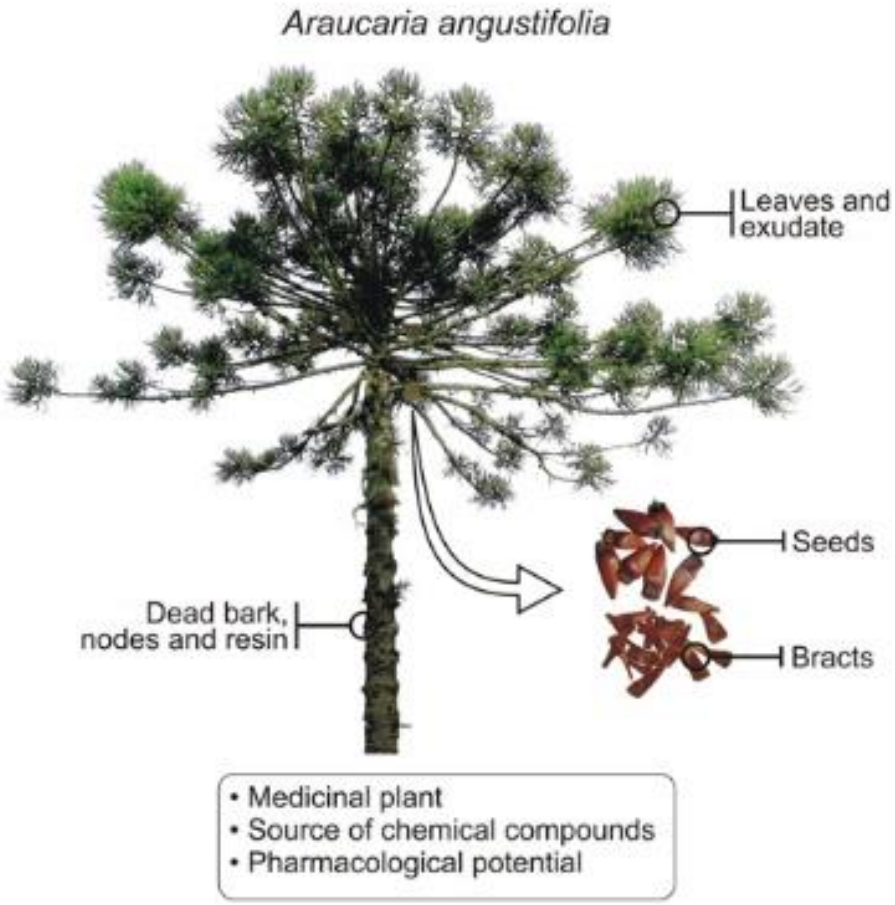
3 RESULTADOS

3.1 CAPÍTULO I

Chemical Constituents and Biological Activities of *Araucaria angustifolia* (Bertol.) O. Kuntze: A Review

Artigo publicado na revista *Organic & Inorganic Chemistry*

Graphical abstract



Review Article

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Chemical Constituents and Biological Activities of *Araucaria angustifolia* (Bertol.) O. Kuntze: A Review

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Abstract

Araucaria angustifolia is a tree that belongs to Araucariaceae family and it is mainly found in Southern Brazil. This plant has a notable therapeutical history in folk medicine holding great socioeconomic and environmental importance. Until now, some studies were conducted to assess its chemical composition, biological and pharmacological properties. The studies have shown that the bark, knot, needles (leaves), seeds and bracts (sterile seeds) contain high concentrations of active compounds and exhibit different biological effects. In the folk medicine the different parts of this plant are used to treat various types of illnesses, such as shingles, respiratory tract infections, sexually transmitted diseases and some types of wounds. Bearing this in mind, this review focuses on all currently chemical and biological effects already reported for *A. angustifolia* and provide a novel perspective and useful information for future research.

Keywords: *Araucaria angustifolia*; Medicinal plant; Chemical compounds; Biological effects

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Introduction

Araucaria is one among three genera that belong to the family Araucariaceae, occupying an isolated position amidst the conifers [1]. The genus Araucaria includes nineteen species and presents the largest geographical range in this family, widespread from South America to Australia and Pacific islands [2]. Although the family Araucariaceae is now restricted to the southern hemisphere [3], fossil evidence shows that it previously occurred also in the northern part of the globe [4]. *Araucaria angustifolia* (Bertol.) O. Kuntze is a subtropical species known popularly as Araucaria or Brazilian pine. Native species can be found in mountain climate throughout southern Brazil, northeastern Argentina and eastern Paraguay [1]. Nowadays, *A. angustifolia* is critically endangered [5] due to long periods of logging for wood and agriculture purposes [1]. The *araucaria* seed, named *pinhão,* is a seasonal product and has great nutritional value being a source of dietary fiber, carbohydrates, proteins and minor nutrients [6-8]. More importantly, they contain higher content of phenolic compounds [9]. In addition to the powerful chemical characterization of the seeds, other parts of the tree are equally important. Studies have reported that the resin (found in the wood and knots), the dead bark (which is naturally discarded by the tree) and the leaves (needles) retain an intriguing chemical composition with dynamic biological activity [10-16]. Our group has importantly contributions regarding *A. angustifolia* biological effects. We reported that bracts (sterile seeds) contain high levels of chemical compounds with important pharmacological actions in several models of study [17-20]. Despite the different extraction conditions employed in distinct studies, phenolic compounds can be regarded as the major constituents in this particular plant. Even though few pharmacological studies have been performed on this plant, there is a notable history of medicinal use by native populations. Infusions of leaves, bark and knots are used to treat anemia, muscle strains, varices, renal and sexually transmitted diseases [21-23]. Moreover, the syrup produced from resin is used to treat respiratory tract infections [24], indicating a therapeutic versatility in the empiric uses of *A. angustifolia*. Although the scientific literature about this plant is scarce, the majority of the studies have attempted to identify their chemical constituents and left aside their role in biological systems. Given this, the purpose of this review is to provide an overview of the main chemical compounds found in *A. angustifolia* and its biological activities, bringing evidences as basis for further research.

Botanical characterization

A. angustifolia is a subtropical gymnosperm pertaining to the family Araucariaceae, order Coniferales. This species was described for the first time by Bertoloni in 1820 as *Columbea angustifolia* Bert. After, it was redescribed by Richard Rich as *Araucaria brasiliana* and rectified by Otto Kuntze as *Araucaria angustifolia* (Bert.) Ktze [1]. It occurs as a major species within the Araucaria moist forests favored by altitudes ranging from 500 to 1,500 m [25]. The tree is tolerant to low temperatures [24] and has physiological adaptability to light and shade shifting conditions of the environment [1]. *A. angustifolia* stands out among other arboreal species due to its large and umbelliform canopy **(Figure 1)**. It features a rectilinear, cylindrical trunk that can reach 25 to 50 m height and may range between 1 to 2 m in diameter [26]. The trunk presents a purplish-brown colored, rough outer shell and an inner shell which is resinous and whitish [24]. As time goes by, the outer shell (dead bark) is naturally discarded by the tree, which may live 200 years on average [25]. The young tree is symmetrical, cone shaped, covered with alternate and grouped branches from base to apex, containing dark-green acicular (needle-shaped) leaves that remain attached to the tree for many years and can reach up to 6 cm long and 1 cm wide [1]. *A. angustifolia* is predominantly dioecious [27], *i.e.,* it features male and female specimens that have their own distinct strobili. The female strobili, known as cone, are globular or ovoid, having closely overlapping scales [28] and bracts inserted on a conic central axis **(Figure 2A)**. The male catkins are elongated, cylindrical, dense and covered by scales **(Figure 2B)**, which arrange themselves in a spiral [28]. The scales from the base open to allow the release of pollen and dissemination occurs through wind. Both strobili develop during summer [29]. The fecundated cone **(Figure 3A)** may measure 10 to 25 cm in diameter and weigh up to 4.7 kg, containing more than 1,000 elements [17], including seeds **(Figure 3B)** and bracts **(Figure 3C).** Bracts occurrence is about five times higher than fertile seeds [17]. The reproductive process of species is long. Pollination occurs during September and October and, once fertilized, the cones mature in 2 to 3 years [1,29]. It usually takes 12 to 15 years for a young plant to start producing seeds. These seeds, popularly known as *pinhão,* are dispersed mainly from May to August [1,25]. They exhibit yellowish-brown coloration, encased in a very resistant dark-brown coat along with an internal adherent membrane **(Figure 4)**. Araucaria seeds are fleshy and have ovate-oblong format, ranging from 3 to 8 cm in length and 1 to 2.5 cm in width, weighing approximately 8 g [6,9].

Figure 1 Photograph of pine *Araucaria angustifolia*. The tree exhibit canopy umbelliform in peculiar shape and large with a rectilinear and cylindrical trunk (photographed by Cátia S. Branco).

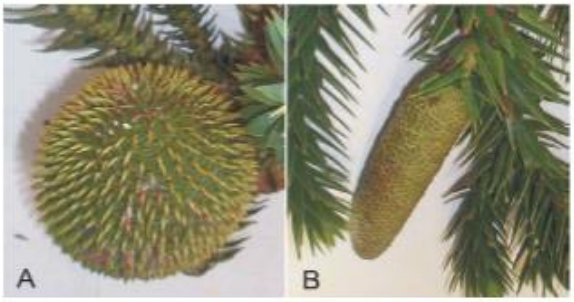

Figure 2 *Araucaria angustifolia* strobili female (A) and male (B) specimens (photographed by Cátia S. Branco).

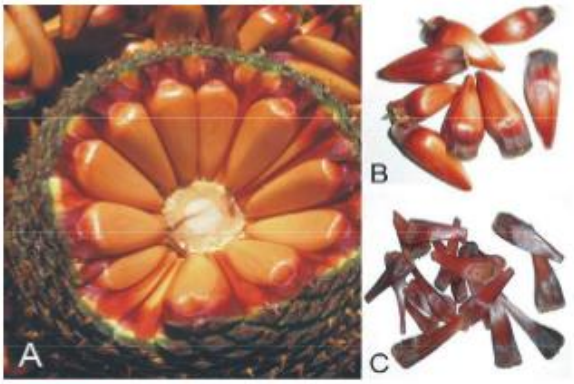

Figure 3 Mature female reproductive structure showing a conic central axis (A), seeds (B), and bracts (C). Photographed by Cátia S. Branco and adapted from Embrapa, Brazil (https://www.embrapa.br/busca-de-imagens/-/midia/685001/pinhas-e-pinhoes).

Ethnopharmacology

The medicinal potential of *A. angustifolia* has been initially explored by indigenous populations. Posteriorly, it began to be widely used in different parts of South America for treatment of several illnesses. Different parts of the tree (bark, resin, knots leaves and seeds) are employed in folk medicine, prepared mainly as infusions or tinctures. In respect to the empirical use of *A. angustifolia* bark, there are some reports about the use of infusions in order to treat topically muscle strains and varices [21]. Besides bark, the knots extracted from old trunk are used orally as infusion for treatment of renal and sexually transmitted diseases [21,22]. Moreover, a syrup produced from bark resin is used to treat respiratory tract infections, mainly bronchitis [24]. *A. angustifolia* leaves are also employed in ethnomedicine. Infusion of leaves (young or senescent) is orally used for the treatment of scrofula, fatigue and anemia, whereas tinctures from them are topically used on dermatological conditions such as dryness

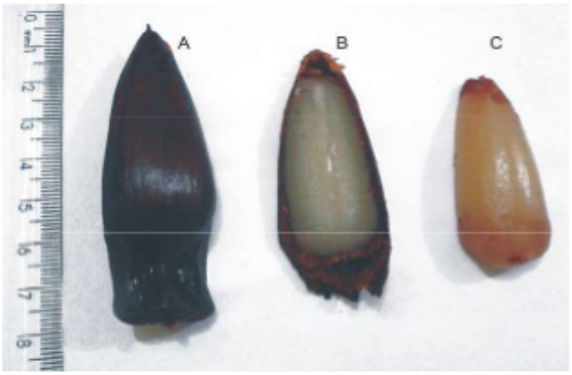

Figure 4 *Araucaria angustifolia* cooked seeds (*pinhão*) showing external coat (A), longitudinal section exhibiting embryo (B) and internal edible portion (C) (Photographed by Cátia S. Branco).

skin and wounds [21,22,24]. The leaves are also used to treat herpes disease, since they exhibit important antiviral activity [15]. Araucaria seeds are medically employed in treatment of heartburn, anemia and tumors [22,23].

Nutritional aspects

Apart from the empiric uses of the seeds, they are considered an excellent source of energy and nutrients, due to its constitution of complex carbohydrates [30] especially starch which displays low glycemic index when compared to white bread [6]. Furthermore, it presents low contents of lipids and proteins, being a source of dietary fiber, magnesium and copper **(Table 1)**. Proteins from *araucaria* seeds has nutritional value comparable to legume seeds that contain lysine and histidine as limiting amino acids [31], and may be used also as a complementary source of protein in food formulations [8]. Cooked seeds are reported as food of low glycemic index. This is mainly due to the high content of amylose in the starch that probably contributes to the formation of resistant starch in the seeds after cooking, generating a slow absorption of glucose by the organism [6]. Consumption of foods that present low glycemic response might play an important role in the prevention and treatment of metabolic chronic diseases and their cardiovascular complications, such as dyslipidemia, obesity and diabetes [32].

Chemical Characterization of *A. angustifolia*

Besides carbohydrates and proteins of nutritional interest, *A. angustifolia* is reported to contain important secondary metabolites. The plant is a rich source of polyphenols, besides steroids and terpenoids. A complete overview of the main chemical compounds described in different parts of *A. angustifolia* is shown in **Table 2** and a description of these constituents is given below.

Polyphenols

Polyphenols are structurally characterized by the presence of a benzene ring bound to one or more hydroxyl (-OH) groups [33]. Among the antioxidant compounds that integrate human diet, polyphenols are majority and great quantities of them are found in fruits, vegetables, nuts, cereals, chocolate and beverages including tea, coffee and wine (for review, see Ref. [34]). Regular consumption of polyphenols brings benefits for health specially reducing the risk of developing age-related degenerative diseases. These beneficial effects are associated to their capacity to scavenge oxidatively generated free radicals, such as those derived from lipids and nucleic acids [35,36]. There are a variety of classification systems for polyphenols, though they are generally divided into two major distinguishable classes regarding their basic chemical structure: flavonoids and nonflavonoids [34].

Flavonoids: Flavonoids may be divided into six subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavan-3-ols [34]. The most common flavonoids found in *A. angustifolia* have their chemical structure presented in **(Figure 5).** Catechin **(Figure 5A)** and epicatechin **(Figure 5B)** are flavan-3-ols, the most complex subclass of flavonoids. This subclass ranges from simple monomers to oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins [34,37]. Quercetin **(Figure 5C)** is a flavonol, whereas apigenin **(Figure 5D)** is a flavone. Seccon *et al.* [12] showed that the hydroalcoholic extract from Araucaria dead bark presents a total phenolic content of 64 mg/g of gallic acid equivalents (including 1.85 mg/g of anthocyanin content and 12 mg/g of proanthocyanidin content) along with the presence of flavonols, mainly quercetin, besides the flavan-3-ols catechin and epicatechin. Biflavonoids (dimeric flavonoids) were identified in *A. angustifolia* leaves [13-15], besides high amounts of proanthocyanidins [15]. Flavonoids are also found in *A. angustifolia* propolis, which is the resinous substance collected from the tree and processed by bees [38,39], and also in the seeds. Cordenunsi *et al.* [6] described that, in the seed, phenolic compounds can migrate from the internal coat to the pulp during heating process, and therefore significant amounts of catechin and quercetin may be

Table 1 Proximate composition of cooked *Araucaria angustifolia* seeds (*pinhão*).

Composition		Value
Moisture (g/100 g)		50.35 ± 0.71
Starch (g/100 g)		34.48 ± 0.72
Protein (g/100 g)		3.31 ± 0.05
Lipid (g/100 g)		1.26 ± 0.09
Ash (g/100 g)		1.41 ± 0.02
Dietary Fiber (g/100 g)	Soluble	0.55 ± 0.18
	Insoluble	5.17 ± 0.25
Calcium (mg/100 g)		16.0
Potassium (mg/100 g)		727.0
Magnesium (mg/100 g)		53.0
Iron (mg/100 g)		0.80
Zinc (mg/100 g)		0.80
Copper (mg/100 g)		0.18

Adapted from Cordenunsi et al. [6] and Brazilian Food Composition Table [30]

Table 2 Overview on the chemical constituents identified in different parts of *Araucaria angustifolia*.

Plant Material	Solvent Used	Analysis Method	Compounds Identified	Reference
Bark	Benzene	Chromatography	(+)-pinoresinol dimethyl ether (eudesmine)[P], sitosterol[S] and sugiol[T]	[50]
Bark	Ethanol/water	LC/UV spectrophotometry/ NMR	Benzoic acid[P], *p*-hydroxybenzoic acid[P], protocatechuic acid[P], quercetin[P], (-)-epiafzelechin protocatechuate[P], (-)-epiafzelechin *p*-hydroxybenzoate[P] and (-)-epicatechin[P]	[12]
Bracts	Water	HPLC/UV spectrophotometry	Catechin[P], epicatechin[P] and rutin[P]	[17]
Bracts	Water	HPLC/UV spectrophotometry	Catechin[P], epicatechin[P], quercetin[P] and apigenin[P]	[18]
Bracts	Water	HRMS (MS, MS/MS mode)	Quinic acid, 4'-methoxytectorigenin[P], 3-glucoside-dihydroquercetin[P] and amentoflavone 4',4'',7,7''-tetramethyl ether[P]	[19]
Knot	Benzene	Chromatography/^{13}C NMR	Secoisolariciresinol monomethyl ether[P] and lariciresinol-4-methyl ether[P]	[48]
Knot	Benzene	Chromatography/^{13}C NMR	Pinoresinol[P], secoisolariciresinol[P], lariciresinol[P], isolariciresinol[P] and isolariciresinol-4'-methyl ether[P]	[49]
Leaves	Methanol	HPLC/MS/NMR	Amentoflavone[P], mono-, di-, tri- and tetra-*O*-methylamentoflavone[P] and ginkgetin[P]	[13,14]
Leaves	Ethanol / water	HPLC/TLC/UV spectrophotometry/NMR	Bilobetin[P], II-7-*O*-methyl-robustaflavone[P] and cupressuflavone[P]	[15]
Leaves	Ethanol	GC/MS	B-sitosterol[S], ent-kaurene[T] and phyllocladene[T]	[16]
Leaves (exudate)	Acetone	TLC/CG-MS	*p*-coumaric acid[P], *E* and *Z* communic diterpenic acids[T]	[43]
Propolis	Ethanol	TLC/CG/CG-MS	5,6,7-trihydroxy-3,4'-dimethoxyflavone[P], kaempferid[P], aromadendrine-4'-methyl ether[P], *E* and *Z* 2,2-dymethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes[P], dihydrocinnamic acid[P], *p*-coumaric acid[P], ferulic acid[P], caffeic acid[P] and di- and triterpenes[T]	[38]
Propolis	Ethanol/water	HPLC/UV spectrophotometry/NMR	Quercetin[P], rutin[P], gallic acid[P], protocatechuic acid[P], chlorogenic acid[P] and its esther derivates, *p*-coumaric acid[P], syringic acid[P], vanillic acid[P], caffeic acid[P] and ferulic acids[P]	[39]
Resin (knot)	Chloroform/methanol	TLC/^{1}H and ^{13}C NMR	Hinokiresinol[P], cryptoresinol[P], secoisolariciresinol[P], isolariciresinol[P], pinoresinol monomethyl ether[P], 2,3-bis-(*p*-hydroxyphenyl)-2-cyclopentene-1-one[P] and 4,4'-dihydroxychalcone[P]	[11]
Resin (wood)	Dichloromethane/ methanol	GC/MS	Lignans[P], 4-hydroxybenzaldehyde[P], hydroquinone[P], *p*-coumaric acid[P], ferruginol diterpene[T]	[10]
Seedling stages	Ethanol	TLC/MS/NMR	*E* and *Z* octadecyl ferulate[P], biflavones[P], benzaldehydes[P] and its derivates, vanillin[P], pinoresinol[P], eudesmin[P], lariciresinol[P], cabreuvine[P], irisolidone[P] and diterpenes[T]	[41]
Seeds	Phosphate-HCl buffer	HPLC	Lectins	[61,62]
Seeds	Methanol	HPLC	Catechin[P] and quercetin[P]	[6]
Seeds	*N*-hexane/water	Chromatography	*N*-acetyl-D-glucosamine-specif lectin	[59]
Seeds	Glycine-HCl buffer	Chromatography	Lectins	[60]
Seeds	ND	Chromatography	Lectins	[65]
Seeds	Ethanol / water	HPLC	Catechin[P], quercetin[P] and gallic acid[P]	[9]
Seeds	Methanol	HPTLC	Flavonoids[P] and proanthocyanidins[P]	[40]
Seeds (coat)	Ethanol / water	Chromatography	Proanthocyanidins[P]	[64]

Analysis Methods: gas chromatography (GC); liquid chromatography (LC); thin layer chromatography (TLC); high performance liquid chromatography (HPLC); high performance thin layer chromatography (HPTLC); high resolution mass spectrometry (HRMS); multiple reaction monitoring mode (MRM); mass spectrometry (MS); nuclear magnetic resonance (NMR); not described (ND).
Compounds Classes: polyphenols[P]; steroids[S]; terpens[T].

found in cooked seed. Similar results were reported in another study [9], showing differences between extracts obtained from cooked and raw seeds regarding flavonoids (11.89 ± 0.26 µg/mg in cooked seeds and 1.16 ± 0.02 µg/mg in raw seeds extract) and proanthocyanidins (40.70 ± 0.59 µg/mg in cooked seeds and 0.45 ± 0.02 µg/mg in raw seeds extracts) contents. Recently, Mota *et al.* [40] investigated two extracts, one from the external coat and other from the inner seed pulp (endosperm and embryo). Both

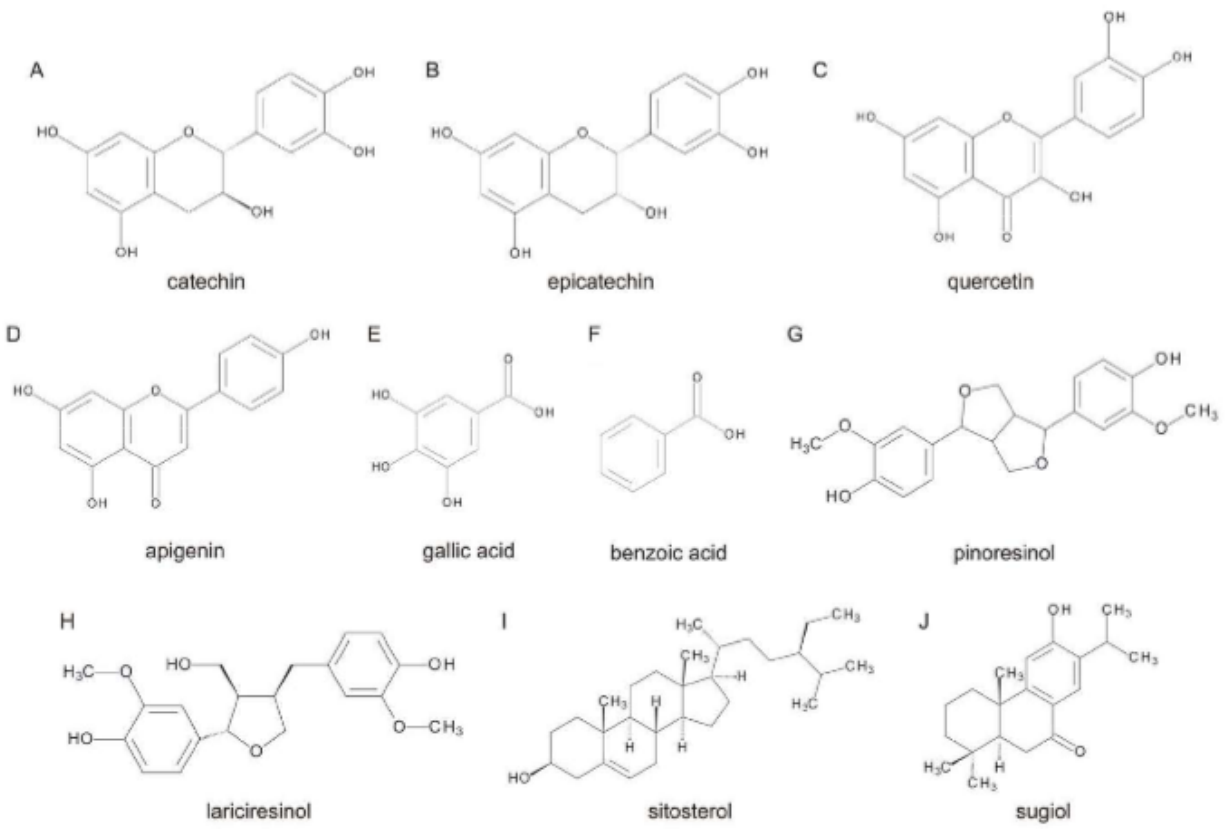

Figure 5 Main chemical compounds found in *Araucaria angustifolia* tree. Images obtained using Symyx Draw 4.0.0 software (Symyx Technologies Inc., Santa Clara, California, USA).

of them presented proanthocyanidins in its constitution, but the coat extract exhibited around tenfold the concentration of proanthocyanidins compared to inner seed extract (148 versus 10 mg cyanidine chlorohydrate/g extract). Taking into account the high levels of flavonoids found in *araucaria* seeds, our research group has focused on the bracts (sterile seeds), demonstrating that this material is rich in flavan-3-ols, flavonols [17], flavones [18] and biflavonoids [19]. Biflavonoids were also reported by Fonseca *et al.* [41] in tissues samples from *A. angustifolia* at different stages of differentiation and development, showing that seedlings stems, which are source of explants, display a variety of apigenin dimers.

Phenolic acids and their derivatives: Phenolic acids, other important class of polyphenols, can be classified as derivatives of either benzoic acid or cinnamic acid. Benzoic acid derivatives include gallic (3,4,5-trihydroxy-), vanillic (4-hydroxy-3-methoxy-) and protocatechuic (3,4-dihydroxy-) acids, whereas *p*-coumaric (4-hydroxy-), caffeic (3,4-dihydroxy-), and ferulic (4-hydroxy-3-methoxy-) acids are cinnamic acid derivatives. In addition, phenolic acids may originate from hydroxycinnamic acid, such as chlorogenic acid (3-caffeoylquinic acid) [42]. It has been shown that *A. angustifolia* species contain phenolic acids **(Table 2)**, being gallic acid **(Figure 5E)** the most common one. This compound was already detected in the seeds [9] and in the propolis [39], whereas benzoic acid **(Figure 5F)** was identified in the bark [12], and also in the propolis [39]. Exudate from leaves presents *p*-coumaric acid [43], which was also found in the propolis [10] along with dihydrocinnamic, ferulic and caffeic acids [38]. Some other phenolic acids, including protocatechuic acid (9.1 µg/mL) and ester derivatives of chlorogenic acid (7.9 µg/mL), were quantified in *A. angustifolia* propolis along with minor amounts (<1.8 µg/mL) of *p*-coumaric, syringic, vanillic, caffeic and ferulic acids [39].

Lignans: Plant secondary metabolites also include lignans, compounds that present carbon skeletons constructed by the oxidative coupling of two or three phenyl propane units and are biosynthesized through the shikimic acid pathway [10,44]. They are classified in five main types: lignans, neolignans, norlignans, hybrid lignans, and oligomeric lignans [45]. These compounds are important components of foods since they occur either freely or in its glycosylated form in plants, mainly wood and resin [46,47]. Some lignans extracted from plants of the genus *Araucaria* have been analyzed, specifically in the species *A. angustifolia* [10,11,41,48-50] and *A. araucana* [51]. Regarding *A. angustifolia*, lignans have been reported mainly in the wood, knots and

resin, and the chemical structures of the most representative compounds are shown in **(Figures 5G-5H).** Fonseca *et al.* [48,49] identified different lignans, such as pinoresinol dimethyl ether, secoisolariciresinol, lariciresinol, isolariciresinol, isolariciresinol-4'-methyl ether, secoisolariciresinol monomethyl ether and lariciresinol-4-methyl ether in the knots of *A. angustifolia* dead trees. Presence of lignans including secoisolariciresinols, lariciresinols and pinoresinols were also detected from the knot [11] and wood [10] resins. Other parts of *A. angustifolia*, including seedling stems [41] and trunk bark [50] also contain lignans (pinoresinols, lariciresinols and eudesmin) in its composition.

Steroids and terpenes

Steroids and terpenes are isoprenoids synthesized *via* the mevalonate pathway. They constitute an important group of small secondary metabolites associate with plant signaling. Terpenic compounds, including di- and triterpenes usually accumulate as conjugates with carbohydrates and other macromolecules, mainly as triterpene glycosides [52]. Bankova *et al.* [43] reported the presence of terpenes, specifically diterpenic acids in the exudate from *A. angustifolia* leaves. Diterpenes were also identified in the wood resin [10]. Besides resin, propolis contain terpenic compounds mostly di- and triterpenes [38]. Steroids and terpenoids were also identified in *A. angustifolia* leaves [16] and bark, mainly the steroid sitosterol **(Figure 5I)** and the diterpene sugiol **(Figure 5J)** [50]. Isoprenoids are synthesized during different stages of plant development. Fonseca *et al.* [41] detected diterpenes from the seedling to roots of *A. angustifolia.*

Biological Activities

Despite its traditional uses as medicinal product, there are few studies aiming to elucidate the pharmacological potential of the different parts *A. angustifolia* tree. Major studies regarding the diverse biological activities are summarized in **Table 3.**

Antioxidant activity

Antioxidant activity is the most reported biological action for *A. angustifolia*. The antioxidant defense system controls the levels of reactive oxygen species (ROS) promoting useful molecular functions minimizing oxidative damage [35]. Taking into account that the misbalance of the antioxidant system lead to the generation of oxidative stress and this disturbance exerts a critical role in aging chronic degenerative diseases and cancer, natural products rich in antioxidants may be considered as health-promoting bioactive agents [53-55]. Corroborating with this evidence, several reports showed that *A. angustifolia* exerts antioxidant effects in different study models **(Table 3)**. An extract from araucaria dead bark demonstrated cytoprotective effects in mouse L929 fibroblasts against oxidative stress induced by hydrogen peroxide (H_2O_2). In a dose-responsive manner, the highest concentration of 1 mg/mL was able to increase cellular protection by 131% [12]. The compound catechin isolated from the bark was able to protect against lipid peroxidation induced by UV radiation (IC_{50} 18 ± 4 µM) and ascorbyl radical (IC_{50} 6 ± 0.25 µM) exposure in rat liver treated microsomes [12]. In a similar way, our group previously reported that an extract obtained from *A. angustifolia* bracts displayed antioxidant effects against ROS-induced oxidative stress in *Saccharomyces cerevisiae*. The concentration of 0.15% avoided cytotoxic effects induced by H_2O_2 and, the concentrations of 0.05, 0.10 and 0.15% exhibited non-mutagenic and antimutagenic activities in the same model. These effects were attributed to the antioxidant capacity of phenolic compounds, which could be responsible for neutralizing H_2O_2 avoiding hydroxyl radical formation, therefore preventing DNA damage [17]. Our group also reported that the bracts extract presented antioxidant and antigenotoxic activities in MRC5 human lung fibroblast cells. In this study, the aqueous extract from araucaria bracts (25 and 50 µg/mL) significantly protected MRC5 cells against H_2O_2-induced cytotoxicity and oxidative damage to lipids, proteins and DNA [18]. Additionally, the bracts extract was able to avoid depletion of superoxide dismutase and catalase activities [18]. Yamaguchi *et al.* [13] reported that a biflavonoid fraction obtained from extract of *A. angustifolia* leaves was effective to protect plasmid DNA against single strand break induced by either singlet oxygen or Fenton reaction at concentrations of 20 to 100 µM. The antioxidant effect of this extract was also evaluated in calf thymus DNA and the biflavonoid fraction was capable of preventing formation of cyclobutane thymine dimer (at concentrations of 0.5 to 2.0 mg) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (at concentrations of 100 and 500 µM), avoiding oxidation of nucleotides [14].

Antiproliferative and cytotoxic activities

There are few studies in the scientific literature regarding the potential anticancer effects of *A. angustifolia*. Búfalo *et al.* [38] reported cytotoxic effects of araucaria propolis on human laryngeal epidermoid carcinoma (HEp-2) cells. In this study, it was observed cytotoxic effects induced by propolis in a dose (5, 10, 25, 50 and 100 µg/well) and time (6, 24, 48 and 72 h) dependent manner against HEp-2 cells *in vitro*. Moreover, changes in cell morphology were observed, including lysis and disorganization of the cellular monolayer. In another study, Meneghelli *et al.* [39] reported the effects of *A. angustifolia* propolis on viability, proliferation, cell migration, capillary tube formation, and angiogenesis using *in vitro* and *in vivo* models. Hydroalcoholic extract of propolis was able to decrease viability in 24 (IC_{50} 297 µg/mL) and 72 (IC_{50} 130 µg/mL) hours, proliferation (at doses of 130 to 180 µg/mL for 72 h) and tubulogenesis (at doses of 150 to 200 µg/mL for 24 h) on human endothelial cells. Furthermore, the angiogenesis and vasculogenesis processes, also evaluated in this study, were inhibited using chorioallantoic and yolk-sac membranes from chick embryos (450 mg propolis.kg^{-1}), indicating that *A. angustifolia* propolis is a potential therapeutic agent for angiogenic diseases, such as cancer. Recently, our group demonstrated that the aqueous extract from *A. angustifolia* bracts present selective antiproliferative effects (IC_{50} 250 µg/mL for 24 h) mediated by mitochondrial dysfunction and apoptosis activation on human larynx HEp-2 cancer cells. The inhibition of cell proliferation was accompanied by pro-oxidant effects, including oxidative damage to lipids and proteins, nitric oxide production, along with depletion of superoxide dismutase and catalase antioxidant activities at concentrations (100, 250 and 500 µg/mL). The bracts extract also generated genotoxic and apoptotic effects, as well as inhibition on complex I of the mitochondrial electron transport chain and reduction on ATP

Table 3 Overview on the biological effects of *Araucaria angustifolia*.

Plant Material	Study Model	Biological Effects	Reference
Bark	*In vivo* (L929 mouse fibroblasts and rat liver microsomes)	Antioxidant activity against H_2O_2-induced oxidative stress; prevention of microsome lipid peroxidation	[12]
Bracts	*In vivo* (cells of *Saccharomyces cerevisae*)	Antioxidant and antimutagenic activities against H_2O_2	[17]
Bracts	*In vitro* (MRC5 human lung fibroblast cells)	Antioxidant and antigenotoxic activities against H_2O_2	[18]
Bracts	*In vivo* (velvetbean caterpillar *Anticarsia gemmatalis*)	Entomotoxic; pro-oxidant and genotoxic activities	[20]
Bracts	*In vitro* (human larynx Hep-2 cancer cells)	Antitumor and pro-apoptotic activities; induction of mitochondrial dysfunction; genotoxicity	[19]
Leaves	*In vitro* (*Escherichia coli* HB 101 / plasmid pBluescript PUC19	Effective to quench singlet oxygen ($^1O_2$); DNA protection against single strand break	[13]
Leaves	*In vitro* (Calf thymus DNA)	Protection against UV-induced thymine dimmers formation	[14]
Leaves	*In vitro* (Herpes Simplex Virus type 1)	Antiviral activity	[15]
Leaves	*In vitro* (*Lactuca sativa* seeds)	Allelopathic activity	[16]
Leaves (exudate)	*In vitro* (*Staphylococcus aureus* and *Candida albicans*)	Antibacterial action but no antifungal effect	[43]
Propolis	*In vitro* (human larynx Hep-2 cancer cells)	Cytotoxicity	[38]
Propolis	*In vitro* and *in vivo* (human endothelial cells and chick embryos)	Antiproliferative, antitubulogenic and antiangiogenic activities	[39]
Seeds	*In vivo* (human volunteers)	Low glycemic response	[6]
Seeds	*In vitro* and *in vivo* (*X. axonopodis* pv. *passiflorae*, *C. michiganensis* subsp. *Michiganensis*, rats and rabbits)	Antibacterial activity against Gram-positive and –negative; anti-inflammatory and hemagglutinating activities	[59]
Seeds	*In vivo* (Swiss and Wistar rats)	Anti- and pro-edematogenic actions	[60]
Seeds	*In vivo* (Swiss rats)	Depressant activity in the central nervous system	[65]
Seeds (coat)	*In vitro* and *in vivo* (human and porcine α-amylase and rats)	Inhibition of human salivary and porcine pancreatic α-amylase; reduction in post-prandial glycemic levels in rats	[64]

production, indicating the potential of bioactive compounds from *A. angustifolia* bracts on the modulation of mitochondrial function on cancer cells [19,56].

Allelopathic and entomotoxic activities

Plants produce and store high levels of secondary metabolites that are subsequently released into the environment, which may affect other plants or animals [57]. Plants also present entomotoxic actions against insect-pests, showing an important potential to be explored mainly due to the emergence of insects resistant to chemical insecticides and to the rise of organic agriculture [58]. There are only two studies in the scientific literature exploring the allelopathic and entomotoxic potentials of araucaria. In a study conducted by Braine *et al.* [16], it was showed that the extract from *A. angustifolia* leaves (187.5 and 250 mg) presents allelopathic potential on germination and growth of *Lactuca sativa* seeds, and the main allelochemical compounds identified in the ethanolic extract were *ent*-kaurene and phyllocladene. This allelopathic potential seems to serve *A. angustifolia* on the successional dynamics of Araucaria Moist Forests. Our group has investigated the entomotoxic effect of an aqueous extract obtained from bracts of *A. angustifolia* on the velvetbean caterpillar *Anticarsia gemmatalis* (Lepidoptera: Erebidae) [20]. This extract, rich in phenolic compounds, was able to increase the number of malformed pupae (0.15, 1.5 and 7.5 mg), along with a decrease in the emergence of the insects (1.5 and 7.5 mg), and these effects were related with the lipid, protein and DNA damage detected in the larvae *via* oxidative stress.

Antiviral and antibacterial activities

In traditional medicine *A. angustifolia* has been used for treatment of infectious diseases caused by pathogenic agents. The antiviral activity of araucaria was showed in a study conducted by Freitas *et al.* [15] with Herpes simplex virus type 1 (HSV-1) model. HSV is a double-stranded DNA enveloped virus, extremely widespread in human populations and responsible for a broad range of human infectious diseases, such as gingivo-stomatitis, genital diseases and encephalitis. The crude hydroethanolic extract (HE) obtained from *A. angustifolia* leaves and some different fractions of this extract were able to induce virucidal activity against HSV-1, exhibiting antiherpetic potential (IC_{50} 32.10 ± 3.65 µg/mL for HE). This effect was associated with the content of biflavonoids and proanthocyanidins present in the leaves.

Antibacterial potential of *A. angustifolia* was investigated in a few studies. Santi-Gadelha *et al.* [59] prepared an aqueous extract from *araucaria* seeds and then purified and characterized a lectin (N-acetyl-D-glucosamine-specific lectin) with antibacterial activity (150 µg/mL) against Gram-negative (*Xanthomonas axonopodis pv. passiflorae*) and Gram-positive (*Clavibacter michiganensis* subsp. Michiganensis) strains at doses of 150 µg/mL. Bankova *et al.* [43] also reported the antibacterial action of leaves exudate (0.4 mg of extract) against strains of the pathogen *Staphylococcus aureus*, which is a major causing agent of nosocomial and community-acquired infections.

5.5 Anti-inflammatory and antiedematogenic activities

Chemical compounds from plants are able to exhibit anti-inflammatory and antiedematogenic activities. It has been shown that *A. angustifolia* seeds may modulate acute inflammation process *in vivo*. Santi-Gadelha *et al.* [59] elucidate the mechanism involved in the anti-inflammatory effect of a lectin (N-acetyl-D-glucosamine-specific lectin) from aqueous extract of *A. angustifolia* seeds in rats. The inflammation was induced in a paw edema model by subcutaneous injection of carrageenan, which is able to release several inflammatory mediators, including biogenic amines, prostaglandins, and nitric oxide. Intravenous injection of lectin (0.01, 0.1 and 1 mg/kg) prior carrageenan reduced paw edema inhibiting the cellular event of acute inflammation *via* carbohydrate site interaction [59]. In another study, Mota *et al.* [60] have described the effects of a lectin (N-acetyl-glucosamine-ligant) from *A. angustifolia* seeds in the same model of paw edema in rats, and the anti-inflammatory and pro-edematogenic actions were evaluated. Intravenous injection of lectin (0.1 and 1 mg/kg) inhibited the dextran-induced edema and vascular permeability, which were prevented by association of the lectin with its binding sugar N-acetyl-glucosamine. Lectin also inhibited edema induced by serotonin. The mechanism associated with anti-inflammatory and pro-edematogenic actions appears to be involved in a common pathway to activation or inhibition of inflammatory mediators from resident mast cells [60].

Actions on metabolism and central nervous system

Amongst the different parts of *A. angustifolia*, the seed has been reported as having effects on modulation of energetic metabolism and central nervous system. Araucaria seeds are rich in lectins [61,62], an important group of glycoproteins widely studied. Lectins are able to induce different actions in various biological systems, including cell agglutination and glycoconjugate precipitation, once they recognize and bind to carbohydrates or other substances derived from sugars [63]. The effect of *A. angustifolia* seeds consumption on the glycemic metabolism was studied for the first time by Cordenunsi *et al.* [6]. The analysis of the carbohydrate availability evaluated in a short-term assay (two weeks) in humans showed that the glycemic responses produced by seeds cooked with the coat were 23% lower when compared to white bread. This result is important because foods that present a low glycemic index are linked to the beneficial effects on preventing and controling chronic non-infectious diseases. In a recent study, Silva *et al.* [64] also investigated the effects of a seed coat extract on glycemic levels of rats and activity of α-amylases (human salivary and porcine pancreatic). The seeds coat extract (250 mg/kg), rich in proanthocyanidins, was effective in diminishing the post-prandial glycemic levels in rats after starch administration. Moreover, the extract was able to inhibit both human salivary (range up to 80 µg/mL) and porcine pancreatic (range up to 50 µg/mL) α-amylases, indicating that araucaria seeds present potential to be used in therapeutic interventions aiming to suppress postprandial hyperglycemia in diabetic patients. The effects of araucaria lectins on central nervous system were investigated in an animal model of epilepsy [65]. Lectin was isolated and purified from *A. angustifolia* seeds, and then dissolved in saline before administration to rats. Seizures were induced with pentylenetetrazol, pilocarpine and strychnine and were monitored during 1 hour. The behavioral profile (locomotor activity) was also evaluated. Lectin (10 mg/kg) increased latency to convulsions and latency to death in both the pentylenetetrazol- and strychnine-induced seizures; however it was not able to protect in the pilocarpine model. In addition, lectin (0.1, 1 and 10 mg/kg) was able to reduce locomotor activity, showing depressant effect similar to the drug diazepam. These effects were attributed to the capacity of lectin from *araucaria* seeds to modulate GABAergic and glycinergic systems.

Conclusions and Future Prospects

This is the first work that has summarized the relevant literature concerning the chemical constituents, biological activities and ethnobotanical aspects of the conifer *A. angustifolia*, an important plant with a long tradition of medicinal and nutritional uses in South America. This review provides many contributions for the natural products research area, since they show the beneficial effects performed by the chemical compounds existent in this plant for prevention and treatment of some human pathologies. Considering the social and ecological importance of *A. angustifolia*, it is essential that conservation programs must be performed and constantly updated. Moreover, it is also important to improve researches involving the development of pharmaceutical products using residual parts of the plant, since the use of bracts, dead barks and needles would not compromise *A. angustifolia* reminiscent populations nor human and animal feed.

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3.2 CAPÍTULO II

Mitochondria and redox homoeostasis as chemotherapeutic targets of *Araucaria angustifolia* (Bert.) O. Kuntze in human larynx HEp-2 cancer cells

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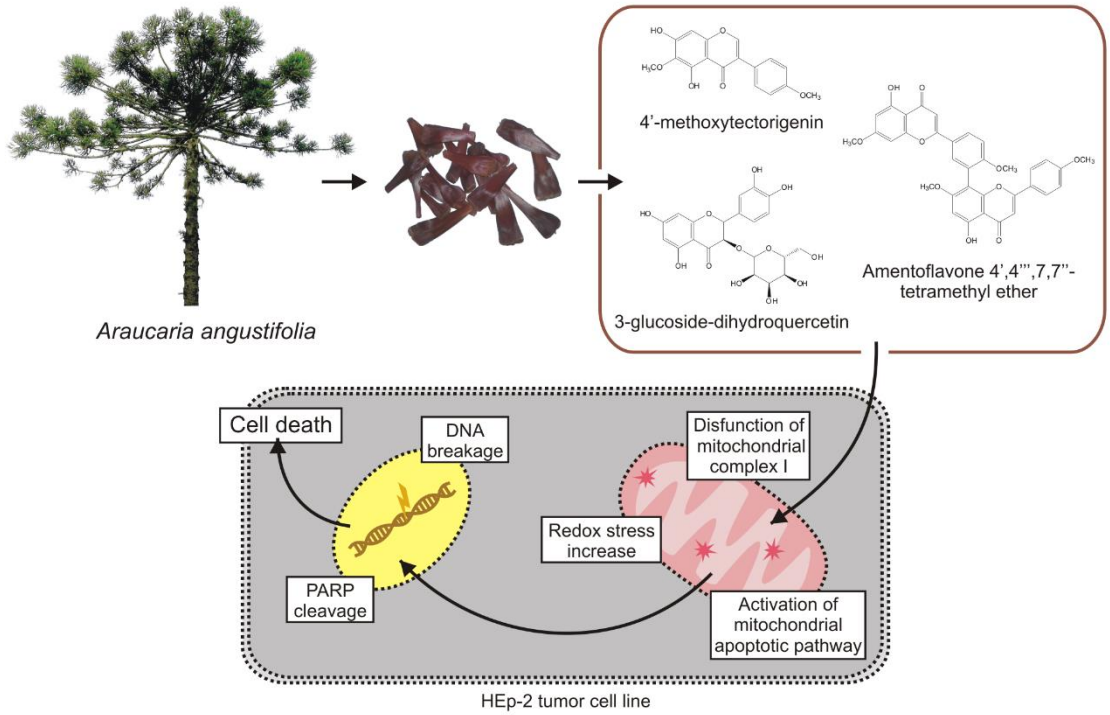
Graphical abstract



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Mitochondria and redox homoeostasis as chemotherapeutic targets of *Araucaria angustifolia* (Bert.) O. Kuntze in human larynx HEp-2 cancer cells

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ABSTRACT

Natural products are among one of the most promising fields in finding new molecular targets in cancer therapy. Laryngeal carcinoma is one of the most common cancers affecting the head and neck regions, and is associated with high morbidity rate if left untreated. The aim of this study was to examine the antiproliferative effect of *Araucaria angustifolia* on laryngeal carcinoma HEp-2 cells. The results showed that *A. angustifolia* extract (AAE) induced a significant cytotoxicity in HEp-2 cells compared to the non-tumor human epithelial (HEK-293) cells, indicating a selective activity of AAE for the cancer cells. *A. angustifolia* extract was able to increase oxidative damage to lipids and proteins, and the production of nitric oxide, along with the depletion of enzymatic antioxidant defenses (superoxide dismutase and catalase) in the tumor cell line. Moreover, AAE was able to induce DNA damage, nuclear fragmentation and chromatin condensation. A significant increase in the Apoptosis Inducing Factor (AIF), Bax, poly-(ADP-ribose) polymerase (PARP) and caspase-3 cleavage expression were also found. These effects could be related to the ability of AAE to increase the production of reactive oxygen species through inhibition of the mitochondrial electron transport chain complex I activity and ATP production by the tumor cells. The phytochemical analysis of *A. angustifolia*, performed using High Resolution Mass Spectrometry (HRMS) in MS and MS/MS mode, showed the presence of dodecanoic and hexadecanoic acids, and phenolic compounds, which may be associated with the chemotherapeutic effect observed in this study.

1. Introduction

Cancer represents the second leading cause of death in occidental countries. Laryngeal carcinoma is one of the most common form of tumor cells that reach the head and neck, and it represents about 30% of all malignancies in these body areas [1]. Although there are effective drugs for treating several types of cancer, laryngeal cancer is one of the many cancers that still lack appropriate treatment, thus giving rise to considerable morbidity and mortality rates in patients. The resistance of tumor cells depend mainly on their capacity to resist to apoptosis and growth-inhibitory signals, thereby leading to tissue invasion and metastasis [2]. Some of these resistance characteristics are dependent on redox homoeostasis alterations in cancer cells [3]. Therefore, it is important to investigate new therapeutic agents, which may present selective properties to induce cytotoxicity through the activation of cell death signaling pathways.

Plant-derived products possess a wide range of pharmacological actions, including chemopreventive, anticancer and apoptotic properties [4–6]. *Araucaria angustifolia* (Bertolini) Otto Kuntze belongs to the Araucariaceae family and is one of the main pine species found in South America. In Brazil, native populations of this species occur essentially in the southern highlands, constituting the Araucaria forests. *A. angustifolia* is a dioecious species, meaning male and female specimens have their own distinct strobili. Female strobilus consists of seeds (the edible part named "pinhão") and bracts, which are undeveloped seeds commonly discarded into the environment. Moreover, *A. angustifolia* presents great relevance in the Brazilian folk medicine. Different parts of the tree (bark,

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needles and resin) are used to treat various illnesses, particularly diseases of the respiratory tract. Recently, our group has characterized an *A. angustifolia* extract obtained from the bracts, which is rich in phenolic compounds such as flavonoids and tannins [7,8].

Phenolic compounds are multifaceted molecules, which have powerful antioxidant abilities. Nevertheless, some of these compounds may promote the formation of reactive oxygen species (ROS) [6,9] mainly through the mitochondria. The effect resulting from ROS production occurs through additional mechanisms of action in which the polyphenols are able to generate *o*-semiquinones and to oxidize NADH, resulting in inhibition of mitochondrial respiration [10]. Mitochondrial dysfunction is often associated with oxidative damage, defective ATP synthesis, and cell death, showing that mitochondria might be an important therapeutic target for cancer treatment [11,12]. Inhibition of mitochondrial respiration has been associated with the ability of polyphenols to modulate the dynamics of cancer cells in order to exert selective anticancer effects [6]. However, the mechanism of action of polyphenols in implicating this effect still remains unclear.

In this context, the objective of this study was to (1) evaluate the antiproliferative activity of an extract obtained from *A. angustifolia* bracts on human laryngeal carcinoma (HEp-2) and non-tumor (HEK-293) cells; (2) explore the cytotoxic mechanism of action of the extract in tumor cells and to; (3) evaluate the chemical composition of AAE to better examine the biological properties of this extract.

2. Materials and methods

2.1. Chemicals

Complete Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), trypsin–EDTA and penicillin–streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Apoptosis antibody sampler kits (CS9915, CS9942 and CS4670) were acquired from Cell Signaling Technology (Danvers, MA, USA). Complex I Enzyme Activity Microplate Assay Kit was purchased from Abcam (Cambridge, MA, USA), and the Cell Titer-Glo® Luminescent Cell Viability Assay Kit from Promega (Madison, WIS, USA). Low-melting point agarose and normal agarose were purchased from Invitrogen (Carlsbad, CA, USA). Acrylamide kit was acquired from Sigma–Aldrich (St. Louis, MO, USA). Other reagents and solvents were obtained from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade.

2.2. A. angustifolia extract

Female strobili of *A. angustifolia* were collected in Caxias do Sul, Rio Grande do Sul (29°9′34.90″S, 51°8′45.34″W); Ibama n° 02001.001127/2013-94, Brazil. Voucher specimens were identified by the Herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (HUCS 40710/40711). Bracts were manually separated from the pine and the extract was obtained under reflux (100 °C) for 15 min using 5 g of bracts in 100 mL of distilled water, as already described [7,8,13]. The extract was filtered in Millipore equipment (pore size, 0.45 μm; SFGS 047LS, Millipore Corp.) and lyophilized (LIOBRAS model L-101) under vacuum pressure to yield a powder, which was stored protected from light until use.

2.3. Mass Spectrometry

The lyophilized extract of *A. angustifolia* was dissolved in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid or 0.1% ammonia hydroxide for ESI(+) or ESI(−) respectively. The solutions were individually infused directly or with HPLC (Shymadzu) assistance into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 μL min^{-1}. ESI(+)-MS, tandem ESI(+)-MS/MS and ESI(−)-MS were acquired using a hybrid high-resolution and high accuracy (5 μL/L) microTOF-QII mass spectrometer (Bruker® Daltonics) under the following conditions: capillary and cone voltages were set to +3500 and +40 V, respectively, with a de-solvation temperature of 100 °C. Diagnostic ions in different fractions were identified by the comparison of exact *m/z* with compounds identified in previous studies. For data acquisition and processing, Hystar software (Bruker® Daltonics) was used. The data were collected in the *m/z* range of 70–800 at the speed of two scans per second, providing the resolution of 50,000 (FWHM) at *m/z* 200. No important ions were observed below *m/z* 150 or above *m/z* 500, therefore data is shown in the *m/z* 180–500 range.

2.4. Cell culture

Human laryngeal cancer cells (HEp-2 line) and human non-tumor cells (HEK-293 line) were obtained from American Type Culture Collection (ATCC) bank. Both epithelial cell lines were cultivated under standardized conditions in DMEM medium, supplemented with 10% heat-inactivated FBS and penicillin–streptomycin (10,000 U/mL). Cells were seeded in culture flasks and maintained in a humidified atmosphere at 37 °C with 5% CO_2. Studies were conducted when the cells reached 80–90% confluency.

2.5. Cell viability assays

In order to determine the antiproliferative activity of the *A. angustifolia* extract (AAE) on HEp-2 cells, 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) [14], and the trypan blue exclusion assays were performed [15]. For the MTT assay, seeded cells (1×10^4 cells/well in 96-well plate) were treated with different concentrations (100, 250 and 500 μg/mL) of AAE, and incubated at 37 °C in 5% CO_2 for 24, 48 and 72 h. After this time, the medium was removed and 1 mg/mL MTT dye in serum-free medium was added to the wells. Plates were incubated at 37 °C for 3 h. Subsequently, MTT solution was removed and the resulting formazan violet product was dissolved in 100 μL dimethylsulfoxide (DMSO), stirred for 15 min, and the absorbance was measured using a microplate reader (Victor-X3, multilabel counter, Perkin Elmer, Finland) at 517 nm. The cell viability in each well was expressed as percentage compared to non-AAE treated control cells. In addition to the MTT assay, trypan blue stain (0.4%) dissolved in Phosphate Buffered Saline (PBS) was used to assess cell viability. Cells (2.5×10^5 cells/well in a six-well plate) were treated with increasing concentrations of AAE (100, 250 and 500 μg/mL) for 24 h. The number of viable and dead cells was counted using an optical microscope. The percentage of viability was calculated by number of unstained cells/total number of cells × 100. Normal human embryonic kidney-293 (HEK-293) epithelial cell line was used as non-tumor control.

2.6. Cell and nuclear morphology analysis

Changes in cellular morphology were observed in culture flasks after 24 h of different AAE treatments (100, 250 and 500 μg/mL). Nuclear morphology evaluation was performed according to Jaganathan et al. [16]. The cells (2×10^5 per well) were grown on 12 mm cover slips in 24-well culture plates and exposed to different concentrations of AAE (100, 250 and 500 μg/mL) for 24 h. The monolayer of cells were washed with PBS and fixed with 3% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and

incubated with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) were analyzed. Images were obtained using an inverted fluorescent microscope (Optiphase-403F, USA).

2.7. Oxidative damage to lipids and proteins, and nitric oxide levels

With the aim to study the cytotoxic mechanisms of the AAE, oxidative damage to lipids and proteins, and nitric oxide (NO) production were assessed in HEp-2 cells (1×10^7) after 24 h treatment with different AAE concentrations (100, 250 and 500 μg/mL). Oxidative parameters were assessed after the incubation of the cells with RIPA lysis buffer for 30 min, and centrifugation at 1500g at 4 °C for 5 min. The supernatants were used for the assays. Lipid peroxidation was monitored by the formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction, according to Wills [17] with modifications. Specifically, 400 μL of supernatant from each sample was combined with 600 μL of 15% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA). The mixture was heated at 100 °C for 20 min. After cooling to room temperature, the samples were centrifuged at 1300g for 10 min. The supernatants were isolated, and their absorbance were measured at 530 nm. Hydrolyzed 1,1,3,3-tetramethoxypropane (TMP) was used as standard, and the results were expressed as nmol TMP/mg of protein. Oxidative damage to proteins were measured based on the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) [18]. For the control, 200 μL of DNPH (10 mM) or 200 μL of HCl (2 M) were added to 50 μL of cell supernatants. The reaction mixture was incubated in the dark for 30 min, and vortexed every 10 min. After, 250 μL of 20% TCA was added to each reaction mixture and centrifuged at 1400g for 8 min. The supernatants from each sample were discarded and the pellets were washed 3 times with ethanol–ethyl acetate (1:1) to remove free reagent. Samples were centrifuged and the pellets were resuspended in 1000 μL of urea solution (8 M) at 37 °C for 15 min. Absorbance was read at 365 nm, and results were expressed as nmol DNPH/mg protein. Nitric oxide production was determined based on the Griess reaction [19]. Taking into account that accurate NO measurements are very difficult to assess in biological specimens, nitrite concentration was estimated as an index of NO production. A standard curve was performed using sodium nitroprusside (SNP) for calibration. Optical density was quantified at 535 nm and the results were expressed as nmol SNP/mg protein. Protein concentration was determined by the Lowry et al. [20] method using bovine serum albumin as standard.

2.8. Superoxide dismutase and catalase activities

After 24 h of treatment with different concentrations of AAE (100, 250 and 500 μg/mL), HEp-2 cells (1×10^7) were incubated with RIPA lysis buffer for 30 min, and centrifuged at 8000g at 4 °C for 10 min. The supernatants were used for both enzymatic assays. Superoxide dismutase (Sod) activity was measured by the inhibition of self-catalytic adrenochrome formation rate at 480 nm, in a reaction medium containing 1 mmol/L adrenaline (pH 2.0) and 50 mmol/L glycine (pH 10.2) at 30 °C for 3 min [21]. Results were expressed as USod (units of enzyme activity)/mg protein. One unit is defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50%. Catalase (Cat) activity was measured according to the method described by Aebi [22]. The assay determines the rate of H_2O_2 decomposition at 30 °C for 1 min in 240 nm. Results were expressed as UCat/mg of protein. One unit is defined as the amount of enzyme that decomposes 1 mmol of H_2O_2 in 1 min at pH 7.4. Protein concentration was determined by the Lowry method [20] using bovine serum albumin as standard. All absorbance were measured in spectrophotometer model UV-1700.

2.9. DNA damage

Single cell gel electrophoresis (Comet assay) was performed to assess the genotoxic effects of AAE on 24 h treated HEp-2 cells, as described by Singh et al. [23]. Cells (2.5×10^5) were washed with ice-cold PBS, trypsinized, and resuspended in complete medium. Slides were prepared by mixing 20 μL of suspended cells and 80 μL of low melting point agarose (0.75%). The mixture was poured onto a frosted microscope slide coated with normal melting point agarose (1%). After solidification, the coverslip was removed and the slides were placed in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM ethylenediaminetetraacetic acid (EDTA), 1% triton X-100, and 10% DMSO, pH 10.0) for 24 h. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH = 12.6) for 20 min. The DNA was electrophoresed for 20 min at 25 V (0.9 V/cm) and 300 mA, and the buffer was neutralized with 0.4 M Tris (pH 7.5). Finally, DNA was

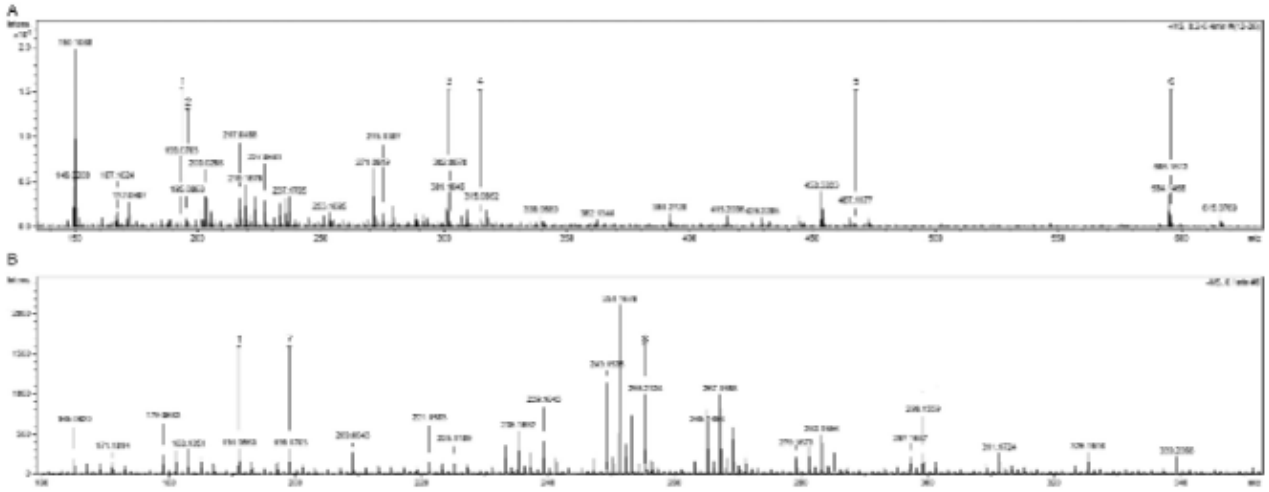


Fig. 1. HRMS full mass spectra of the *Araucaria angustifolia* extract in positive (A) ESI (+) and negative (B) ESI (−) mode.

Table 1
Identified compounds from *Araucaria angustifolia* by HRMS ESI-MS (+) and (−).

Entry	Precursor ion *m/z* (%)	Identification	Elem. comp.	Diff. ppm	Refs.
ESI(+) $[M+H]^+$					
	193.0703	1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid	$C_7H_{12}O_6$	4.73	[26]
	195.0868	3-*O*-methyl-D-*chiro*-inositol	$C_7H_{14}O_6$	0.32	[27]
	302.0870	4-Nitrophenyl-β-D-glucopyranoside	$C_{12}H_{16}NO_8$	1.95	[28]
	315.0862	4′-Methoxytectorigenin	$C_{17}H_{14}O_6$	2.11	[29]
	467.1177	3-Glucoside-dihydroquercetin	$C_{21}H_{22}O_{12}$	1.18	[30]
	595.1598	Amentoflavone 4′,4‴,7,7″-tetramethyl ether	$C_{34}H_{27}O_{10}$	1.04	[31]
ESI(−) $[M-H]^-$					
	191.0560	1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid	$C_7H_{10}O_6$	2.28	[26]
	199.1703	Dodecanoic acid	$C_{12}H_{22}O_2$	2.48	[32]
	255.2334	Hexadecanoic acid	$C_{16}H_{30}O_2$	3.90	[33]

stained with silver nitrate. Images of 100 randomly selected cells (of four replicated slides) were analyzed from each sample. DNA damage was visually scored according to tail size into five classes, from no tail (0) to maximal (4) long tail. Therefore, a group damage index (DI) could range from 0 (all cells with no tail, 100 cells × 0) to 400 (all cells with maximally long tails, 100 cells × 4). The frequency (%) of the different classes of DNA damage was also evaluated.

2.10. Immunoblotting analysis

HEp-2 cells (6×10^5) treated with AAE during 24 h were harvested and mixed with Laemmli sample buffer, denatured by boiling (100 °C) for 5 min. Briefly, 3000 cells/μL (20 μL) were loaded and the proteins were separated on 7.5–15% SDS–PAGE gels. After, the proteins were electro-transferred onto nitrocellulose membranes (Amersham Hybond™-C Extra). The membranes were blocked with 5% non-fat dry milk and were stained overnight at 4 °C with primary antibodies. Specifically, cleaved and total Poly-(ADP-ribose) polymerase (PARP), Apoptosis Inducing Factor (AIF), p53, cleaved caspase-3, Bax, Bad, and Bim were labeled with primary antibodies, and β-actin was used as a loading control. The following dilutions were used: PARP and AIF (1:2000), p53 (1:500), cleaved caspase3, Bax, Bad, Bim and β-actin (1:1000). After incubation with primary antibodies, the membranes were washed with PBS-T (PBS-buffered saline containing 0.1% Tween-20) to remove unbound primary antibodies. The membranes were then stained with anti-Rabbit IgG conjugated–peroxidase or Mouse IgG secondary antibodies (1:2000) for 1 h at room temperature. The membranes were washed repeatedly. Protein detection was performed by using a chemiluminescence protocol (Amersham Bioscience). Protein band images were captured using Image Scanner™ III (GE Healthcare) and pairwise comparisons of the protein bands on the immunoblot were performed using Image-J 1.45 software.

2.11. Mitochondrial complex I activity

HEp-2 cells (1×10^7) were grown in culture flasks and treated with 100 μg/mL of AAE for 24 h. After AAE-treatment, cells were washed with PBS, scraped and homogenized with ice-cold PBS. Mitochondrial complex I activity was assayed in the cell extracts using the Complex I Enzyme Activity Microplate Assay Kit, by following the manufacturer's instructions. Mitochondrial complex I activity was measured based on the oxidation of NADH to NAD^+ at 450 nm in the microplate reader (Victor-X3, Perkin Elmer, Finland). Positive control was obtained with cells exposed to rotenone (20 μM, a complex I inhibitor) for 30 min. Mitochondrial complex I activity was also evaluated in HEK-293 cells treated with AAE (100 μg/mL) for 24 h, using rotenone (20 μM; 30 min.) as positive control. Data were presented as percentage of control (%).

2.12. ATP quantification

HEp-2 cells (5×10^4 cells/well in opaque-walled 96-well plate) were incubated with 100 μg/mL of AAE for 24 h. ATP levels were measured by using the Cell-Titer-Glo® Assay Kit, according to the manufacturer's instructions. Luminescence was recorded using a multi-mode microplate reader (Victor-X3, Perkin Elmer, Finland). Positive control was obtained with cells exposed to rotenone (20 μM, a complex I inhibitor) for 30 min. ATP levels were also

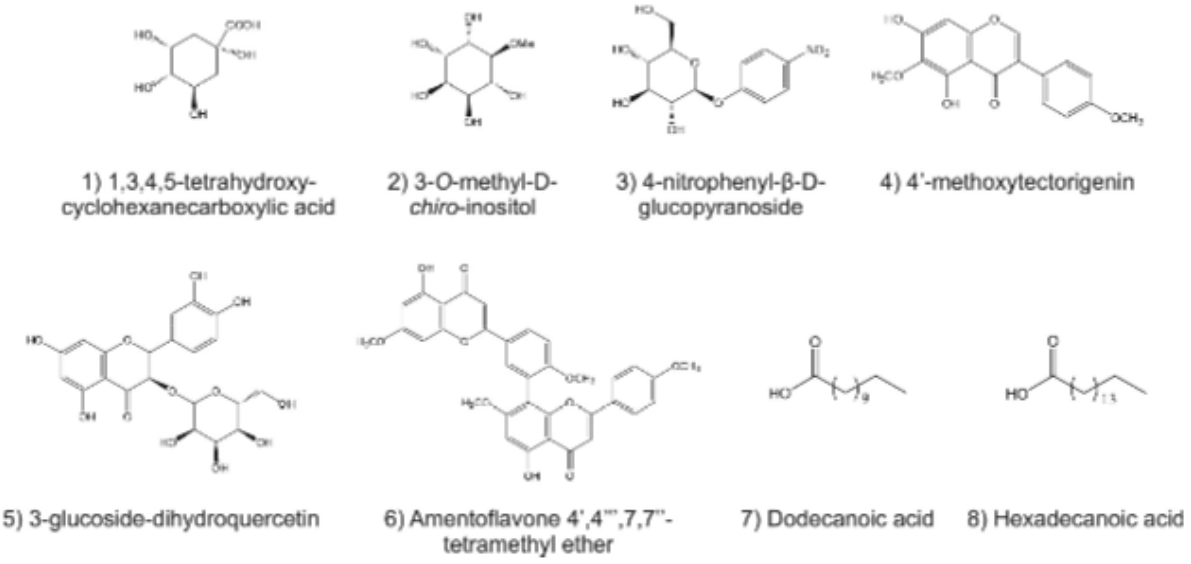


Fig. 2. Chemical structures of the compounds identified in *Araucaria angustifolia* extract.

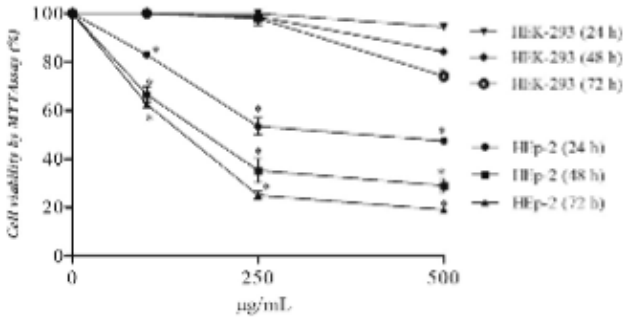


Fig. 3. Antiproliferative activity on HEp-2 cells treated with *Araucaria angustifolia* extract for 24, 48 and 72 h by MTT assay. % viability = [(number of cells at time of observation/number of control cells) × 100]. Values were averaged and expressed with SD values. *Significant difference between the cell lines according to ANOVA and Tukeýs post hoc test ($p \leqslant 0.05$). HEK-293 (non-tumor cells) was used as control. Both lines present epithelial morphology.

quantified in HEK-293 cells treated with AAE (100 µg/mL) for 24 h, using rotenone (20 µM; 30 min.) as positive control. Results were expressed as percentage of control (%).

2.13. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 21.0) for Windows (Illinois, USA). Kolmogorov–Smirnov test was used to assess for the parametric distribution of data. Statistical significance was evaluated using one-way analysis of variance (ANOVA), followed by Tukeýs post hoc test. Results are deemed significant if *p*-value was less than 0.05.

3. Results

3.1. Phytochemical characterization

To investigate the chemical composition of AAE, High Resolution Mass Spectrometry (HRMS) was used with Q-TOF-II (Bruker® Daltonics). The electrospray ionization (ESI) technique allows for the analysis of a wide range of compounds. Thus, HRMS-ESI has been elected as one of the most powerful tools for the analysis of complex mixtures such as plant extracts and have been used by our group to rapidly analyze natural products [24]. In accordance, the positive (A) and negative mode (B) were tested

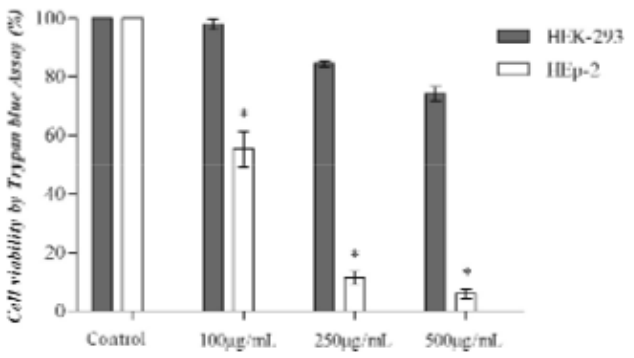


Fig. 4. Antiproliferative activity of *Araucaria angustifolia* extract on HEp-2 cells for 24 h of treatment measured by trypan blue stain exclusion assay. The percentage of viability was calculated by number of unstained cells/total number of cells × 100. *Significant difference between the cell lines according to ANOVA and Tukeýs post hoc test ($p \leqslant 0.05$).

using 0.1% of formic acid or 0.1% or ammonia hydroxide, respectively (Fig. 1).

According to a set of information provided by HRMS as exact *m/z*, isotopic ratio, fragmentation pathway (MS/MS), it is possible to confirm the chemical structures of compounds already indicated in literature. The widely accepted accuracy threshold for confirmation of elemental compositions was established as 5 ppm [25], since it usually provides highly reliable identification of the targeted compounds. For direct comparison with predetermined to different *Araucaria* samples, some compounds were identified in the crude extract used in this study (Table 1). They belong to different classes, such as flavanols, glucosides and carboxylic acids (Fig. 2).

Other peaks (*m/z*) could not be recognized, and future studies will be necessary. In addition, the isolation of these compounds and identification of their chemical structures by other spectroscopic methods are necessary since there is no further information about this species.

3.2. AAE exhibits antiproliferative activity

The sensitivity of HEp-2 cell line to AAE treatment was evaluated by the MTT assay (Fig. 3) and trypan blue exclusion method (Fig. 4). Non-tumor (HEK-293) cell line was used as a control. It was observed that AAE treatments induced a significant time-dependent antiproliferative activity in HEp-2 cells. The concentrations of 250 and 500 µg/mL were able to inhibit tumor cell growth by approximately 50% in 24 h of treatment. In 48 h, tumor reduction reached 65 ± 4.9% and 70 ± 2.8% for 250 and 500 µg/mL, respectively. In 72 h, higher cytotoxicity rate was observed as tumor reduction reached approximately 80% in the treatment with 250 and 500 µg/mL, indicating a dose-dependent effect only in treatments below 250 µg/mL. It is important to observe the selective effect of AAE, which presented no significant cytotoxicity for non-tumor HEK-293 cells treated with 250 µg/mL of AAE. Trypan blue assay (Fig. 4) confirmed the results found in MTT assay, reinforcing the selective antiproliferative activity of AAE to tumor cells.

3.3. AAE induces morphological alterations in HEp-2 cells

Microscopic analysis of HEp-2 cells treated with different concentrations of AAE (100, 250 and 500 µg/mL) for 24 h of treatment demonstrated a decrease in cell number, along with characteristic structural changes, including vacuolization of the cytoplasm and permeabilization of the plasma membrane. In addition, AAE changed the symmetry of the cells, reducing their growth and cell adherence (Fig. 5A). To assess the nuclear morphology, the cells were stained with DAPI, a DNA-specific fluorescent dye, and were then examined by fluorescent microscopy. HEp-2 cells treated with 250 and 500 µg/mL of AAE exhibited condensed and fragmented nuclei when compared to control cells, which showed clear and interphase nuclei (Fig. 5B).

3.4. AAE pro-oxidants effects

To evaluate the possible mechanisms by which AAE leads to cell death, oxidative damage markers to biomolecules, production of NO and enzymatic antioxidant activities were assayed. As shown in Table 2, AAE was able to induce oxidative damage to lipids and proteins, and to increase the production of NO. Antioxidant activities of Sod and Cat enzymes were significantly decreased under all treatments, indicating that AAE could be generating high levels of superoxide anion radical ($O_2^{\cdot-}$), thus increasing redox imbalance in tumor cells.

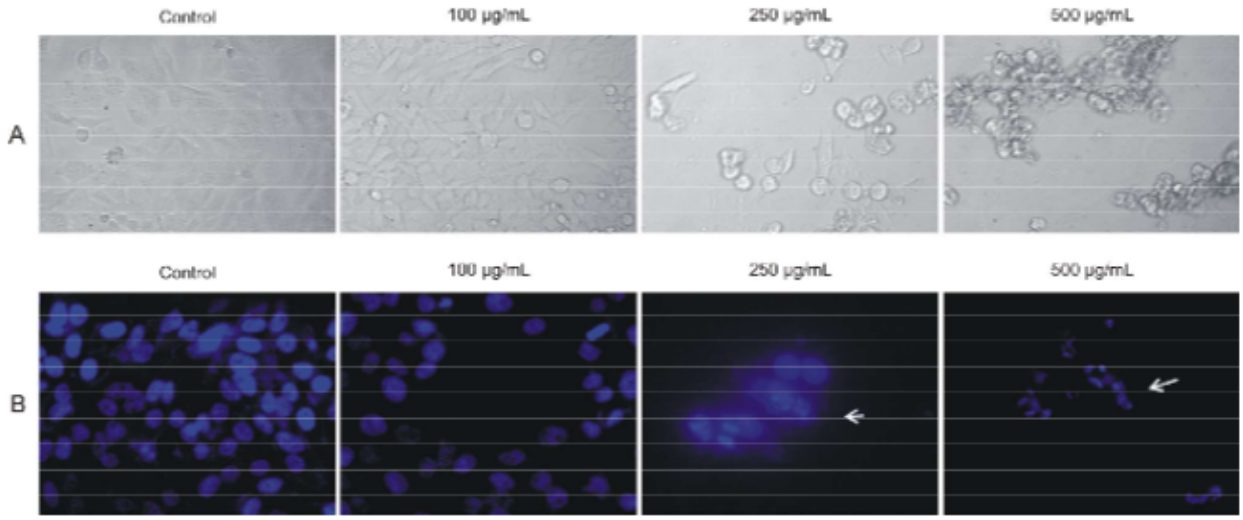


Fig. 5. Morphological and nuclear alterations of HEp-2 cells in the presence or absence of *Araucaria angustifolia* extract (100, 250 and 500 µg/mL) for 24 h. The photomicrographs show a more representative image for each treatment. (A) Cells were grown in culture flasks as described in Section 2, and then photographed (magnification 20×). (B) Cells were grown on 12 mm cover slips in 24-well culture plates stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI), then photographed (magnification 20× and 40×). Arrows indicate condensation of chromatin and DNA fragmentation.

Table 2
Redox stress markers in HEp-2 tumor cells treated with *Araucaria angustifolia* extract.

	TBARS (nmol TMP/mg protein)	Carbonyl protein (nmol DNPH/mg protein)	Nitrite concentration (nmol SNP/mg protein)	Sod activity (USod/mg protein)	Cat activity (UCat/mg protein)
Control	$2.98 \pm 0.09^{a*}$	8.0 ± 1.0^{a}	3.6 ± 0.1^{a}	9.32 ± 0.15^{a}	33.2 ± 0.6^{a}
AAE (100 µg/mL)	3.92 ± 0.35^{ab}	13.2 ± 0.6^{b}	8.4 ± 0.2^{b}	2.44 ± 0.02^{b}	10.5 ± 2.3^{b}
AAE (250 µg/mL)	4.02 ± 0.56^{ab}	23.0 ± 0.3^{c}	13.0 ± 1.9^{bc}	2.34 ± 0.07^{b}	9.6 ± 0.8^{b}
AAE (500 µg/mL)	4.42 ± 0.01^{b}	24.1 ± 1.9^{c}	14.0 ± 1.4^{c}	1.25 ± 0.33^{c}	9.8 ± 0.5^{b}

Results are expressed as mean ± SD.
*Different letters indicate statistically different values in each parameter evaluated, according to ANOVA and Tukey's post hoc test ($p \leq 0.05$).

3.5. AAE induces DNA damage

The genotoxic effect of AAE on HEp-2 cells was determined through Single cell gel electrophoresis assay (Fig. 6). AAE treatment was able to increase DNA damage levels in all tested concentrations when compared to non-treated control group. DNA damage indexes were 158.0 ± 11.31, 176.0 ± 16.18, and 280.0 ± 14.43 for 100, 250 and 500 µg/mL of AAE, respectively (Fig. 6A). AAE treatments increased the frequency of classes 2, 3, and 4 of DNA damage in a dose-dependent manner, with high levels of the maximal DNA damage (class 4) (Fig. 6B).

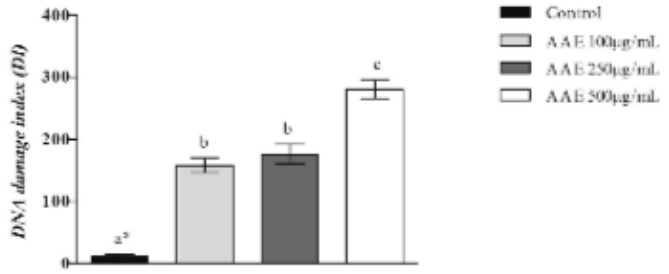
3.6. AAE modulates apoptotic proteins expression

To assess the involvement of apoptotic signaling molecules following AAE treatment, the expression levels of cleaved and total PARP, AIF, p53, cleaved caspase-3, Bax, Bad, and Bim were examined. In AAE-treated HEp-2 cells (100, 250 and 500 µg/mL), the expression levels of total PARP were found to be 3, 5, and 9 times higher than control, respectively (Fig. 7A). In addition, our results showed that AAE treatment with 500 µg/mL was able to induce PARP cleavage, which is an important substrate for apoptosis. Expression levels of AIF were also upregulated by AAE treatment, as shown in Fig. 7B. Similar results were observed for cleaved caspase-3 expression levels, which showed upregulation after AAE treatment (Fig. 7D). AAE treatment did not influence the expression of p53 (Fig. 7C), suggesting an induction of p53-independent apoptosis. Proteins of the mitochondrial intrinsic pathway (Bax, Bad and Bim) were also analyzed. Bax expression was significantly increased following treatment with AAE, only at higher concentrations (Fig. 7E). On the other hand, the expression levels of Bad and Bim were not modulated by AAE treatment (Fig. 7F and G).

3.7. AAE induces mitochondrial dysfunction

In order to evaluate the possible effects of AAE on the mitochondrial electron transport chain complex I, the lowest cytotoxic concentration (100 µg/mL) of the extract was used. In HEp-2 cells AAE treatment was able to reduce mitochondrial complex I activity by 55.05% (Fig. 8A) when compared to control group. Rotenone, a specific inhibitor of complex I, caused a significant reduction in complex I activity (76.36 ± 5.14%; Fig. 8A) following 30 min of exposure. In addition, solvent control (DMSO) did not inhibit complex I activity (data not shown). Furthermore, HEp-2 cells exposed to AAE treatment showed decreased levels of ATP production when compared to untreated cells (20.86 ± 3.37%), and this decrease was more accentuated than those observed for rotenone exposure (57.26 ± 1.03%; Fig. 8B). In order to assess the effect of AAE on mitochondrial function in normal cells, mitochondrial complex I activity and ATP levels were evaluated in HEK-293 cell line. It was observed that rotenone presented higher inhibition on complex I activity (around 75% of inhibition related to control group) and consequently lower production of ATP (around 60% in comparison to control group). Moreover, HEK-293 cells treated with AAE presented less inhibition on mitochondrial complex I activity (around 35% of inhibition in relation to control group) along with no significant reduction on ATP levels (around 92% of production compared to control) (Fig. 8A and B).

(A)



(B)

	Undamaged cells	Damage class one	Damage class two	Damage class three	Damage class four
	Treatments				
Control	$80.75 \pm 6.60^{a*}$	9.00 ± 0.82^{b}	2.80 ± 0.84^{b}	1.00 ± 0.01^{b}	1.00 ± 0.01^{b}
AAE (100µg/mL)	35.50 ± 6.36^{a}	24.00 ± 1.41^{ab}	20.50 ± 3.54^{bc}	10.50 ± 2.12^{bc}	9.50 ± 0.71^{c}
AAE (250µg/mL)	17.50 ± 3.51^{b}	17.00 ± 2.00^{b}	19.50 ± 2.12^{b}	30.00 ± 2.00^{a}	31.50 ± 0.71^{a}
AAE (500µg/mL)	0.00 ± 0.00^{c}	15.50 ± 2.65^{b}	16.50 ± 3.42^{b}	17.25 ± 4.27^{b}	50.75 ± 5.12^{a}

Fig. 6. DNA damage index by comet assay in HEp-2 cells after 24 h treatment with AAE. Each cell was allocated to one of the five classes from 0 (undamaged) to 4 (maximally damage), according to the size and shape of the tail. The values obtained for each group could range from 0 (0 × 100) to 400 (4 × 100) (A). Frequency (%) of different classes of DNA damage in HEp-2 cells (B). Results are expressed as mean ± SD. *Superscript letters indicate significant differences according to ANOVA and Tukey's post hoc test ($p \leq 0.05$).

4. Discussion

Some polyphenols [6,34] and fatty acids [35,36] found in plants have already been described as chemotherapeutic agents; however, the mechanisms implicated in this effect have not been clearly defined. Therefore, the study of chemical composition and biological effects of plants with chemotherapeutic potential is useful to the search of new compounds for cancer treatment. In this study, we investigated the chemical composition, antiproliferative activity and cytotoxic mechanism of action of *A. angustifolia*, an important native species from South America, on HEp-2 cells.

From the full mass spectrum and in accordance with a set of characteristics for each ion, some compounds were identified by ESI-MS (+) and ESI-MS (−). In this work, for example, the presence of 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid (determined by *m/z* 193.0703 and 191.0560 in positive and negative mode respectively) is a fragment indicative of 5-p-cis-coumaroylquinic acid presence, which had its chemical structure recently determined by Chen et al. [26]. Among the compounds identified by HRMS analysis, it could be highlighted the presence of 4′-methoxy-tectorigenin, 3-glucoside-dihydroquercetin and biflavonoid amentoflavone 4′,4‴,7,7″-tetramethyl ether, important derivatives of polyphenols; phenolic compounds that were previously reported to exhibit anticancer and apoptosis-inducing properties in cell lines and animal models [6,37]. Moreover, two important fatty acids, dodecanoic acid (lauric acid) and hexadecanoic acid (palmitic acid) were identified, both of which present antiproliferative actions, capacity to modulate apoptosis, and ability to regulate the cell cycle of human colon cancer cells [35,36,38].

In the present study, AAE showed a selective capacity to inhibit the proliferation of HEp-2 cells, with no cytotoxicity to normal epithelial cells. After 72 h of treatment, the cytotoxicity rate induced by 250 µg/mL AAE reached around 80% of the tumor cells, however it was not cytotoxic to non-tumor cells. This selectivity is an important factor in cancer therapy and natural products that present this particular selectivity are promising candidates to be developed into new chemotherapeutic agents. Cancer cells utilize high amounts of glucose to produce lactate even in the presence of oxygen ("Warburg effect"), differently from normal cells [39]. This metabolic choice reduces concentration of pyruvate destined to mitochondrial oxidative phosphorylation, and is probably related to the overexpression of pyruvate kinase isoenzyme M2 (PKM2), which is able to inactivate the pyruvate dehydrogenase complex (PDHC) [40–42]. PDHC is a key enzyme that catalyzes the oxidative decarboxylation of pyruvate in order to produce acetyl-CoA, linking glycolysis to the tricarboxylic acid cycle [43], and thereby promoting mitochondrial respiration. Amongst the possible mechanisms associated with selectivity elicited by AAE, we hypothesized that AAE is able to inhibit PKM2, therefore activating PDHC. Similar mechanisms have been already described to dichloroacetate, which is able to induce a metabolic switch from glycolysis to mitochondrial respiration in tumor cells (for review, see [44]).

Besides the assumption that AAE could interfere with cytosolic metabolism, our experimental data demonstrated that AAE may act as a mitochondrial complex I inhibitor, with consequent ATP depletion in HEp-2 tumor cells. In this sense, the AAE exerts a biphasic effect, first reversing the "Warburg effect" on HEp-2 cells and posteriorly inhibiting the mitochondrial activity in these cells. Tumor cells present a high proliferative index and require more ATP generation [45,46], being more sensitive to inhibition of oxidative phosphorylation than non-tumor cells.

It has already been shown that some phenolic compounds are able to inhibit mitochondrial respiration, resulting in the overproduction of ROS (for review, see [10]). Furthermore, it has been shown previously that polyphenols diffuse easily through the biological membranes and enter into the mitochondria [47–49]. Flavonoids, an important group of polyphenols, exhibit chemical

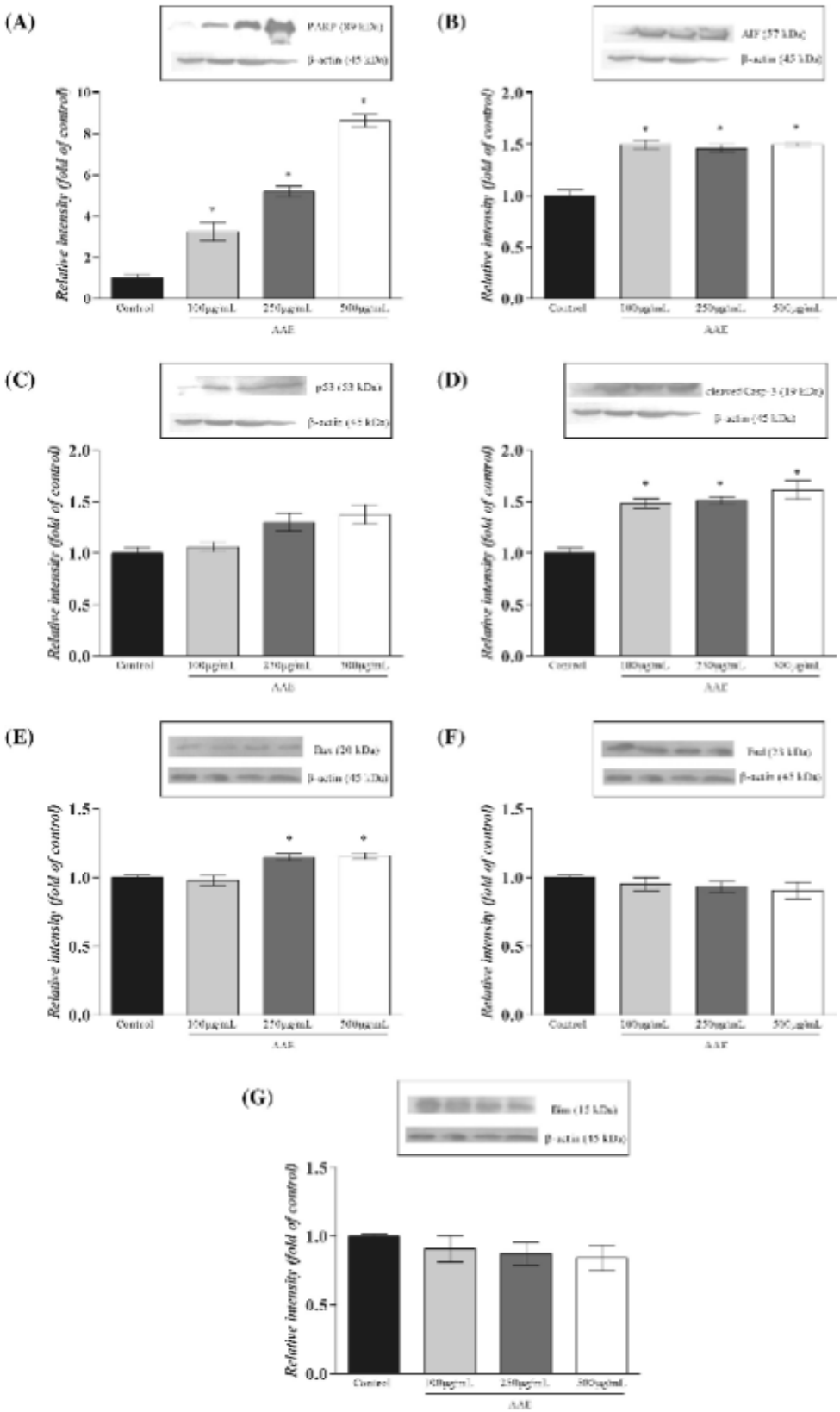


Fig. 7. Effect of AAE on protein expression of apoptotic cascade in HEp-2 cells. Protein expression evaluation of PARP (Poly-(ADP-ribose) polymerase) (A); AIF (Apoptosis Inducing Factor) (B); p53 (C); cleaved caspase-3 (D); Bax (E); Bad (F); Bim (G). The cells were treated with AAE at different concentrations of 100, 250 and 500 μg/mL for 24 h. Western blot data are quantified using β-actin as an internal control. Expression levels of proteins are expressed as the relative intensity of the bands. $^{*}p \leq 0.05$ compared to the respective control.

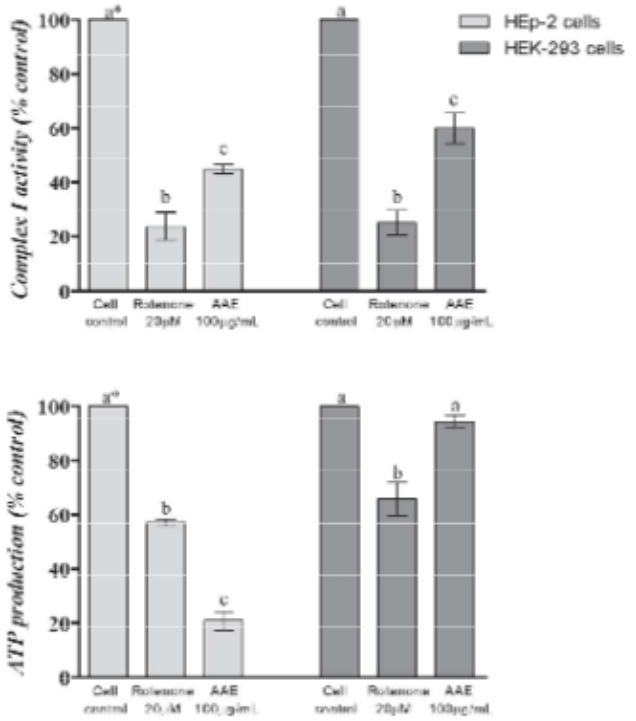


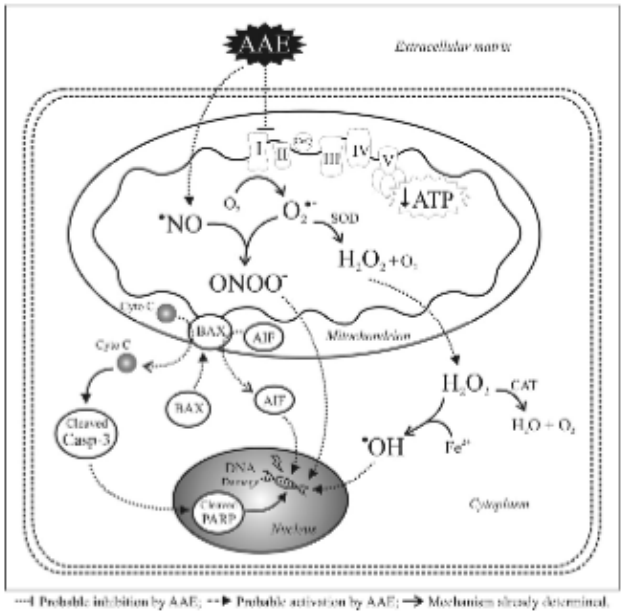
Fig. 8. Effects of AAE treatment (100 μg/mL) on complex I mitochondrial activity (A) and ATP production (B) in HEp-2 and HEK-293 cells. Results are expressed as mean ± SD. Superscript letters indicate significant differences among groups in each cell line according to ANOVA and Tukey's post hoc test ($p \leq 0.05$).

homology with the quinone moiety of coenzyme Q, suggesting a competitive inhibition through binding to the quinone sites in the mitochondrial electron chain [50]. Similarly, three phenolic compounds found in AAE (4′-methoxytectorigenin, 3-glucoside-dihydroquercetin, and amentoflavone 4′,4‴,7,7‴-tetramethyl ether) present chemical homology with the quinone moiety of coenzyme Q. This may explain the inhibition of mitochondrial complex I observed in treated HEp-2 cells.

Complex I of the mitochondrial electron transport chain is one of the major sites of superoxide radical ($O_2^{\bullet-}$) production [51]. Superoxide anion radical can be dismutated by the enzyme Sod, producing H_2O_2. Increase in ROS levels along with reduced activities of the antioxidant enzymes observed in this study could lead to tumor cell death. Along with the depletion of enzymatic antioxidant defenses (Sod and Cat), we found high levels of lipid peroxidation, protein carbonyl contents, and NO in AAE-treated HEp-2 cells. This redox imbalance may lead to a loss of cell integrity and consequent cell death. Nitric oxide has been implicated in the modulation of different cancer-related events [52] by being able to induce cytostatic and/or cytotoxic effect on tumor cells through caspase signaling [53]. NO can freely diffuse from the cytosol to mitochondria, or can be formed in the mitochondria [54]. Although the NO is a very unreactive radical, its interaction with $O_2^{\bullet-}$ forms peroxynitrite ($ONOO^-$), a potent cytotoxic oxidant with multiple biological actions, including oxidation of thiols or thioethers, nitration of tyrosine residues, and oxidation of nucleic acids [55,56]. It has already been shown that $ONOO^-$ generation may also occur in mitochondria [54,57,58], resulting in redox stress and alterations in mitochondrial physiology, such as inhibition in energy metabolism and opening of the permeability transition pores [54,59]. Besides NO, the hydroxyl radical ($^{\bullet}OH$) produced from H_2O_2 through Fenton reaction, is one of the most reactive chemical species known, and it is able to induce high rates of DNA damage [60]. In this study, AAE was able to induce a significant DNA damage index in HEp-2 cells. Additionally, DNA breakage was confirmed by nuclear microscopic analysis, which showed that AAE-treated cells exhibit condensed and fragmented nuclei.

Cells show a wide range of responses upon exposure to ROS, ranging from prevention of cell division, senescence or apoptosis [60]. The morphological changes associated with apoptosis process include chromatin condensation, nuclear fragmentation, membrane blebbing and cell shrinkage [61], as observed in this study. We found that AAE treatment significantly upregulated the expression of cleaved caspase-3, an important effector involved in the irreversible process of apoptosis. In addition, we also observed that the expressions of Bax and AIF were also highly upregulated in AAE-treated HEp-2 cells. On the other hand, Bad (Bcl-2-associated death promoter) and Bim (Bcl-2-interacting mediator of cell death) were not upregulated by AAE treatment, suggesting that the extract have other target molecules in the mitochondrial intrinsic pathway.

Another important target in cancer therapeutics is PARP. Our study demonstrated that AAE was able to increase total PARP expression and induce PARP cleavage. When the levels of DNA damage are irreparable, PARP plays a decisive role in the apoptotic process, becoming a key substrate for the degradation of damaged DNA by caspases [62,63]. In fact, our results showed upregulation in the levels of cleaved caspase-3 and PARP, indicating that AAE induces apoptosis also via substrate cleavage. Additionally, expression of protein p53, related to maintaining genomic stability had no significative change in HEp-2 cells. Similar results were found in previous reports [16,64] that showed induction of apoptosis independent of p53 function.



Scheme 1. Hypothetical model of *Araucaria angustifolia* extract (AAE) mechanisms of action on HEp-2 cells. AAE inhibit mitochondrial complex I activity leading to ATP depletion and redox stress by ROS generation. Superoxide radical anion ($O_2^{\bullet-}$) can be dismuted by action of the mitochondrial superoxide dismutase (Sod) enzyme and/or may react with NO generating $ONOO^-$, a potent oxidant that can diffuse into the cytosol, cleaving DNA. H_2O_2 is easily diffusible through biological membranes, reacting with the catalase (Cat) enzyme or form $^{\bullet}OH$ via Fenton reaction. Cellular stress and genotoxic insults increase Bax expression levels, which translocates from the cytosol to the mitochondria, facilitating cytochrome *c* and AIF release. Released cytochrome *c* is associated with up-regulation of cleaved caspase-3 expression, which in turn cleaves nuclear proteins, such as PARP, leading to apoptosis by mitochondrial death pathway.

The study of cell death signaling in different families of proteins is very important in the discovery of new potential anticancer agents. In fact, AAE displays a diverse range of molecular targets (Scheme 1), mainly through mitochondrial dysfunction and redox stress generation, demonstrating an important antitumor potential. Howsoever, it is important to evaluate the same molecular parameters in non-tumor cells. Moreover, the theoretical explanation on how AAE may be modeling the PKM2 and PDHC activities needs to be further investigated, along with other regulatory enzymes of cytosolic and mitochondrial carbohydrate metabolism.

Although future studies are needed, our results brings forward opportunities for future investigations that will lead to the development of new therapeutic or adjuvant agents in the cancer research field.

5. Conclusions

The findings of our study provide evidence that the chemical composition of AAE demonstrates selective cytotoxicity and pro-apoptotic activity in HEp-2 tumor cells. The cytotoxic mechanisms of AAE include inhibition of the mitochondrial complex I activity, induction of redox stress and DNA breakage. The apoptosis pathway activation occurs via Bax-triggered, along with AIF and cytochrome *c* release, and it is independent of p53 incitement. Lastly, this study showed that mitochondria and redox homoeostasis are chemotherapeutic targets of AAE in human larynx HEp-2 cancer cells.

Conflict of Interest

The authors declare no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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3.3 CAPÍTULO III

Polyphenols-rich extract from *Araucaria angustifolia*: Differential mechanisms on cancer and normal cells

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RESEARCH HIGHLIGHT

Polyphenols-rich extract from *Araucaria angustifolia*: Differential mechanisms on cancer and normal cells

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Cancer cells present differential metabolism compared to normal cells. Multiple molecular mechanisms converge to alter cellular metabolism, and some of these include a process of metabolic reprogramming which provides advantages to tumor cells in energy generation, growth and proliferation. Tumor energy production is basically dependent on glucose driven to glycolysis (Warburg effect), but it also happens by means of fatty acids and glutamine metabolism. Among the current challenges in cancer therapy, the tumor cell resistance and the absence of selectivity of anti-cancer agents stand out. It has been already shown that polyphenols are able to exert differential effects on normal and tumor cells. However, the exact mechanisms of these actions are not fully understood. In our previous study, we showed that a polyphenols-rich extract (PE) from *Araucaria angustifolia* held a selective capacity to inhibit the proliferation of human larynx HEp-2 cancer cells with minimal cytotoxicity to normal epithelial cells. We hypothesized that the effect presented by PE have happened by reversing the "Warburg effect" on cancer cells and inhibiting the mitochondrial electron transport chain complex I activity along with ATP depletion on these cells. In this research highlight we will discuss the effects of the PE on mitochondrial metabolism and their possible role in the modulation of mitochondrial sirtuin 3 (SIRT3) on tumor (HEp-2) and normal (HEK-293) cells, which may help to clarify the tumor selectivity exhibited by polyphenols.

Keywords: Cancer; mitochondria; redox status; SIRT3; polyphenols

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Cancer cells microenvironment and metabolism differ significantly from normal cells. In relation to their microenvironment, cancer cells are able to alter surrounding tissue or adjacent stroma in order to build a supportive environment for tumor progression and metastasis [1, 2]. The high proliferation rate exhibited by tumor cells depends on many factors, including changes in the checkpoint controls that regulate normal cellular division [3] and failure in the activation of apoptosis, a critical process for cell death induction, which are key factors in carcinogenesis development and metastatic progression of tumors [4].

Regarding cancer cell metabolism, as observed by Otto Warburg [5], these cells display dysfunctional mitochondrial respiration, taking up high amounts of glucose to produce lactate. This metabolic reprogramming represents an important adaptation to microenvironment conditions exhibited by tumor cells [6]. The high demand of glycolysis displayed by these cells provides metabolites for other metabolic pathways also used by tumors to increase biomass and boost proliferation rates [7–9]. These atypical metabolic characteristics in energy generation provide advantages to cancer cells, representing an important limitation in therapeutic approach. Besides cancer cell resistance, the selectivity is another central factor to be considered when designing and developing new chemotherapeutic agents [10].

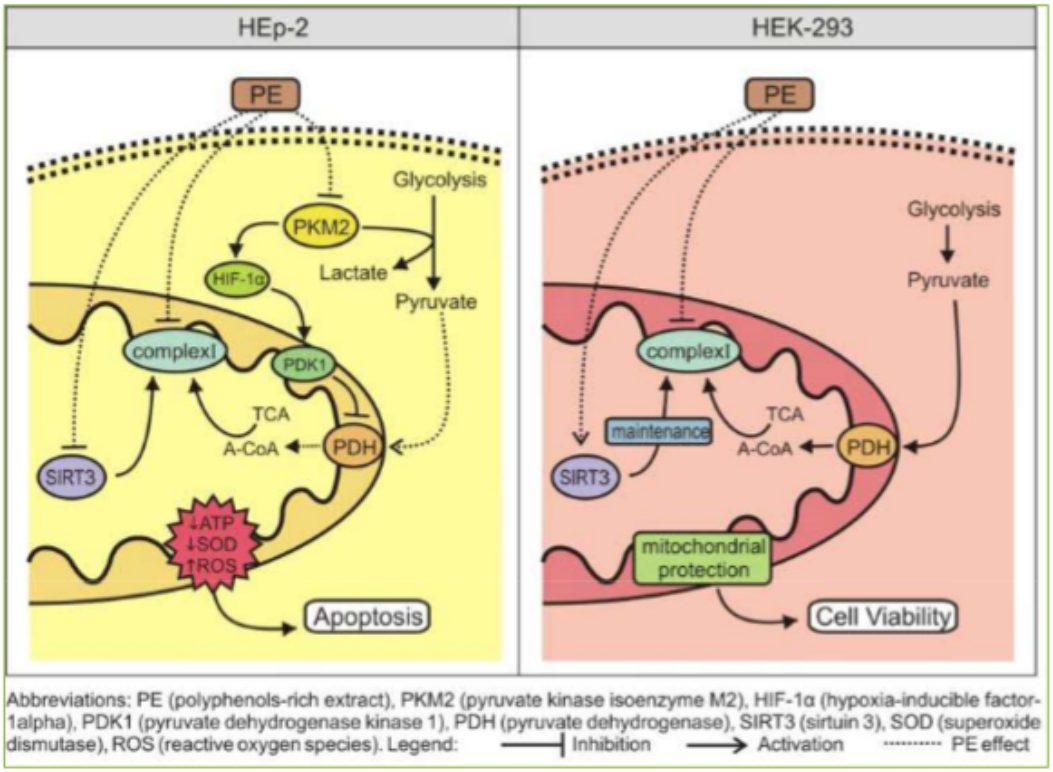

Fig 1. Proposed action mechanisms of a polyphenols-rich extract (PE) from *Araucaria angustifolia* on cancer and normal cells. On HEp-2 cancer cells, PKM2 enzyme regulates glucose metabolism and lactate production, and acts as a transcriptional co-factor of HIF-1α, which increases PDK1 activity, thus inhibiting PDH. On HEK-293 normal cells, PKM2 is absent or downregulated, therefore PDH is functional, producing A-CoA which is used in the TCA cycle. PE inhibits PKM2 enzyme on cancer cells, consequently downregulating HIF-1α and PDK1, thus activating PDH and restoring mitochondrial respiration. PE also inhibits mitochondrial complex 1 activity along with SIRT3, which cannot restore complex 1 function, generating high levels of superoxide anion radical, causing mitochondrial dysfunction, ATP depletion and cell death. On normal cells, PE activates SIRT3, which is able to maintain mitochondrial complex 1 activity and ATP levels, avoiding mitochondrial damage and preserving cell viability.

For example, paclitaxel has good antiproliferative effects on human breast cancer MCF-7 cell; however, it is able to damage normal cell seriously, as shown by Zhao *et al* [11]. In this sense, natural or chemical compounds that display selective cellular targets are promising candidates in the anti-cancer therapeutics research.

Due to the central role of mitochondrial activity in critical cellular processes, such as metabolism and apoptosis, mitochondria have been considered an important target for cancer therapy [12–14]. Mitochondrion is an important bioenergetics organelle of the cell. Besides ATP generation and control of redox homeostasis, it also modulates calcium flux throughout the cell and mediates the intrinsic pathway of apoptosis [15–17]. Mitochondrial function depends on their assembly, maintenance and dynamics, and the occurrence of disruptions in this balance may cause primary and secondary mitochondrial dysfunction, being hence deleterious to the cell [16]. Typically, the primary dysfunction is associated with mutations to nuclear genes encoding mitochondrial proteins, whereas secondary dysfunction is generally caused by events from other etiology [16, 18, 19]. Often, the dysfunction of mitochondria is related with calcium homeostasis disruption, deficient ATP generation and enhanced reactive oxygen species (ROS) formation, mainly through the electron transport chain (ETC), which induces the formation of the mitochondrial permeability transition pore (MPTP) leading to mitochondrial damage [16, 20]. The complex I of ETC, also namely NADH: ubiquinone oxidoredutase is among the major sites of superoxide anion radical ($O_2^{\cdot-}$) generation [20], one of the most common form of ROS. Superoxide anion radical can be dismutated by the action of the superoxide dismutase (SOD) antioxidant enzyme, producing hydrogen peroxide. Mitochondria contain their own SOD, specifically MnSOD, found into the mitochondrial matrix [20, 21], however, high levels of ROS as well as other toxic stimuli, can trigger the intrinsic apoptosis process, which is a

desirable effect in cancer therapy. Intrinsic apoptosis, mediated by mitochondria, is initiated with the release of cytochrome *c* (Cyt *c*) into the cytosol, which occurs often as result of mitochondrial membrane permeabilization induced by MPTP formation, and lead to initiation of the apoptotic process and cell death [22]. In this context, mitochondria restoration is an interesting strategy to be explored in cancer research field [23].

Recently, our research group showed that a polyphenols-rich extract (PE) derived from natural source (*Araucaria angustifolia)* generates high cytotoxicity in human larynx HEp-2 cancer cells, with minimal effect to normal HEK-293 cells [24]. This extract contains several polyphenols belonging to the flavonoids class, including catechin and epicatechin (flavan-3-ol subclass); rutin and quercetin (flavonol subclass); apigenin (flavone subclass); 4'-methoxytectorigenin (isoflavone); 3-glucoside-dihydroquercetin (dihydroflavonol subclass), and the biflavonoid amentoflavone 4',4''',7,7''-tetramethylether [24–26]. All of these are phenolic compounds that hold recognized anti-cancer properties (for review, see Asensi *et al*. [27]). We found that the PE inhibited the activity of the complex I of the ETC, and consequently depleted ATP production on cancer cells, leading the cell to death via apoptosis. In contrast, PE administration did not reduce ATP levels on normal cells, although it had minimally diminished complex I activity, and consequently the cell viability was maintained (Figure 1). Considering these data we showed that PE had the ability to alter the metabolism of cancer cells, reversing the Warburg effect and then restoring mitochondrial function in these cells. We postulated that this effect is possible due to the inhibition of the pyruvate kinase isoenzyme M2 (PKM2) on tumor HEp-2 cells, which is a key regulator of the glycolytic pathway, reprogramming the flux of glucose that supplies the metabolic demand of growing cells [28]. This enzyme is one of the four isoforms of pyruvate kinase [29], and acts as a transcriptional co-factor of hypoxia-inducible factor-1alpha, which upregulates pyruvate dehydrogenase kinase 1, causing an inhibition of the pyruvate dehydrogenase [30]. The silencing of PKM2 allows the formation of pyruvate and its conversion to acetil-CoA, consequently activating cellular respiration. Although PKM2 is encountered in few types of normal cells [28, 31], it is present at high levels in tumor cells [3], being an important target for cancer therapy.

The inhibition of mitochondrial complex I by PE showed in our work generated high levels of ROS and ATP depletion, starting the activation of mitochondrial death pathway, through upregulation of Bax expression. Bax proteins translocate from the cytosol to the mitochondrion, facilitating Cyt *c* and AIF release. Escape of Cyt *c* is associated with upregulation of cleaved caspases-3, which cleave nuclear proteins, such as PARP, leading to the irreversible process of apoptosis [24].

In our study [24], PE presented minimal cytotoxicity in HEK-293, possibly through the differential modulation on a group of proteins named sirtuins. Sirtuins activity have been implicated in numerous biological processes including cancer, and are messenger molecules that mediate the metabolic status of the cell in response to stress conditions [32]. Mitochondrial sirtuins (SIRT3, SIRT4, SIRT5) are able to regulate cellular metabolism via post-translational modifications [33], and specifically SIRT3 is responsible for coordinating the maintenance of mitochondria in order to produce energy, mediating the oxidative metabolism and redox homoeostasis [34-36]. Moreover, SIRT3 activates the ETC by deacetylating NDUFA9, a necessary component of electron transport complex I, and it is also involved with the maintenance of ATP levels [34, 37]. The expression of Cyt *c*, another important component of the ETC, is also dependent of SIRT3 [38], as well as the activation of MnSOD antioxidant enzyme [39, 40]. Therefore, we hypothesized that the data shown in our previous study in cancer cells [24] is due to PE downregulation and/or inhibition of SIRT3, resulting in decreased antioxidant response and increased ROS, thus inhibiting ETC and cell growth. On the other hand, in normal cells the antioxidant response was maintained, preventing the cells from oxidative damage and cell death (Figure 1). Similar mechanism was shown for (-)-epigallocatechin-3-gallate, a compound similar to those found in our PE, which was able to suppress the expression and activity of SIRT3 on cancer cells and to increase SIRT3 levels on normal cells [41]. These experimental and hypothetical data evince the need to perform further studies to understand the differential mechanisms of the polyphenols on cancer and on normal cells, which could contribute to find selective targets in cancer treatment.

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Conflicts of Interest

The authors declare no conflict of interest.

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3.4 CAPÍTULO IV

Araucaria angustifolia extract induces mitochondrial dysfunction and global DNA hypomethylation in HEp-2 cancer cells

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Araucaria angustifolia extract induces mitochondrial dysfunction and global DNA hypomethylation in HEp-2 cancer cells

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Abstract

Araucaria angustifolia extract (AAE) is a polyphenol-rich extract that has gained interest as a natural anticancer agent. Recent work suggests that AAE induces oxidative damage and apoptosis through its action on decreasing complex I activity of the mitochondrial electron transport chain (ETC). In the present study, we aimed to further examine the specific targets by which AAE exerts pro-apoptotic effects in HEp-2 cancer cells by means of: 1) levels of pyruvate dehydrogenase (PDH) was assessed by ELISA assay; 2) levels of reactive oxygen species (ROS) generation was determined using a fluorometric assay; 3) levels of mitochondrial ETC complexes, focusing on complex I at the gene transcript and protein level relevant to ROS generation was evaluated by multiplex ELISA followed by qRT-PCR and immunoblotting; 4) mitochondrial membrane potential ($\Delta\Psi m$) was assessed by MitoTracker Red CMXRos; and 5) chemical variations on DNA was evaluated by dot-blotting in HEp-2 cells. Results demonstrated that AAE increased protein levels of PDH, switching energy metabolism to oxidative metabolism. Protein expression levels of complex I and III were found decreased in AAE-treated HEp-2 cells, leading to increased intracellular ROS. Analyzing the subunits of complex I relevant to ROS generation, decreased protein and gene transcript levels of NDUFS7 were found. Increased ROS levels were also accompanied by a loss of $\Delta\Psi m$, DNA hypomethylation and decreased DNA (cytosine-5)-methyltransferase 1 activity. Our data demonstrate for the first time that AAE exhibits a therapeutic potential for cancer prevention and treatment by inducing mitochondrial dysfunction and epigenetic changes in HEp-2 cancer cells.

Keywords: polyphenols; mitochondria; Complex I; methylation; hydroxymethylation

Introduction

Head and neck cancer includes tumors of the pharynx, larynx and the oral cavity[1]. Laryngeal cancer is the most common head and neck cancer with rates of morbidity, mortality, and estimated new cancer cases increasing globally every year if left untreated[2]. In the early stage, laryngeal cancer is often curable with conventional treatments including surgery, radiotherapy and chemotherapy; however, a majority of patients with the advanced stage have not achieved an improved outcome in the last two decades despite therapeutic advancements[3]. This underscores the need for developing complementary and alternative therapeutic approaches to minimize the morbidity and mortality involved in this cancer. Epidemiological studies have suggested that dietary phytochemicals found naturally in plants reduces the risk of several cancers, including head and neck cancers[4,5]. Some of the phytochemicals that has been extensively studied for cancer prevention and intervention for the past years are polyphenolic compounds[6]. Polyphenols are plant-derived compounds that are increasingly recognized to exhibit a variety of biological activities including antioxidative, anti-inflammatory and anticancer effects[7-10]. One such plant that have exceptionally rich source of polyphenols is *Araucaria angustifolia*[11]. *Araucaria angustifolia* (Bertolini) Otto Kuntze is a dioecious conifer species in the family Araucariaceae found in the Araucaria moist forests of Southern Brazil[11]. As a dioecious species, each tree contains either male or female conifer cones, also known as strobili[11]. Female strobilus originates the pinecone, which contains edible seeds and undeveloped seeds, commonly known as bracts[11]. These bracts are usually discarded into the environment and thus many investigators have sought to investigate the potential health benefits that these seeds hold[11]. Our group has recently characterized the phenolic compounds in *A. angustifolia* extract obtained from the bracts, which includes catechin, epicatechin, quercetin, and apigenin as its main components[11]. Emerging evidences, including those from our group have found that polyphenolic compounds exert anticancer effects through modulating mitochondrial function[12].

Mitochondria are organelles responsible for producing energy by transferring electrons through the electron transport chain (ETC) complexes I-IV in a process called oxidative phosphorylation (OXPHOS)[13]. Substantial body of evidence indicates that cancer cells reprogram their energy metabolism from OXPHOS to anaerobic glycolysis, a process that generates less energy in the absence of oxygen to promote their survival, rapid growth and proliferation[14]. Due to altered metabolism, cancer cells frequently display elevated levels of reactive oxygen species (ROS)[15]. The largest contributor to

ROS generation is the mitochondrial ETC, mainly through complex I and III[16]. Though low levels of ROS are important for cellular signaling, excessive ROS production can lead to irreversible oxidative damage to DNA that drive the initiation and progression of cancer[17]. Intriguingly, while it is believed that ROS contribute to cancer progression, excessive ROS production can also act as cellular toxicants and place cancer cells in a vulnerable state leading to growth arrest and apoptosis[18]. These events are further exacerbated when cancer cells are exposed to compounds that further stress their ability to control redox homeostasis[19]. Oxidative damage to DNA can also cause epigenetic instability[18,19]. A major type of epigenetic change to DNA is methylation[20]. Accumulating evidence have shown that too little or too much methylation on DNA can significantly alter expression of key genes involved in cell cycle regulation, DNA repair and apoptosis[21]. These changes are thought to be important driving factors in the development of cancer[21]. Therefore, compounds that could effectively target these altered metabolic, redox and epigenetic pathways could serve as novel therapeutic approach to prevent cancer cell progression.

Accumulating body of evidence have demonstrated that polyphenolic compounds targets OXPHOS, redox and epigenetic pathways[22,23]. Green tea and coffee polyphenols have been shown to inhibit DNA methyltransferase, an enzyme involved in adding methyl groups on DNA, leading to inhibition of DNA methylation in human breast cancer cells[24]. Using the same breast cancer cell line, polyphenol-rich *Vitis labrusca* seed extract have been shown to induce DNA damage, upregulate pro-apoptotic factors, and inhibit Her-2 oncoprotein expression[25]. A recent study has demonstrated that epigallocatechin-3-gallate (EGCG), a polyphenol found in green tea induce a profound increase in ROS and apoptosis in oral cancer cells but exerts antioxidant and non-cytotoxic effects in non-cancer human fibroblast cells[26,27]. In support of these findings, our group have demonstrated that AAE, a polyphenol-rich extract exerts selective cytotoxic effects in HEp-2 cancer cells by inhibiting mitochondrial complex I activity, increasing oxidative damage to proteins and lipids, and subsequently inducing apoptosis in HEp-2 cancer cells[28]. These effects were not observed in non-cancer epithelial cells[28]. Taken together, the above described data suggest that mitochondrial dysfunction and epigenetic alterations induced by polyphenols play an important role in inhibition of cancer cells.

Because many of these consequences are interdependent, the precise mechanisms by which AAE prevents cancer cell proliferation remains inadequately understood. In the present study, we aimed to further examine the specific targets and mechanisms by which

AAE exerts pro-apoptotic effects in HEp-2 cancer cells. Specifically, we will examine the effect of AAE on the: 1) levels of pyruvate dehydrogenase, an enzyme that forms the link between glycolysis and mitochondrial oxidative metabolism; 2) levels of ROS generation; 3) levels of mitochondrial ETC complexes, focusing on complex I alterations at the gene transcript and protein level relevant to ROS generation; 4) mitochondrial membrane potential and 5) chemical variations on DNA in HEp-2 cells.

2. Materials and Methods

2.1. Preparation of *A. angustifolia* extracts

A. angustifolia extract (AAE) was obtained from non-sterile seeds (bracts) collected from mature female conifer cones (strobili) of *A. angustifolia* in Caxias do Sul, Rio Grande do Sul (29°9'34.90"S, 51°8'45.34"W); Ibama nº 02001.001127/2013-94, Brazil. Voucher specimens were identified by the Herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (HUCS 40710/40711). Bracts were manually separated from the pinecone. The extract was prepared by adding 5 g of bracts in 100 mL of distilled water under reflux at 100 °C for 15 min. The mixture was sterilized by filtering through a Sterflip equipment with a 0.45 μm pore size (SFGS 047LS, Millipore Corp.) and then lyophilized (LIOBRAS model L-101) to yield a powder. The freeze-dried extract was stored at -20°C and protected from light until use. The major phenolic compounds found in AAE, including catechin, epicatechin, rutin, quercetin, apigenin, 4'-methoxytectorigenin, 3-glucoside-dihydroquercetin and the biflavonoid amentoflavone 4',4''',7,7''-tetramethyl ether, were previously determined by high resolution mass spectrometry[11,28].

2.2. Cell culture

Human laryngeal epithelial cell line HEp-2 (ATCC, VA, USA) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma® St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen; Canadian origin), 1% penicillin–streptomycin (10,000 U/mL), and 1% amphotericin B (both from Sigma®). Cells were maintained in 75 cm^2 flasks in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C until experimentation.

2.3. Cell treatment and preparation for experiments

To assess for markers related to the mitochondria, cells underwent two treatments: AAE and rotenone (an established mitochondrial complex I inhibitor to serve as a positive control). First, cells were treated with three different concentrations of AAE dissolved in cell culture media (50, 75 and 100 µg/mL) or in AAE-deficient media for 24 h at 37 °C and 5% CO_2. The 24 h treatment period with AAE was selected based on our previous study that tested the effects of this compound on the growth curve of the cells *in* vitro[28]. Following 24 h, cells underwent a washout period in serum-free media (DMEM without FBS) for an additional 24 h before harvesting for experiments. Next, cells were treated with rotenone at a concentration of 20 µM for 30 min or in rotenone-deficient media. Then, the treatment media was removed and cells underwent a washout period by being cultured in serum-free media (DMEM without FBS) for 24 h prior to being harvested for experimentation. All experiments were performed in triplicates for each treatment condition.

2.4. Cell viability measurement

HEp-2 cells were seeded in 96-well plates at a density of 1×10^4 cells per well with 200 uL medium (DMEM+10% FBS) and grown for 24 hours. Cells were then treated with AAE (50, 75 and 100 µg/mL) for 24 h and rotenone (20 µM) for 30 min, and underwent a washout period in serum-free culture media (DMEM without FBS) the following day. Cell viability in each well was measured by 3-(4,6- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. The MTT assay is based on the reduction of tetrazolium salt to formazan by mitochondrial dehydrogenases (Denizot & Lang, 1986). Briefly, 1 mg/mL of MTT dye was added to each well containing treated- and untreated-cells and incubated for 3 h at 37 °C. Following incubation, 100 µL of dimethylsulfoxide was dissolved in the resulting formazan violet product, stirred for 15 min, and the absorbance was measured at 517 nm with a microplate reader. The cell viability was expressed as percentage compared to AAE-untreated control cells.

2.5. Measurement of pyruvate dehydrogenase (PDH) levels

Pyruvate dehydrogenase (PDH) levels were measured using the PDH Profiling ELISA Kit (Abcam; Cambridge, MA, USA) according to the manufacturer's instructions. Briefly, HEp-2 cells were grown to 80% confluency in 75 cm^2 flask. Cells were treated with AAE as described above, harvested by centrifugation at 125 x *g* for 5 min, and

suspended in 9 volumes of ice-cold PBS. Cell lysates were prepared by adding detergent solution provided by the kit at the optimal protein concentration of 50 µg/200 µL. The amount of PDH immunocaptured was measured at 600 nm for 20 min in a microplate reader. Results were expressed as percentage of relative amount of PDH compared to AAE-untreated control cells.

2.6. Measurement of protein levels of mitochondrial electron transport chain complexes

Protein levels of all mitochondrial ETC complex components were measured in HEp-2 cancer cells treated with AAE or rotenone using the Human Oxidative Phosphorylation (OXPHOS) Magnetic Bead Panel - Cellular Metabolism Multiplex Assay (EMD Millipore, H0XPSMAG-16K) according to the manufacturer's protocol. Briefly, cell lysates were prepared with lysis buffer provided by the kit and were centrifuged at 14000 x g for 20 min. Samples containing 5 µg of total protein were incubated with antibody-coated magnetic beads followed by detection antibodies and streptavidin-phycoerythrin. Luminex Magpix (EMD Millipore) was used to read the plates. Analysis was performed with the xPONENT software, where results were expressed as percentage of median fluorescence intensity (MFI) compared to AAE-untreated control cells.

2.7. Measurement of reactive oxygen species levels

Intracellular reactive oxygen species (ROS) levels were determined by 2',7' – dichlorofluorescin diacetate (DCFDA) fluorometric assay as previously described (Esposti, 2002). Briefly, AAE- and rotenone-treated HEp-2 cells were resuspended in PBS buffer and were loaded into a 96-well plate at a density of 1×10^5 cells per mL. Cells were then incubated with DCFH-DA (Sigma-Aldrich-D6883; St. Louis, MO, USA), a reagent that measures hydroxyl, peroxyl and other ROS within the cell, for 1 h at 37 °C. Following DCFH-DA diffusion into the cell, the compound gets oxidized by ROS into 2', 7' –dichlorofluorescein (DCF), which is highly fluorescent. The fluorescent intensity of DCF was measured at an excitation rate of 488 nm and an emission rate of 525 nm in a microplate reader. Fluorescence was directly proportional to the rate of ROS produced.

2.8. Measurement of protein expression levels of the core complex I subunits

Protein expression levels of the core mitochondrial complex I subunits (NDUFS7, NDUFS8, NDUFV1 and NDUFV2) were evaluated by standard immunoblotting

analysis. First, HEp-2 treated and untreated cells were harvested and prepared in Laemmli sample buffer, and then denatured by boiling at 100°C for 5 min. Next, 3 µg/µL (10 µL) were loaded onto a 12% acrylamide sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Ltd., Ontario, Canada). After the membranes were blocked with 5% albumin for 1 h, blotting was performed using primary antibodies against NDUFS7 (sc-98644, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 2 h, 1:1000), NDUFS8 (ab96123), NDUFV1 (ab96227) and NDUFV2 (ab96117) (Abcam; Cambridge, MA, USA, 2 h, 1:1000), followed by a secondary goat anti-Rabbit IgG (#ab97051) or anti-Mouse IgG (#ab6789; both from Abcam Inc, Cambridge, MA, USA; 1 h; 1:2000) antibody conjugated to horseradish peroxidase. For loading control, mouse monoclonal antibody against beta-actin (#3700 Cell Signaling Technology, Danvers, MA, USA, 1 h, 1:1000; secondary antibody – 1:2000, 30 min) was used. Immunoreactive bands were detected and imaged by incubating with ECL reagents using Versa Doc (Bio-Rad Laboratories Ltd., Mississauga, Canada). Protein bands were normalized by beta-actin and were quantified using the Image Lab 3.0 software.

2.9. Measurement of gene expression levels of the core complex I subunits

Gene expression levels of the core mitochondrial complex I subunits, NDUFS7, NDUFS8, NDUFV1 and NDUFV2 were evaluated by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). First, total RNA was extracted from the AAE- and rotenone-treated HEp-2 cells (as described above) using Trizol® reagent (Thermo Fischer-15586026), followed by spectrophotometric measurement of concentration and purity using the NanoDrop™ 1000 Spectrophotometer System® (Thermo Scientific, Wilmington, DE, USA). Next, complementary DNA (cDNA) was synthesized from 500 ng of total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen-205311) according to the manufacturer's instructions. An aliquot of each cDNA products (100 ng) were mixed with 2x QuantiFast SYBR Green PCR Master Mix, 10X Quantitect Primer Assay (NDUFS7, NDUFS8, NDUFV1 or NDUFV2) and RNase-free water to yield a total reaction volume of 25 uL/well in a 96-well PCR plate. Quantitative real-time PCR was performed with Bio-Rad CFX96 (Bio-Rad Laboratories, Inc.) with the following cycling conditions: 1) initial PCR activation for 5 min at 95 °C; 2) denaturation for 10 s at 95°C and; 3) combined annealing/extension step for 30 s at 60

°C. The cycle was repeated 40 times. Relative expression levels of NDUFS7, NDUFS8, NDUFV1 and NDUFV2 gene transcripts for each sample were calculated by using the ΔC_t method, where gene expression was normalized to the housekeeping b-actin gene.

2.10. Visualization of the mitochondria

Mitochondria were stained using MitoTracker Red CMXRos (M7512, Invitrogen, Carlsbad, CA, EUA) according to the manufacturer's instructions. HEp-2 cells were grown and treated with AAE as described above on coverslips placed in a tissue culture plate at a density of 2×10^5 cells per mL. After cells reached 80% confluency, they were washed twice with PBS and incubated with prewarmed culture media (37 °C) containing 0.5 μg/ml MitoTracker CMXRos for 45 min at 37 °C. Accumulation of the MitoTracker CMXRos probe depends on the membrane potential. After staining is complete, staining solution was removed and replaced with prewarmed media (37 °C). Mitochondria were visualized and images were acquired using a fluorescence microscope Nikon Eclipse Ti-U.

2.11. Measurement of DNA oxidation and methylation levels by dot-blotting

Genomic DNA was extracted from AAE-treated and –untreated HEp-2 using the GenElute mammalian genomic DNA miniprep kit (G1N70, Sigma-Aldrich, Saint Louis, MO) according to the manufacturer's instructions. DNA samples (50 ng) were denatured at 95°C for 5 min and were spotted onto a wet PVDF membrane (Millipore Ltd., Ontario, Canada), fixed with UV irradiation (Stratalinker 1800, auto crosslink-mode; Stratagene, Tokyo, Japan), and blocked with 5% bovine serum albumin in TBS-T (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20). The membranes were then incubated with primary antibodies against 5-methylcytosine (5-mC; 1:500; #Mabe176, clone AB3/63.3, Millipore Ltd., Ontario, Canada), 5-hydroxymethylcytosine (5-hmC; 1:500; ab73938, Abcam, Cambridge, MA, USA) or 8-hydroxy-2' –deoxyguanosine (8-OHdG; 1:500; ab10802, Abcam, Cambridge, MA, USA) for 30 min at room temperature, followed by incubation with anti-rabbit or anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (1:2000) for 30 min at room temperature. Dot signals were visualized with the ECL reagent using VersaDoc (Bio-Rad Laboratories Ltd., Mississauga, Canada) and were normalized for each AAE treatment condition by negative control (AAE-untreated cells) using the Image Lab 3.0 software.

2.12. Measurement of DNA (cytosine-5)-methyltransferase 1 (DNMT1) activity

HEp-2 cells were untreated or treated with the highest dose of AAE (100µg/mL) as described above. After 24 h of washout period, nuclear and cytosolic fractions were separated using the EpiQuik Nuclear Extraction Kit (Epigentek, USA) and colorimetrically assayed for DNMT1 activity using the DNMT1 Assay Kit (ab113469, Abcam, Cambridge, MA, USA) as per manufacturer's instructions. Briefly, DNMT1 contained in the nuclear and cytosolic fractions bound to the DNMT substrate pre-coated on the well, which were probed with a specific DNMT1 antibody and colorimetrically quantified at an absorbance of 450 nm on a microplate reader. Results were expressed as percentage compared to AAE-untreated control cells.

2.13. Statistical analysis

Results were expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using the SPSS 21.0 for Windows software (SPSS Inc., Chicago, IL). Parametric distribution of the data was determined by using the Kolmogorov–Smirnoff test. Differences between the group means were assessed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for separate comparisons of the treatment groups with the control group. When comparison involved only two groups, the data was assessed by independent sample t-test. Differences were considered significant if *p*-value was less than 0.05.

3. Results

3.1. AAE decreased HEp-2 cell viability

An overall significant difference in HEp-2 cell viability was found between the treatment groups $F(4, 35) = 37.84$, $p < 0.0001$ (Figure 1). HEp-2 cell viability was significantly decreased in rotenone (20 µM)-treated cells compared to AAE-untreated control (30 %). While it was found that 50 and 75 µg/mL of AAE were able to maintain cell viability around 100% (97.38 ± 5.95 and 95.75 ± 3.77 %, respectively) the dose of 100 µg/mL of AAE significantly decreased HEp-2 viability (91.88 ± 3.00) by 10% when compared to AAE-untreated control ($p < 0.05$).

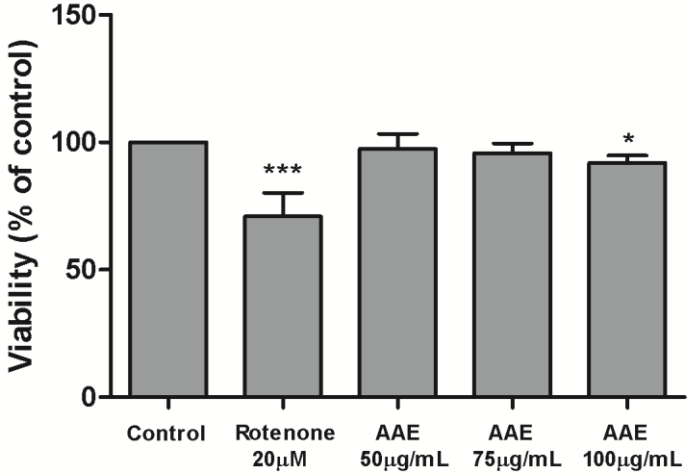

Figure 1. Cell viability measurement by MTT assay in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE) for 24 h. % viability = [(number of cells at time of observation/number of control cells) × 100]. Legend: Statistical significance in relation to control group for *

3.2. AAE altered pyruvate dehydrogenase levels in HEp-2 cells

An overall significant difference in PDH levels was found between the treatment groups, $F(3, 8) = 28.45$, $p < 0.0001$ (Figure 2). Specifically, AAE treatments at 50 and 75 µg/mL significantly augmented PDH levels in HEp-2 cells (33% and 25% of increment, respectively) when compared to untreated HEp-2 control cells. No significant difference was found in HEp-2 cells treated with the highest concentration of AAE (100 µg/mL) when compared to untreated control cells.

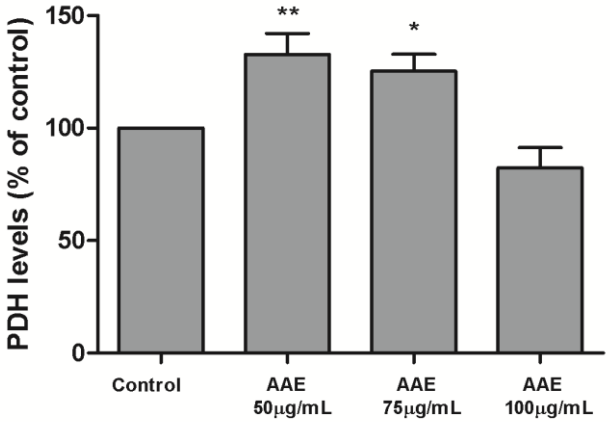

Figure 2. Pyruvate dehydrogenase (PDH) levels in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE). Legend: Statistical significance in relation to control group for * ($p<0.05$), ** ($p<0.01$) using one-way ANOVA followed by Tukey's post hoc test.

3.3. AAE induced specific changes in protein levels of the mitochondrial electron transport chain complexes in HEp-2 cells

We next evaluated whether the levels of complex I, II, III, IV, and V of the mitochondrial electron transport chain differed between the AAE treatment conditions (Figure 3). An overall significance was found between the treatment groups for complex I, $F(4, 10) = 59.24$, $p < 0.0001$; complex II, $F(4, 10) = 50.09$, $p < 0.0001$; complex III, $F(4, 10) = 10.81$, $p = 0.0012$; complex IV, $F(4, 10) = 14.58$, $p < 0.0004$ and complex IV, $F(4, 10) = 29.10$, $p < 0.0001$. Tukey's multiple comparison test revealed that rotenone (20μM), a specific complex I inhibitor, produced a 50% decrease in complex I levels and a 60% increase in complex V levels in HEp-2 cells compared to AAE-untreated control. While AAE at all tested concentrations (50, 75, and 100 μg/mL) induced a significant decrease in complex I (around 40%) and complex III (around 20%) levels in HEp-2 cells, a significant increase in complex IV levels was also observed (11% increase for 50 μg/mL; and 9% increase for both 75 and 100 μg/mL). In addition, HEp-2 cells treated with the highest dose of AAE (100 μg/mL) significantly decreased complex II levels by 50%, and increased complex V levels by 66% in comparison to untreated control. Since complex I and III are a major endogenous source of ROS production and because AAE induced a marked reduction in complex I and III protein levels in a similar way to rotenone, levels of reactive oxygen species production were next evaluated.

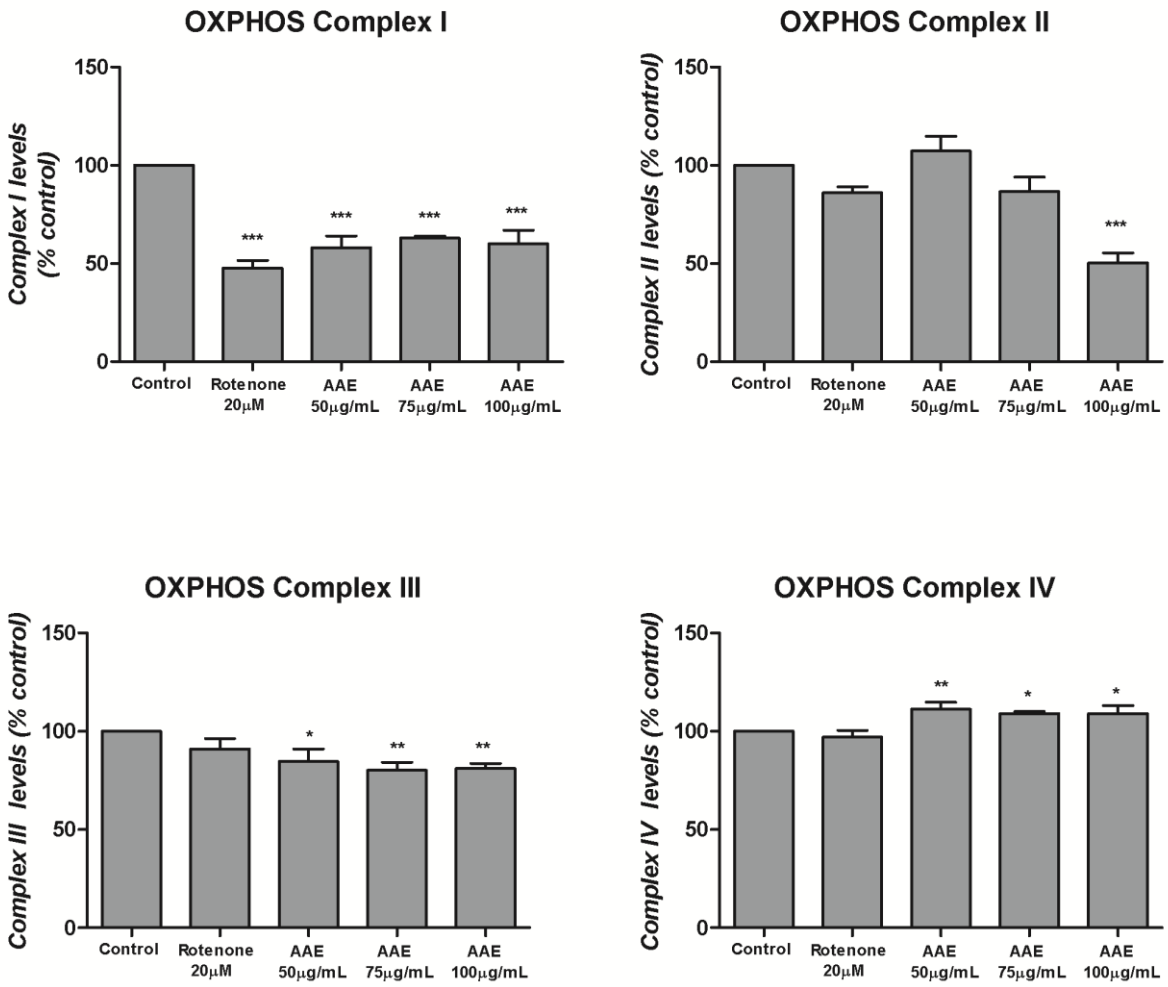

Figure 3. Levels of complex I, II, III, IV, and V of the mitochondrial electron transport chain in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE). The results were normalyzed by NNT (nicotinamide nucleotide transhydrogenase) levels. Legend: Statistical significance in relation to control group for * ($p<0.05$); ** ($p<0.01$); ***($p<0.001$) using one-way ANOVA followed by Tukey's post hoc test.

3.4. AAE induced reactive oxygen species production in HEp-2 cells

There was an overall significant difference in the production of ROS between the treatment groups, $F(4, 15) = 18.67$, $p < 0.0001$ (Figure 4). The highest concentration of AAE (100 µg/mL) induced a significant increase in intracellular ROS by 36%, in a similar manner to rotenone which produced a 42% increase in ROS production when compared to AAE-untreated control. While significance was not reached for cells treated with 50 and 75 µg/mL of AAE, a concentration-dependent increase in ROS levels was observed. As AAE was observed to increase ROS production, the protein expression levels of the core complex I subunits involved in electron transfer and ROS generation were next measured.

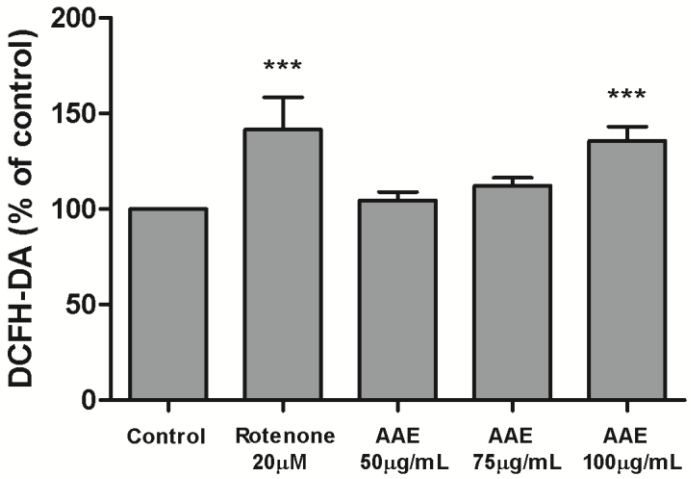

Figure 4. Total levels of ROS measured by redox sensitive fluorophore DCFH-DA in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE). Legend: Statistical significance in relation to control group for ***($p<0.001$) using one-way ANOVA followed by Tukey's post hoc test.

3.5. AAE altered protein expression levels of the core complex I subunits in HEp-2 cells

To determine the effect of AAE on protein levels of the core complex I subunits (NDUFS7, NDUFS8, NDUFV1 and NDUFV2) involved in electron transfer and subsequent ROS generation, standard immunoblotting analysis was used (Figure 5). Between group differences were found only for NDUFS7, $F(4, 15) = 14.50$, $p < 0.0001$ and NDUFV2, $F(4, 15) = 33.03$, $p < 0.0001$ in cells treated with AAE. Further analysis showed a significant decrease in NDUFS7 levels by 0.5, 0.6 and 0.5 times respectively in HEp-2 cells treated at all concentrations of AAE (50, 75 and 100 µg/mL) when compared to AAE-untreated control, while a significant increase in NDUFV2 levels (around 1.5 times) were found in cells treated with 75 and 100 µg/mL of AAE. NDUFS8 and NDUFV1 levels did not differ between the AAE treatment groups. In addition, rotenone

was found to significantly decrease NDUFS7 and increase NDUFV2 levels similarly to AAE, however a decrease in NDUFS8 was also observed.

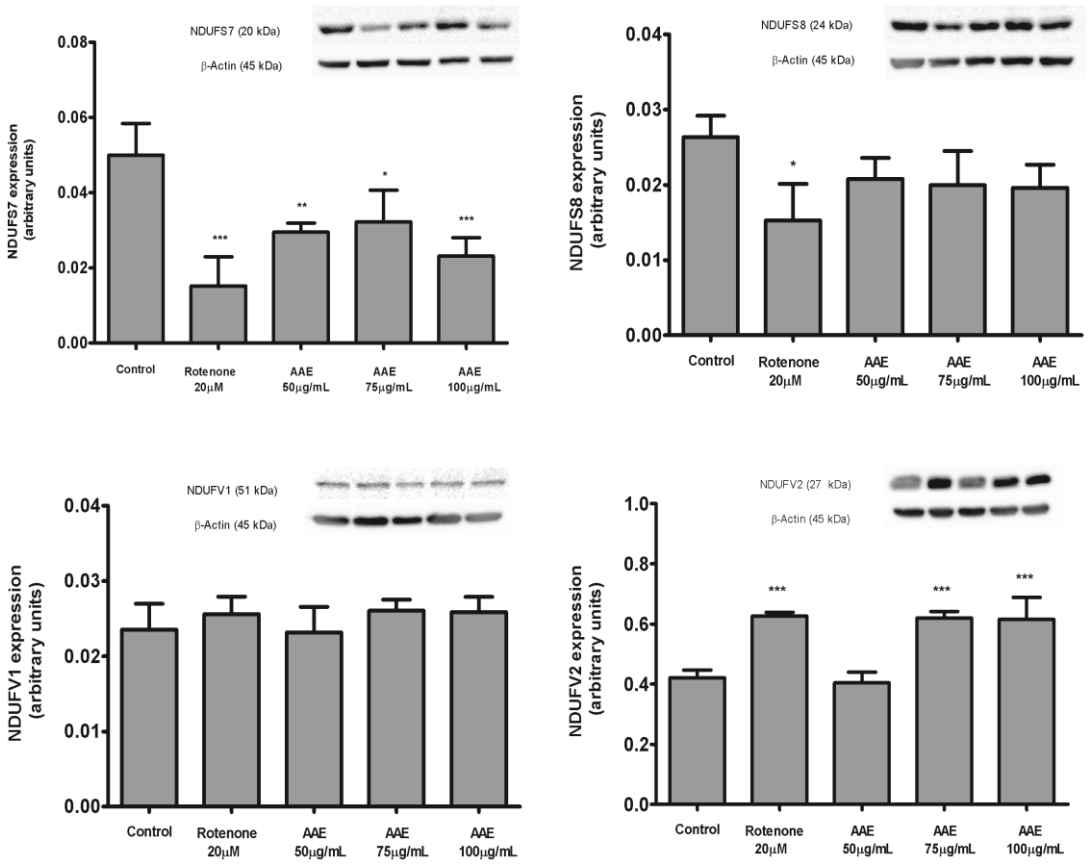

Figure 5. NDUFS7, NDUFS8, NDUFV1 and NDUFV2 protein expression in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE) by Western blot assay normalized by beta-actin. Legend: Statistical significance in relation to control group for * ($p<0.05$); ** ($p<0.01$); ***($p<0.001$) using one-way ANOVA followed by Tukey's post hoc test.

3.6. AAE altered gene expression levels of the core complex I subunits in HEp-2 cells

To complement core complex I subunits analysis, gene expression of NDUFS7, NDUFS8, NDUFV1 and NDUFV2 was assessed using qRT-PCR. An overall significance in NDUFS7 and NDUFV2 gene transcript levels between the groups was found, with a significant downregulation of NDUFS7 by 15% and an upregulation of NDUFV2 by 20% found in HEp-2 cells treated with the highest AAE concentration (100 µg/mL) compared to AAE-untreated control (Figure 6). Rotenone, however, was found to significantly decrease NDUFS7 and NDUFS8 gene transcript levels by around 20%.

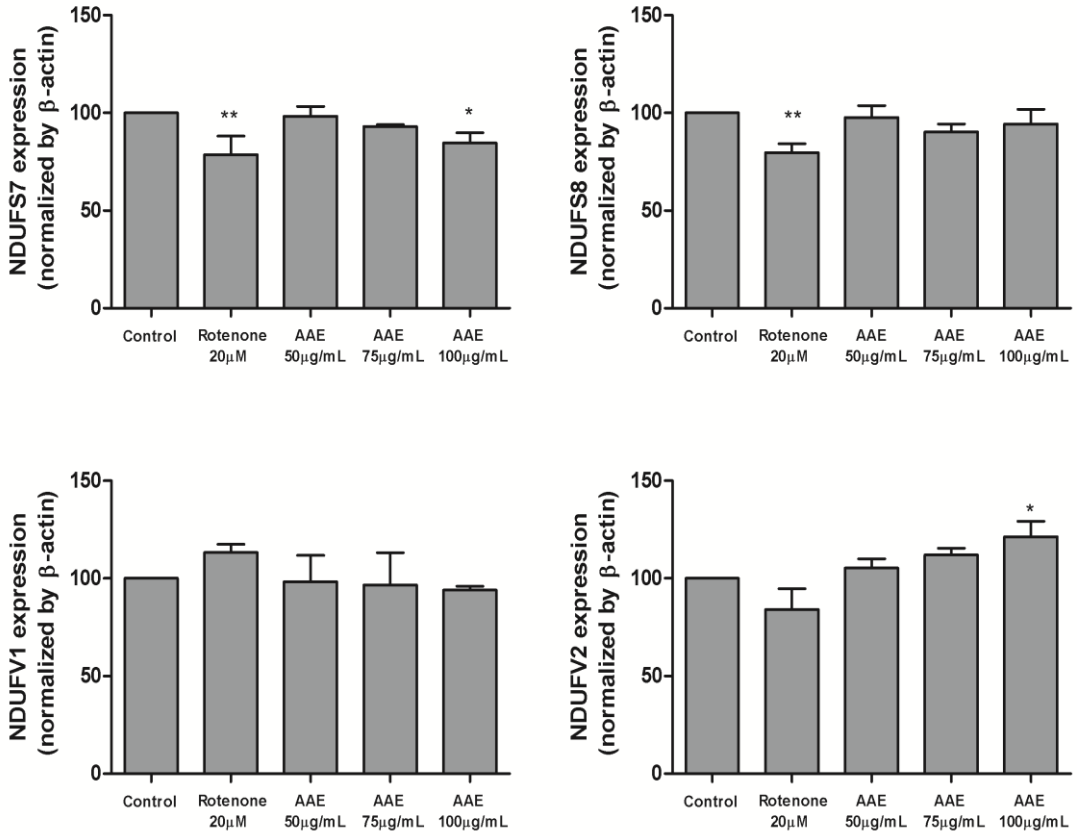

Figure 6. NDUFS7, NDUFS8, NDUFV1 and NDUFV2 gene expression in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE) by qRT-PCR assay normalized by beta-actin. Legend: Statistical significance in relation to control group for * ($p<0.05$), ** ($p<0.01$) using one-way ANOVA followed by Tukey's post hoc test.

3.7. AAE induced changes in mitochondrial membrane potential in HEp-2 cells

To further explore whether AAE alters mitochondrial function, AAE-treated and untreated HEp-2 cells were stained with MitoTracker CMXRos, which measures mitochondrial morphology and mass in a manner that is dependent on the mitochondrial membrane potential (Figure 7). In AAE-untreated control, mitochondria were of round-shaped, closely localized and encircled the nucleus, and brightly stained by the red dye, suggesting maintenance of membrane potential. Similar aspect was observed to HEp-2 cells treated with 50 μg/mL of AAE. In AAE-treated HEp-2 cells (75 and 100 μg/mL), however, mitochondria were abnormally enlarged, scattered in the cytosol, and showed a reduction in membrane polarization relative to that of AAE-untreated HEp-2 cells, as evidence by reduced red intensity.

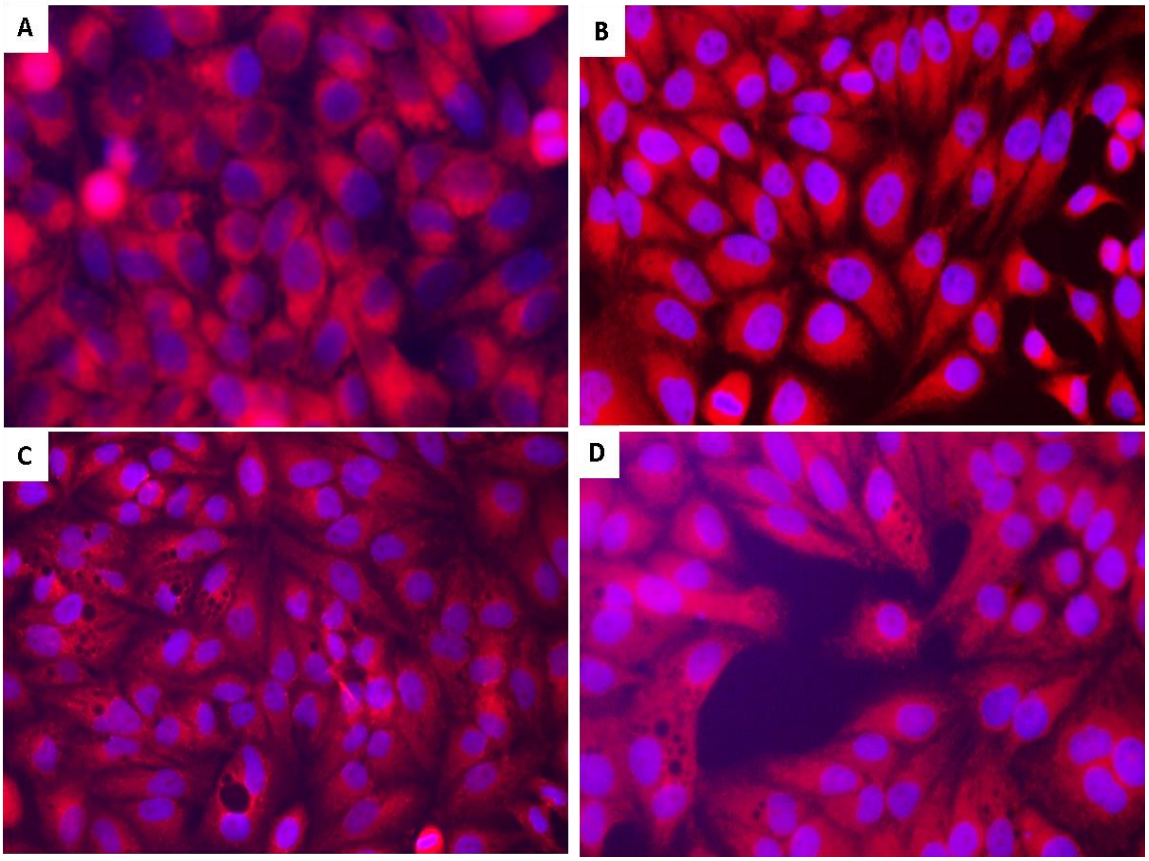

Figure 7. Mitochondrial membrane potential (ΔΨm) in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE). (A) AAE-untreated control cells; (B) AAE 50 µg/mL; (C) AAE 75 µg/mL, and (D) AAE 100 µg/mL. Cells were grown on 12 mm cover slips in 24-well culture plates as described in Section 2, and then photographed (magnification 20×).

3.8. AAE did not affect DNA oxidation in HEp-2 cells

To determine whether DNA is a target for oxidation by AAE, 8-hydroxy-2'–deoxyguanosine (8-OHdG), a marker of DNA oxidative damage, was measured in untreated- and treated-HEp-2 cells. No significant difference was found for 8-OHdG levels between the AAE treatment groups (Figure 8).

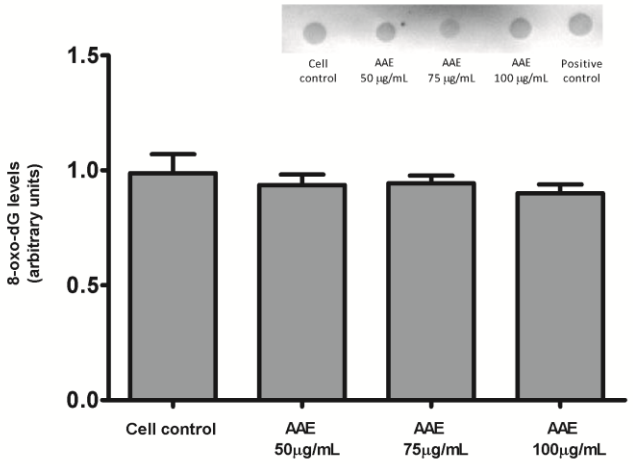

Figure 8. Levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) evaluated by dot-blotting in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE). Results were normalyzed by positive control levels. Legend: Cell control (cells that did not received AAE treatment).

3.9. AAE decreased DNA methylation and increased hydroxymethylation in HEp-2 cells

To further investigate the effect of AAE on DNA chemical modifications, 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) was measured in HEp-2 cells. An overall significant difference in 5-mC, $F(3, 8) = 33.8$, $p < 0.0001$ (Figure 9) and 5-hmC, $F(3, 8) = 18.74$, $p = 0.0006$ (Figure 10) levels were found between the groups. AAE (50, 75 and 100 µg/mL) produced a significant dose-dependent decrease in 5mC levels, and a dose-dependent increase in 5hmC levels (with significance at 75 and 100 µg/mL of AAE) when compared to AAE-untreated control.

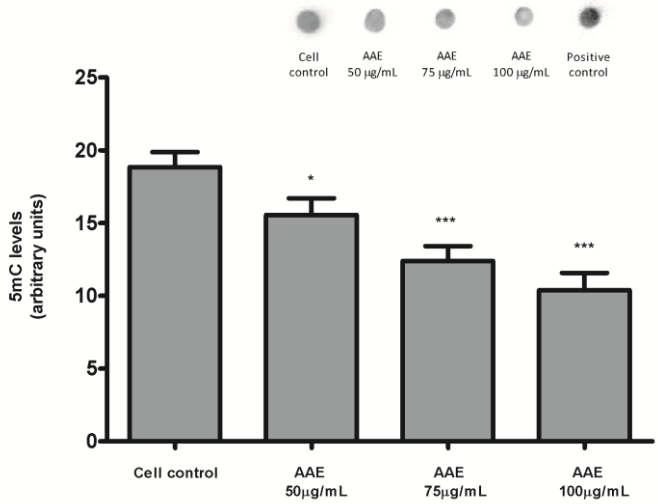

Figure 9. Levels of methylation (5mC) evaluated by dot-blotting in HEp-2 cells treated with *Araucaria angustifolia* extract (AAE). Results were normalyzed by positive control levels. Legend: Cell control (cells that did not received AAE treatment). Statistical significance in relation to control group for * ($p<0.05$), *** ($p<0.001$) using one-way ANOVA

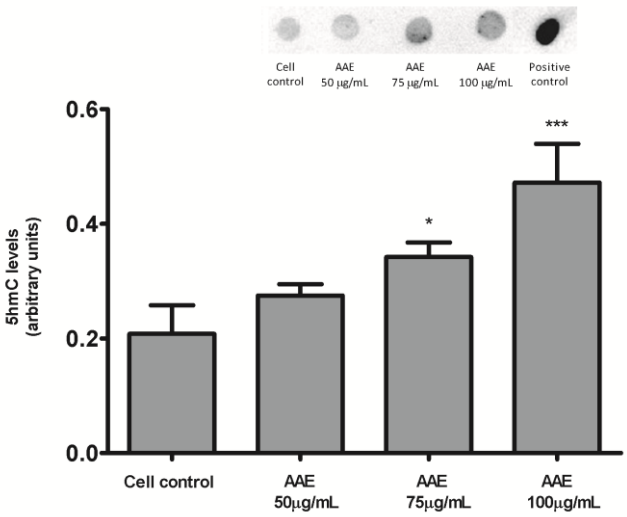

Figure 10. Levels of hydroxymethylation (5hmC) evaluated by dot-blotting in HEp-2 cells treated with Araucaria angustifolia extract (AAE). Results were normalyzed by positive control levels. Legend: Cell control (cells that did not received AAE treatment). Statistical significance in relation to control group for * ($p<0.05$), ***

3.10. AAE decreased DNMT1 activity in HEp-2 cells

As DNMT1 is responsible for regulating DNA methylation levels, the activity of DNMT1 was assessed in the nuclear and cytosolic fractions of HEp-2 cells treated with the highest concentration of AAE (100 µg/mL). To determine whether DNMT1 differed between AAE-untreated and AAE (100 µg/mL)-treated HEp-2 cells, independent samples t-test was used. Results revealed that DNMT1 activity was significantly reduced in AAE (100 µg/mL)-treated HEp-2 cells in both the nuclear, $t(7) = 7.23$, $p = 0.0002$, and the cytosolic, $t(7) = 3.18$, $p = 0.015$, fractions compared to AAE-untreated control (Figure 11).

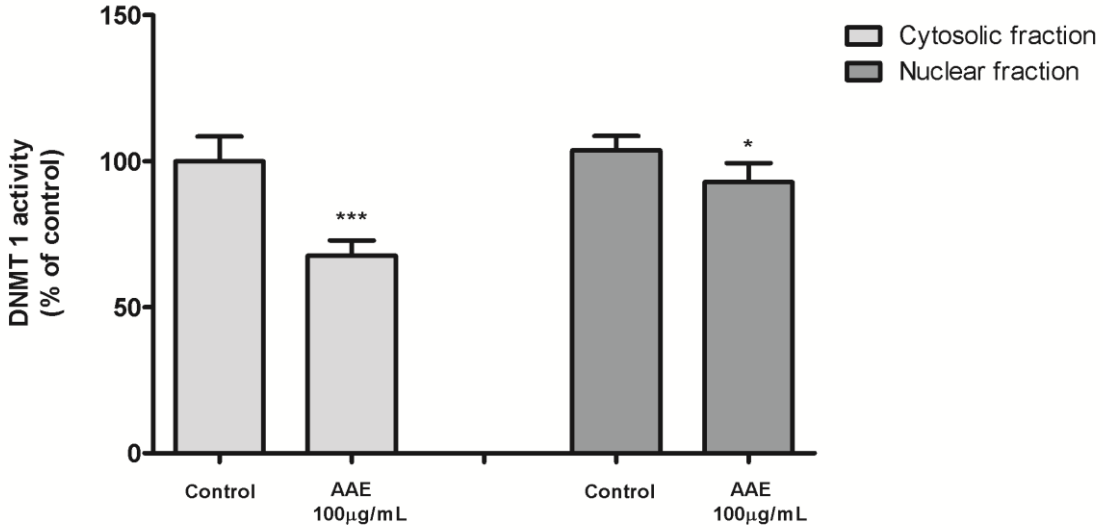

Figure 11. Activity of DNA (cytosine-5)-methyltransferase 1 (DNMT1) in HEp-2 cells treated with *Araucaria angustifolia* extract. Legend: Statistical significance in relation to control group for * ($p<0.05$) and ***($p<0.001$) using one-way ANOVA followed by Tukey's post hoc test.

Discussion

We previously demonstrated that AAE treatment inhibits mitochondrial complex I activity leading to oxidative damage to proteins and lipids and subsequent apoptosis in HEp-2 cancer cells[28]. In the present study, we further explored the underlying mechanisms of action of apoptotic death in HEp-2 cancer cells. Results demonstrated that AAE increased protein levels of PDH, switching energy metabolism to oxidative metabolism. Protein expression levels of complex I and III were found decreased in AAE-treated HEp-2 cells, leading to increased ROS generation. Analyzing the subunits of complex I relevant to ROS generation, decreased protein and gene transcript levels of NDUFS7 in HEp-2 cancer cells were found. Increased ROS levels were also accompanied by decreased mitochondrial membrane potential, and decreased methylation of DNA in HEp-2 cells. There is growing evidence that these mitochondrial and epigenetic impairments can be targeted by AAE polyphenols to inhibit cancer cell growth.

Pyruvate dehydrogenase, the rate limiting enzyme involved in the conversion of pyruvate to acetyl-coA is frequently suppressed in many cancer types leading to increased glycolysis[29]. In patients with head-and-neck cancer, increased lactate concentration, a product of anaerobic glycolysis, was found to clinically associate with tumour progression and metastasis[30]. Increased lactate production in cancer cells suggest that the pathway involved in feeding pyruvate, the final product of aerobic glycolysis into the mitochondria for oxidative metabolism is altered. Herein, we found that AAE concentrations (50 and 75 µg/mL) significantly increased PDH levels in HEp-2 cancer cells, suggesting that AAE may be preventing HEp-2 cancer cells from using anaerobic glycolysis to maintain its rapid proliferation by increasing the influx of pyruvate, the final product of aerobic glycolysis into the mitochondria for oxidative metabolism. Currently there are no studies evaluating the role of polyphenols on PDH in cancer cells. However, several studies demonstrated that positive modulators of PDH presents anticancer effects[29]. For example, dichloroacetate, a small molecule that activate PDH in cancer cells, was shown to increase influx of pyruvate into the mitochondria, increase apoptosis and subsequently suppress tumour growth[31]. These results suggest that targeting PDH by AAE could be a major mechanism of inhibiting growth and inducing apoptosis in HEp-2 cancer cells.

As switching of anaerobic metabolism to oxidative metabolism can trigger apoptosis by activating PDH, we next evaluated the effect of AAE on the protein expression levels of the mitochondrial ETC complexes in HEp-2 cancer cells. We found

that AAE at all tested concentrations decreased protein expression levels of complex I and III and increased complex IV and V. This supports our previous study in which decreased complex I activity was found in AAE-treated HEp-2 cells[28]. The effect of AAE is particularly interesting, because a major effect of ETC inhibition, mainly at complex I and III, is the induction of ROS generation resulting in the selective killing of cancer cells, as they are more vulnerable than normal cells to oxidative stress[32]. Subjecting HEp-2 cells with the highest concentration of AAE (100 µg/mL) was found to induce a significant increase in intracellular ROS, suggesting that induction of cancer cell death by AAE may be mediated in part, by inhibition of mitochondrial complex I and III. Currently, there are very few studies assessing the effect of polyphenols on the levels of mitochondrial ETC complexes in cancer cells. However, a recent study demonstrated that grape seed extract induced mitochondrial dysfunction by inhibiting complex III activity, but did not affect complex I activity in head and neck squamous cell carcinoma, leading to increased intracellular ROS and subsequent apoptosis[33]. The generation of ROS appears to be an important event in facilitating AAE-induced apoptosis in HEp-2 cancer cells.

Increased ROS production is associated with deficits in several protein subunits of the mitochondrial ETC, particularly complex I and III[16]. Complex I is the first and largest complex of the ETC and is a major site of ROS production[34]. It is composed of 45 protein subunits containing 7 nuclear DNA-encoded core subunits clustered into two functional modules: 1) an electron input module (N-module) and 2) an electron output module (Q-module)[35]. The N-module contains a site for oxidation of NADH while the Q-module receives electrons from the N-module and transfers them to the electron-accepting ubiquinone molecule[35,36]. The transfer of electron starts at NDUFV1, NDUFV2, and NDUFS1 subunits of the N-module responsible for oxidizing NADH, followed by NDUFS2, NDUFS3, NDUFS7, and NDUFS8 subunits of the Q module responsible for electron transfer to ubiquinone[34,35]. Herein, we found that AAE induced a significant increase in NDUFV2 in the N-module and a decrease in NDUFS7 in the Q-module at both the gene transcript and protein levels in HEp-2 cells. An increase in NDUFV2 and decrease in NDUFS7 levels suggests that while AAE is able to increase the ability of HEp-2 cells to oxidize NADH, the ability to transfer electrons to ubiquinone is reduced. Reduced electron-transferring ability increases the rate of electrons escaping from complex I and if persist for enough time, will react with molecular oxygen to produce superoxide anion, a major form of ROS involved in cancer cell apoptosis[37,38]. To our knowledge, this is the first study examining the effect of AAE on specific nDNA-

encoded core subunits of complex I in HEp-2 cancer cells and identifies NDUFS7 as a key target of AAE.

ROS accumulation can promote oxidative damage to DNA and mitochondrial membrane, and consequently trigger cancer cell apoptosis[39,40]. Previously, we have shown that AAE induced significant global DNA damage and fragmentation in HEp-2 cells[28]. To assess whether ROS overload induced by AAE was also accompanied by increased oxidative damage to DNA, 8-hydroxy-2' –deoxyguanosine (8-OHdG), a major product of DNA oxidation, was measured. Herein, we demonstrated that AAE did not affect 8-OHdG levels in HEp-2 cells. Increased global DNA damage in the absence of 8-OHdG in AAE-treated HEp-2 cells suggests that DNA guanine base is not a target for oxidation by high levels of ROS and that damage to DNA induced by AAE may occur through different mechanisms, which warrants further studies. ROS could contribute to DNA damage and cancer progression by additional mechanisms including damage to DNA repair enzymes[41], impairment to DNA polymerase leading to increased error rate during replication[42], or reaction of lipid peroxidation products with DNA bases forming a variety of mutagenic adducts[43]. Furthermore, DNA oxidation is not only limited to 8-OHdG. Several other DNA oxidation products such as 2-hydroxyadenine, 5-hydroxycytosine, formyluracil, and 5-hydroxyuracil can lead to mutagenic lesions in DNA[44]. Further studies are needed to examine these specific markers of DNA damage in AAE-treated HEp-2 cells.

A consequence of ROS overload and DNA damage is change in the mitochondrial membrane potential[40,45]. Mitochondrial membrane potential ($\Delta\Psi m$) is an important indicator of functional mitochondria due to its role in maintaining the function of the ETC to generate energy[46]. A significant loss of $\Delta\Psi m$ renders cells depleted of energy and is one of the early events leading to apoptosis[47]. To decipher the implications of increased ROS production and DNA damage following AAE treatment, we next assessed the effect of AAE on $\Delta\Psi m$ using a mitochondrial-targeted probe, Mitotracker Red CMXRos, which accumulates in mitochondria based on its membrane potential. In HEp-2 cells treated with AAE (75 and 100 µg/mL), the fluorescence intensity of the probe decreased, indicating a loss in $\Delta\Psi m$. Loss of $\Delta\Psi m$ is associated with increased permeability of the outer mitochondrial membrane, release of cytochrome c and apoptotic-inducing factor (AIF) into the cytosol, activation of caspase-9 (initiator of apoptosis) leading to activation of caspase-3,6,7 (executioners of apoptosis)[47,48]. In our previous study, we found that AAE increased protein expression levels of pro-apoptotic factors, including AIF and caspase-

3[28]. Previous studies have shown that polyphenols presenting anticancer activities, such as curcumin disrupts ΔΨm and mediates oxidative stress leading to apoptosis in many cancer models[49,50]. Therefore, our data suggests the possibility that AAE exert anticancer effects through ROS accumulation and DNA damage leading to increased mitochondrial membrane permeability and decreased ΔΨm, triggering the apoptotic pathways and death in HEp-2 cancer cells.

Uncontrolled production of ROS could also disrupt epigenetic processes[51,52]. Epigenetic processes involve chemical modifications of DNA to control level of gene expression[20]. DNA methylation, a common epigenetic marker, is the process of adding a methyl group to the cytosine or adenine nucleotide of DNA, leading to silencing of gene expression[53]. Evidence have demonstrated that a large number of human malignancies are associated with high levels of 5-methylcytosine on tumour suppressor genes that are transcriptionally silent[21,54]. In the present study, we demonstrated that AAE decreased 5-mC levels in a concentration-dependent manner in HEp-2 cancer cells. We further showed that AAE decreased the activity of DNMT1, a predominant human methylating enzyme. These findings suggest that AAE may be promoting DNA demethylation in HEp-2 cancer cells. DNA demethylation is also associated with the conversion of 5mC to 5hmC[55]. Low levels of 5hmC was found to associate with myeloid malignancies[56]. Herein, AAE was also found to increase 5hmC levels in HEp-2 cells in a dose-dependent manner. Several studies have shown that the production of ROS can directly oxidize the methyl group on 5mC leading to demethylation processes in several model systems[57-59]. In line with our findings, a study demonstrated that (-)-epigallocatechin-3-gallate could inhibit DNMT1 activity and decrease methylation at specific genes involved in regulating cell cycle division, including p16, RARb and MLH[60]. Following EGCG treatment, cell growth was found inhibited and ultimately many damaged and polynuclear cells were observed – a sign that the cancer was being stopped[60]. Decreased DNMT1, reduced DNA methylation and increased hydroxymethylation found in this study may be attributed to increased intracellular ROS and suggests that AAE may be reactivating tumour suppressor genes, which is critical for preventing cancer cell growth and survival. While these results are very interesting, future studies are needed to determine which specific genes are targets of methylation by AAE.

While interpreting our results, it is important to discuss several limitations from our methods and highlight future directions. First, little efforts have been made to evaluate the role of individual polyphenol constituents of AAE on its pro-apoptotic effects. Further

studies are necessary to examine which active constituent(s) of AAE are involved in mitochondrial dysfunction and epigenetic changes in HEp-2 cancer cells. Secondly, we only analyzed 4 nuclear-encoded genes of complex I as they are most relevant to electron transfer and ROS generation. Given that electron transfer also occurs through complex III and can be affected by genetic alterations in the mitochondrial DNA, we could not exclude the possibility that AAE also play a role in influencing these changes. Furthermore, several markers such as PDH and DNMT1 were measured either at the protein or activity level. More studies involving the combination of gene, protein, and activity levels, as well as identifying key transcriptional regulators are needed in order to fully understand the mechanisms of AAE.

Conclusion

In conclusion, we further provided compelling evidence that AAE shows pro-apoptotic activity against HEp-2 cancer cells. This pro-apoptotic activity was associated with increased PDH levels which was accompanied by increased intracellular ROS. Increased ROS appeared to originate from decreased NDUFS7 levels of complex I, which triggered loss of mitochondrial membrane potential, DNA hypomethylation and induction of apoptosis in HEp-2 cancer cells. The results demonstrate the potential of AAE to be used as a therapeutic agent for cancer for laryngeal carcinoma.

Conflict of interest

The authors declare no conflict of interest.

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3.5 CAPÍTULO V

Development of polycaprolactone (PCL) nanospheres in the presence or absence of polyphenols-rich extract and evaluation of PCL toxicity in human cells

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DEVELOPMENT OF POLYCAPROLACTONE (PCL) NANOSPHERES IN THE PRESENCE OR ABSENCE OF POLYPHENOLS-RICH EXTRACT AND EVALUATION OF PCL TOXICITY IN HUMAN CELLS

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DEVELOPMENT OF POLYCAPROLACTONE (PCL) NANOSPHERES IN THE PRESENCE OR ABSENCE OF POLYPHENOLS-RICH EXTRACT AND EVALUATION OF PCL TOXICITY IN HUMAN CELLS

Nanoparticles have great potential as drug delivery; however, there is few studies on their possible toxicity. In this study we developed nanospheres using poly(ε-caprolactone) (NPCL) and evaluated its mechanisms of action in tumor (HEp-2) and non-tumor (MRC5) cells. The possibility to associate a polyphenols-rich extract (PE) to the formulation was also investigated. Cell viability was assessed by MTT assay; and oxidative and morphological parameters were evaluated as NPCL's mechanisms. The pH of NPLC in presence or absence of PE was stable between a range of 5.9 and 6.4; diameter was around 200 nm and both samples exhibited narrow distribution (polydispersity index up to 0.19) with zeta potential from -9.6 to -10.6 mV. Low association rate for NPCL *plus* PE (efficiency of 19.05 %) was found; therefore, further studies are needed to improve PE encapsulation. NPCL reduced viability with different level of toxicity (IC_{50} values 460 ± 25 µg.mL^{-1} for HEp-2 and 573 ± 51 µg.mL^{-1} for MRC5 cells; p=0.008, *t*-test). This reduction was associated to alterations on redox metabolism. Changes on symmetry and cell adherence at highest doses were observed in both cells. Results suggest that, depending on the applied concentrations and the cell type, NPCL *per se* may interfere on cellular response.

Keywords: nanotechnology; cytotoxicity; redox metabolism, tumor cells

INTRODUCTION

Nanomedicine is among one of the most promising fields, combining the pharmaceutical and biomedical sciences with nanotechnology. In the last decades, the number of different applications using nanoparticle materials on drug delivery or as drugs themselves (*per se*) has increased [1–3]. Nanocarriers are systems developed at the nanoscale (< 1 μm) and includes several different forms already approved by Food and Drug Administration (FDA) such as microcapsules and nanospheres [4–6]. The nanospheres are systems able to incorporate both hydrophilic and lipophilic substances, thus increasing the bioavailability of poorly soluble substances [7]. Among the aliphatic polyesters commonly used as drug carriers, the poly(ε-caprolactone) (PCL) has stood out due to their diverse applications [2]. Some of the main advantages of using PCL are a slow degradation rate, the absence of acidity during degradation and its high permeability to small drug molecules [8,9], showing broad pharmacological applicability.

The increasing number of diseases worldwide highlights the importance of the development of new pharmacological alternatives, and the polymeric systems for drug delivery have gained attention. Diseases like cancer have driven studies seeking to develop new nanotechnology-based therapeutics [10,11]. Nowadays, more than 50 % of the drugs used on cancer treatment is based on natural products [12] which contain phytochemicals, potent modulators of cellular signaling pathways [13–16]. Taking into account the low bioavailability of these compounds, their instability, and target specificity, the nanotechnology represents an innovative and promising approach. Our research group has been studying a polyphenols-rich extract (PE) derived from natural source, the *Araucaria angustifolia* (for review, see [17]), which possess important and selective antitumor activity [18,19], being therefore a promising candidate for encapsulating. Polyphenols are compounds chemically labile and encapsulating is a valuable way to preserve its chemical characteristics and hence the stability. In fact, there are studies reporting the use of the nanocarriers to incorporate chemically labile substances, such as lipoic acid [20], quercetin [21,22], and catechin [22].

Until now, there are few data in the literature showing employment of nanotechnology for association of plant extracts, since encapsulate these phytochemicals without changing their genuine characteristics is a challenge to be overcome. Indeed, it is need to know the physicochemical parameters of the chosen carrier, its stability and compatibility to phytochemicals naturally present in plant extracts. Furthermore,

understanding the biophysicochemical interactions of nanocarriers without active substance is very important in order to keep the safety and effectiveness.

In view of this, the aim of this study was to prepare a formulation of nanospheres using poly(ε-caprolactone) (NPCL) and evaluate its mechanisms of action in tumor (HEp-2) and non-tumor (MRC5) cells. Moreover, the possibility to associate a polyphenols-rich extract (PE) to the formulation was also investigated.

EXPERIMENTAL

Chemicals

Poly(ε-caprolactone) (PCL) (Mw = 14,000 g mol^{-1}) and sorbitan monoesterate (Span 60 ®) were supplied by Aldrich® (Strasbourg, France). Polysorbate 80 was obtained from Henrifarma® (São Paulo, Brazil). Acetone was acquired from Nuclear® (Diadema, Brazil). The chemicals used in cell culture were Complete Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), trypsin–EDTA and penicillin–streptomycin, all were purchased from Gibco BRL® (Grand Island, NY, USA). Catechin and other reagents and solvents were obtained from Sigma® (St. Louis, MO, USA). All chemicals were of analytical grade.

Preparation of NPCL

NPCL were synthesized by interfacial deposition of preformed polymer [23]. The organic phase composed of PCL, sorbitan monoesterate and acetone was prepared at 40 °C. Separately, polysorbate 80 was dissolved in distilled water (aqueous phase). The organic solution was injected into the aqueous solution, and maintained for 10 min under magnetic stirring, at room temperature. After complete homogenization, the acetone was eliminated and the suspension was concentrated under reduced pressure at 40 °C (Rotavapor Buchi ®, model R210). For preparation of NPCL containing the polyphenols-rich extract (PE) from *Araucaria angustifolia* sterile seeds (bracts), two different approaches were employed: a) it was used an aqueous extract obtained using 5 g of bracts in 100 mL of distilled water (under reflux at 100 °C) for 15 min; and b) it was used a lyophilized powder obtained from PE as described above, under vacuum conditions at -54 ± 5 °C (Lyophilizer LIOBRAS® model L-101); both were added to the aqueous phase (polysorbate 80 and distilled water) containing, respectively, 1.43 mg mL^{-1} and 1.27 mg mL^{-1} of catechin equivalents. PE is rich in phenolic compounds including catechin,

epicatechin, rutin, quercetin, apigenin, 4'-methoxytectorigenin, 3-glucoside-dihydroquercetin and the biflavonoid amentoflavone 4',4''',7,7''-tetramethyl ether, as described previously by our group [18,24,25]. As catechin is the major compound present in PE, it was used as a marker to represent the total content in the colloidal structure.

Chemical characterization of NPCL suspensions

The physico-chemical characterization of the suspensions was performed through the determination of the pH, particle size, analysis of polydispersity index (PDI) and zeta potential (ZP). The pH values of the suspensions were measured without previous dilution using a calibrated potentiometer (TECNOPON model Mpa-210). Quantifications of particle diameters and PDI were performed by dynamic light scattering using a Zetasizer ZS (Malvern, UK). Measurements of ZP were carried out by electrophoretic mobility technique using a Zetasizer ZS (Malvern, UK). To assess the morphology and surface characteristics of NPCL, the suspensions were previously dried by lyophilization (Lyophilizer LIOBRAS® model L-101) and analyzed by scanning electron microscopy (SEM) using a Shimadzu SSX 550, equipped with a CCD camera. In order to quantify levels of catechin (marker) in the NPCL formulation containing PE, an aliquot of the suspension was combined with methanol, and submitted to ultrasonic extraction to promote the dissolution of the nanospheres. The solution was then filtered in Millipore equipment (pore size, 0.22 μm; Millipore® Corp.) and analyzed by High Performance Liquid Chromatography (HPLC). To assess the encapsulation efficiency of the PE, it was used a microcentrifuge tube coupled with filter (Amicon® Ultra 0,5 10.000 NMWL; Millipore Corp.). The sample was centrifugated at 12 000 rpm, 4 °C for 15 min to separate aqueous and polymeric portions. The lipophilic (polymer) portion was analyzed by HPLC in order to quantify the amount of catechin. The chromatographic analyzes were carried out using HPLC model HP 1100 UV/VIS (Palo Alto, CA) by Zorbax SB C18 column (250 x 4 mm) with a flow rate of 0.5 ml min^{-1}. The samples were eluted (20 μL injection volume) using an isocratic mobile phase 90% acidic water (5% acetic acid) and 10% acidic methanol (5% acetic acid), monitored by UV absorbance at 280 nm at 45 min. The concentration of catechin was estimated from standard curves obtained by the analysis of various doses of standard compound. The results were expressed as $mg.mL^{-1}$ of catechin.

Assays with mammalian cells

To study the effects of NPCL in human cells, the laryngeal tumor cells (HEp-2) and lung fibroblast cells (MRC5) were used, both acquired from American Type Culture Collection (ATCC bank; VA, USA). Cells were cultured in DMEM medium, supplemented with 10 % FBS, and 1 % penicillin–streptomycin (10,000 U mL^{-1}), and were maintained in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C until experimentation.

Analysis of cytotoxicity of NPCL – MTT assay

The possible cytotoxicity of the NPCL was evaluated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- 2H-tetrazolium bromide] assay [26]. This assay reflects the activity of mitochondrial dehydrogenases by viable cells in convert MTT salt. The HEp-2 and MRC5 cells were seeded (1×10^4 cells mL^{-1}), and incubated at 37 °C in 5 % CO_2 for 24 h to attach. After this time, the medium was removed and replaced by fresh complete medium containing 0; 0.03; 0.3; 3; 30; 300 and 750 µg mL^{-1} of PCL nanospheres for 24 h. MTT (1 mg mL^{-1}) was then added to the wells, and incubated for 3 h. Subsequently, MTT solution was removed and the resulting formazan violet product was dissolved in dimethylsulfoxide (DMSO), stirred for 15 min, and the absorbance was measured using a microplate reader (Victor-X3, multilabel counter, Perkin Elmer, Finland) at 517 nm. The cell viability was expressed as percentage (%) of the control from five independent experiments. The IC_{50}, *i.e.* the inhibitory concentration that is needed to reduce 50 % of the cell proliferation was also quantified.

Redox metabolism evaluation

Oxidative damage to lipids and proteins, and enzymatic and non-enzymatic antioxidant defenses were assessed in HEp-2 and MRC5 cells (1×10^7 cells mL^{-1}) treated with 3; 30; 300 and 750 µg mL^{-1} of NPCL. The concentrations were chosen from MTT assay, taking into account the minimal doses able to reduce cell viability. Oxidative damage to lipids was monitored by the formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction [27]. Results were expressed nmol TBARS per mg of protein. Oxidative damage to proteins were measured based on the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) [28]. The results were expressed as nmol DNPH per mg of protein. Enzymatic antioxidant defenses were assessed through Superoxide dismutase (SOD) and Catalase (CAT) activities [29,30]. SOD activity was

determined spectrofotometrically and results were expressed as USOD per mg of protein. One unit of SOD is defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50 %. CAT activity was determined by hydrogen peroxide (H_2O_2) decomposition rate. The values were expressed as UCAT per mg of protein. Protein sulfhydryl content was quantified as non-enzymatic defense and it was determined by a reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [31]. Results were expressed as mmol DTNB per mg of protein. The protein concentration was measured by Lowry method, using bovine serum albumin as the standard [32]. All assays were performed in triplicate.

Cellular morphology analysis

Alterations on cellular morphology were analyzed in HEp-2 and MRC5 culture flasks after treatment with NPCL (3; 30; 300 and 750 µg mL^{-1}) for 24 h. The changes on symmetry along with increase or decrease on cell number, growth and cell adherence, were analyzed. The images were obtained using an inverted microscope (Optiphase - 403F, USA).

Statistical analysis

Results were expressed as the mean ± standard deviation (SD) from at least three independent experiments. The data were determined to be parametrical by using the Kolmogorov–Smirnoff test. Statistical significance was evaluated using a one-way analysis of variance (ANOVA) and Tukey's post hoc test. Relationships between the continuous variables were assessed using the Pearson's correlation coefficient. Results are deemed significant if *p*-value less than 0.05. The software SPSS 21.0 for Windows (SPSS Inc., Chicago, IL) was used for statistical analysis.

RESULTS AND DISCUSSION

NPCL characterization

The first approach of NPCL preparation involving the addition of the PE in the liquid form was unsatisfactory; there was no interaction between the liquid extract and the raw materials used in the preparation; therefore it was not possible to obtain and characterize it. More studies are needed to investigate the inability to associate the liquid extract to the system.

For the formulation using PE in the powder form, it was possible to obtain and characterize the samples. The results about main characteristics of formulations of NPCL in the presence or absence of PE are shown in the Table 1. Both evaluated formulations exhibited macroscopically homogeneous aspect. The NPCL formulation showed an opaque white color whereas the NPCL *plus* PE had a slightly brownish appearance (very characteristic of the presence of proanthocyanidins on *A. angustifolia* extract).

Table 1. Physico-chemical parameters evaluated on **N**PCL formulations

Sample	pH value	Particle size (nm)	PDI	Zeta potential (mV)
NPCL	6.04 ± 0.20	189.6 ± 1.0	0.12 ± 0.01	-10.6 ± 1.0
NPCL *plus* PE	5.94 ± 0.29	196.3 ± 1.0	0.19 ± 0.02	-9.6 ± 0.5

NPCL (nanospheres of polycaprolactone); PE (polyphenols-rich extract); PDI (polydispersity index).

The pH found was around 6.0 for both formulations, and it was stable for a period of 30 days with the samples stored under refrigeration (4 °C). According the dynamic light scattering technique, the average particle size for NPCL spheres was around 190 nm, and for NPCL *plus* PE was almost 200 nm. In addition, it was found low PDI values (up to 0.19), which is within the expected parameters for the system. As previously shown, the PDI values of 0.1 to 1 are considered suitable from the standpoint of uniformity in particle distribution [33]. Regarding zeta potential, we found values ranged from -9.6 to -10.6 mV in NPCL *plus* PE and NPCL *per se*, respectively. Zeta potential represents the electrokinetic potential of nanoparticle surface and is a very important marker of interaction between nanosystems and biological membranes [34]. The negative values of ZP obtained in our study are in agreement with previous studies, which also found a negative superficial charge in nanospheres using PCL [35,36] and in nanoparticle containing PEG [1].

For NPCL *plus* PE samples, we also evaluated the total levels of catechin, major compound of the extract (Figure 1), on whole solution. Moreover, in order to assess the association rate (incorporation of PE to the polymer phase) the levels of catechin were also quantified on spheres. The results revealed the total catechin levels found in the whole solution were 0.42 mg mL^{-1}, being greater than 80% of the total content added.

The analysis of the association rate showed only 0.08 mg mL^{-1} of catechin on spheres, indicating an association efficiency of 19.05 %. Previous studies have found different profiles on entrapment of bioactive compounds using plant aqueous extracts in nanosystems [37]. A study using extract from *Phoenix dactylifera* reached an encapsulation efficiency of 70–78% in nanocapsules [38]. The anthocyanin-rich extract of *Hibiscus sabdariffa* was associated to liposomes reaching an association efficiency around 70% [39]. On the other hand, the association efficiency for extract from Green tea (*Camellia sinensis*) in PCL nanoparticles was around 30% [40]. Some factors may influence the association rate, including the nature of the polymer and the physico-chemical characteristics of the drug, and one of the most relevant is the difference of polarity between the drug and the polymer [41]. Alonso et al. (1991) improved the association efficiency in 55% by changing the molar ratio of drug and surfactant to increase the intrinsic drug solubility in nanoparticles [41]. In our work, we have a similar profile, which the PE is polar and the carrier is hydrophobic. Therefore, our data show us the employed polymerization conditions must be changed in order to reach high levels of incorporation using PCL spheres as a vehicle for a polyphenols-rich extract from *A. angustifolia*.

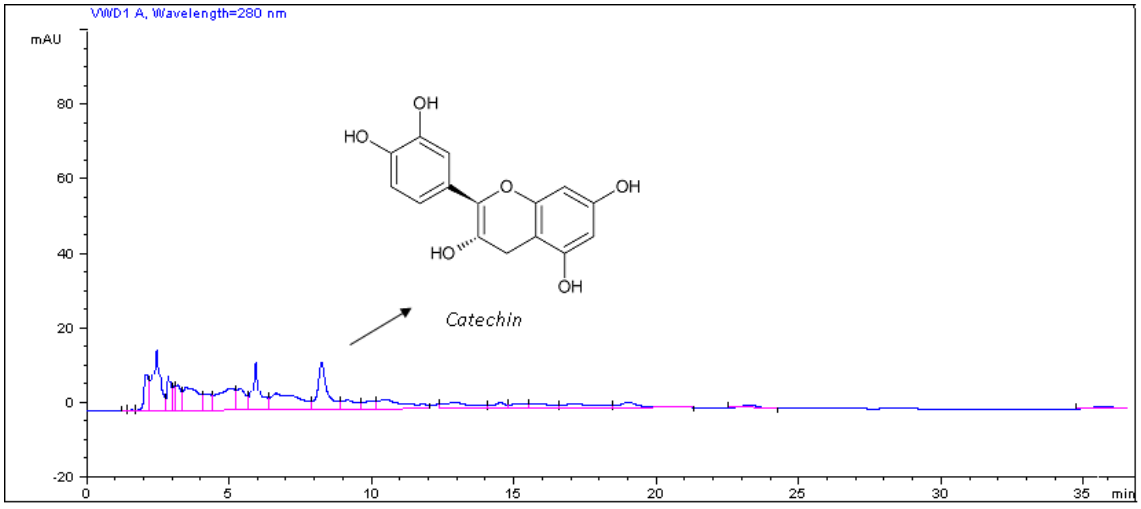


Figure 1. *Chromatograms (HPLC) for tannins at 280 nm showing the presence of catechin in polyphenols-rich extract from A. angustifolia.*

Taking into account the low association rate we have obtained on NPCL *plus* PE formulation, we decided to continue the biological experiments using only NPCL samples, since it is necessary to know the possible toxicity of these systems *per se*. By SEM analysis, the formulation exhibited structures with spherical appearance, size heterogeneity and empty inside, seemingly (Figure 2 A-B), being considered adequate for

in vitro experimentation. According to Gaumet et al. [33], SEM is an appropriated technique to detect particle size from 50 nm to 100 µm. Regarding size, studies with PCL nanoparticles already showed that different diameters, such as 363 nm [42] and 449.6 nm [36] can be found, depending on the employed measurement technique.

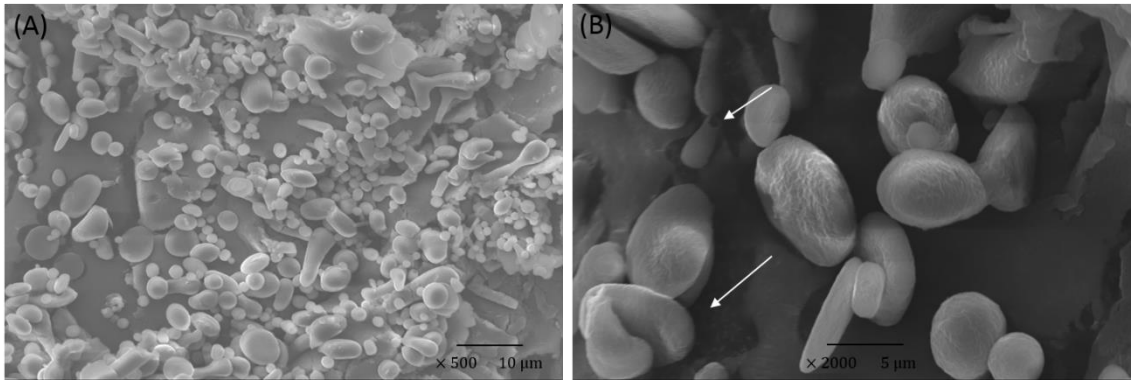


Figure 2. *Representative TEM images of PCL spheres. (A) general aspect of the particles showing size heterogeneity; (B) image showing the formation of hollow structure (arrows). Samples were prepared as described in Section 2, and then photographed.*

NPCL cytotoxicity in mammalian cells

Although polymers generally employed in the preparation of nanosystems are regarded as nontoxic [43], few studies reporting the real effects of its administration on human cells were conducted so far. For that reason, we decide to investigate the possible effects of NPCL on metabolism in tumor (HEp-2) and non-tumor (MRC5) cells, by assessing the cell viability (MTT assay). Both cell lines were chosen due their characteristics in order to explore the differential cellular response in tumor and normal cells. Previously, cells were treated with increased concentrations of NPCL formulations (0.03; 0.3; 3; 30; 300 and 750 µg mL^{-1}) for 24 h. Low doses of NPCL (0.03 and 0.3 µg mL^{-1}) did not significantly reduce the viability of both cells. On the other hand, it was observed that NPCL treatments were able to significantly decrease the cell proliferation from the concentrations of 3 and 30 µg mL^{-1} ($p<0.001$; Figure 3) for HEp-2 tumor and MRC5 non-tumor cells, respectively. The PCL toxicity was already discussed in few studies. In recent study, it was showed time-dependent PCL cytotoxicity (from 2, 4 and 6 days) in all tested doses (25, 100 and 200 µg mL^{-1}) in retinal pigment epithelium and retinal vascular endothelial cells [44]. Similar results were found in hepatocyte after an exposure to PCL at highest doses (300 and 1000 µg mL^{-1}) after 72 h of treatment [42]. In opposition, no significant reduction on hepatocyte viability was found in 24 h of exposition to the PCL

at concentrations up to 1 % (v/v) [2]. With a different level of cytotoxicity, the IC_{50} found in our study was 460 ± 25 µg mL^{-1} for HEp-2 and 573 ± 51 µg mL^{-1} for MRC5 cells being significant statistically (p=0.008; *t*-test), indicating that tumor cells are more sensitive to NPCL treatments than non-tumor cells. Indeed, tumor cells exhibit differential metabolism compared to normal cells, especially on energy generation and maintenance to cell growth and proliferation [45-50]. In order to understand the mechanisms behind the differential reduction on cell viability revealed by NPCL, the cytotoxic concentrations (3; 30; 300 and 750 µg mL^{-1}; 24 h) were chosen to verify possible redox alterations on tumor and non-tumor cells.

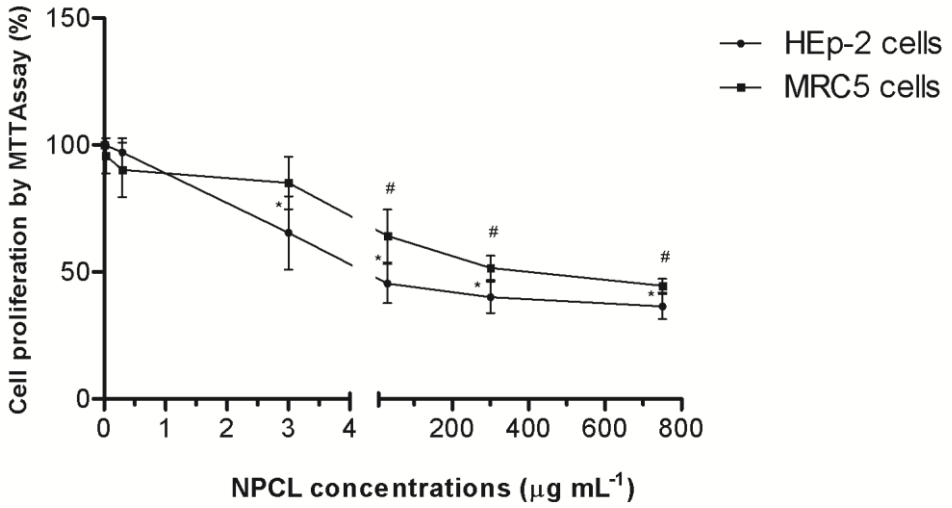


Figure 3. *Viability of tumor (HEp-2) and non-tumor (MRC5) cells treated with increasing concentrations of nanospheres of polycaprolactone (NPCL) for 24 h by MTT assay. % viability = [(number of cells at time of observation/number of control cells) × 100]. Values are expressed as mean ± standard deviation (SD). The symbol # indicates a significant difference from the non-treated cells for MRC5 and * for HEp-2. Statistical significance according to analysis of variance (one-way ANOVA) and Tukey's post-hoc test (p ≤0.001).*

Oxidative stress and cell redox status

With the aim to study the cytotoxic mechanisms of the NPCL, we decide to evaluate oxidative stress levels, once reactive oxygen species (ROS) production has been identified as one of the earliest toxicity mechanisms for polymeric nanoparticles [42]. The oxidative stress evaluation was assessed through the membrane oxidative lipid damage (TBARS assay) and oxidative damage to proteins (Carbonyl assay). The treatment using cytotoxic concentrations (3; 30; 300 and 750 µg mL^{-1}) of NPCL formulations was able to induce high levels of lipid peroxidation at doses of 300 and 750 µg mL^{-1} in HEp-2 tumor

cells when compared to control non-treated ($p<0.001$) and to MRC5 non-tumor cells ($p<0.05$; Figure 4 A).

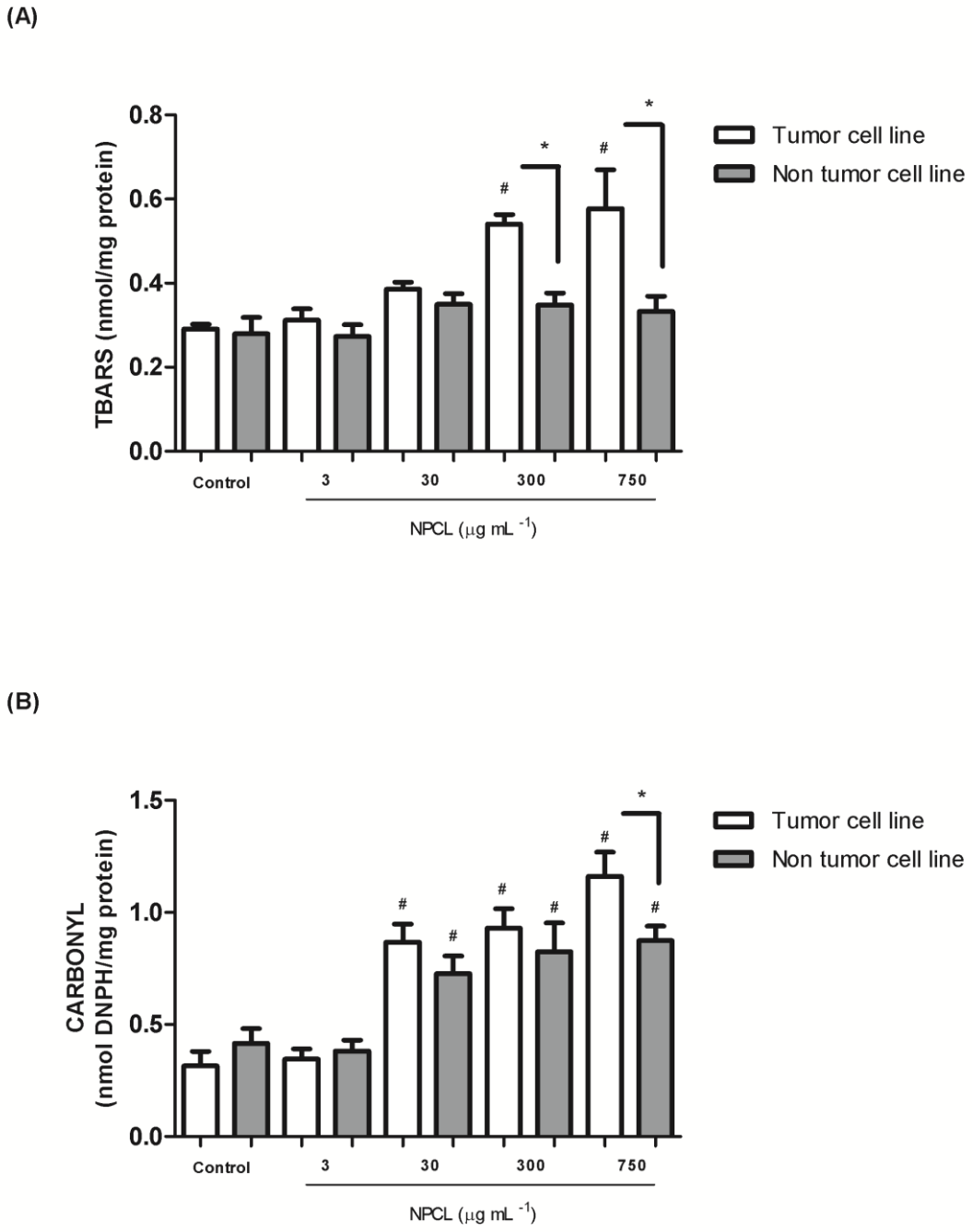
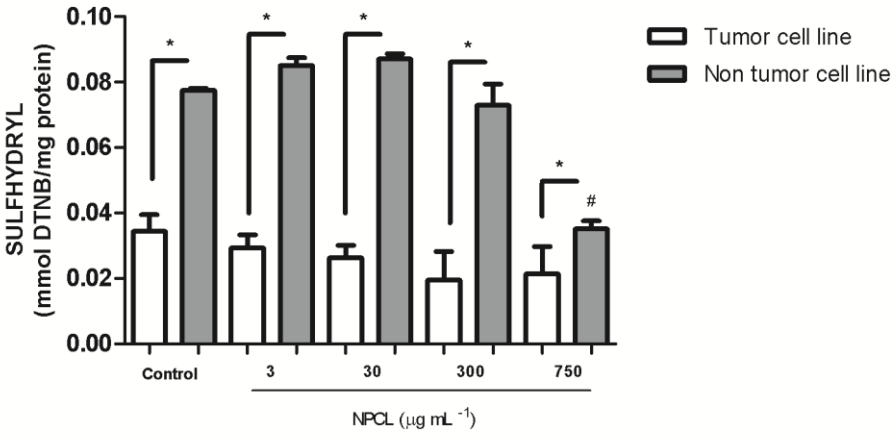


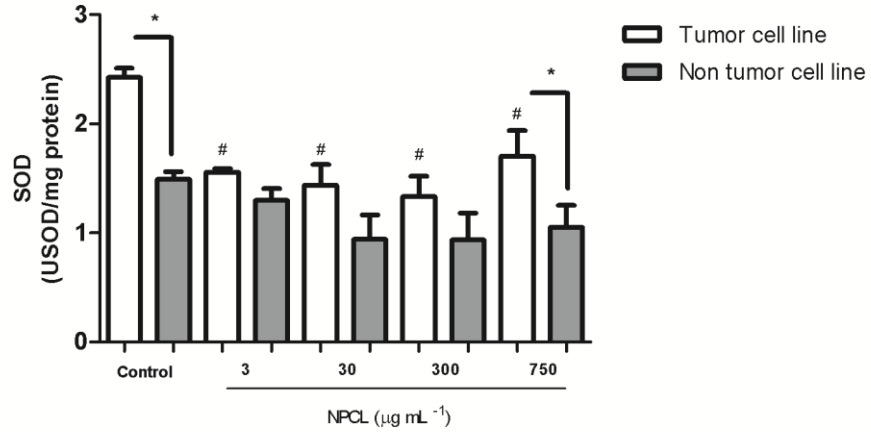
Figure 4. *Oxidative damage to lipids (A) and proteins (B) in tumor (HEp-2) and non-tumor (MRC5) cells treated with different concentrations of nanospheres of polycaprolactone (NPCL) for 24 h. Values are expressed as mean ± standard deviation (SD). The symbol # indicates a significant difference from the controls, and * indicates difference between the cell lines by analysis of variance (one-way ANOVA) and Tukey's post-hoc test.*

Additionally, the treatments were not able to induce lipid peroxidation in non-tumor cells. The doses at 30; 300 and 750 µg mL^{-1} induced a significative increase on carbonyl levels in both tumor and non-tumor cells in relation to the controls ($p<0.001$). The highest concentration (750 µg mL^{-1}) was able to significantly increase carbonyl levels in HEp-2 ($p<0.05$; Figure 4 B) in relation to MRC5 cells. Oxidative damage to lipids and proteins observed is associated with production of reactive oxygen species (ROS) along with alterations on redox defense systems. We assessed total thiol levels measured by sulfhydryl content as a non-enzymatic antioxidant marker and found this parameter significantly altered in all NPCL tested doses (3 to 750 µg mL^{-1}) in HEp-2 tumor cells ($p<0.001$; Figure 5 A) than compared to MRC5 non-tumor cells. Moreover, it was observed that HEp-2 cells present lower levels of sulfhydryl even on control non-treated. Although the higher dose has reduced sulfhydryl levels on non-tumor cells ($p<0.001$) when compared to their respective control, in general these cells exhibit increased levels of this marker, indicating normal cells present enhanced ability to neutralize ROS. Besides sulfhydryl content, we also evaluated activities of superoxide dismutase (SOD) and catalase (CAT), the first line of defense against oxidative injury. The SOD enzyme catalyzes the dismutation of radical anion superoxide ($O_2^{\cdot -}$) producing H_2O_2, which can be eliminated by the action of CAT. The results showed HEp-2 tumor cells naturally present high levels of SOD on control non-treated ($p<0.001$; Figure 5 B) than compared to MRC5 non-tumor cells. In addition, at the highest PCL dose (750 µg mL^{-1}), HEp-2 cells also exhibited increased levels of SOD in relation to MRC5 cells ($p<0.05$). We also observed that in tumor cells the treatments at all doses reduced the SOD activity when compared to their respective control ($p<0.001$). This data seems to indicate that NPCL may induce generation of $O_2^{\cdot -}$, the main ROS produced by mitochondria [51-53]. Besides SOD, we also quantified CAT activity in NPCL-treated cells, however no significant differences were found in both HEp-2 and MRC5 cells (Figure 5 C).

(A)


SULFHYDRYL
(mmol DTNB/mg protein)
0.10
0.08
0.06
0.04
0.02
0.00
Control
3
30
300
750
NPCL (μg mL -1)
Tumor cell line
Non tumor cell line

(B)


SOD
(USOD/mg protein)
3
2
1
0
Control
3
30
300
750
NPCL (μg mL -1)
Tumor cell line
Non tumor cell line

(C)

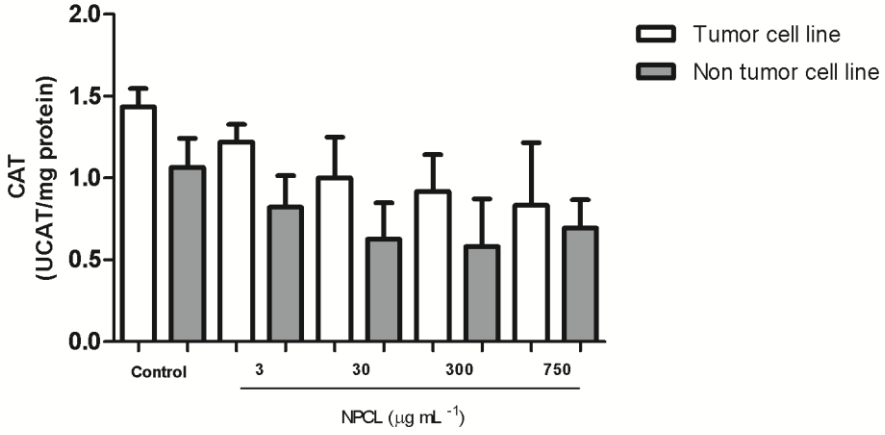

CAT
(UCAT/mg protein)
2.0
1.5
1.0
0.5
0.0
Control
3
30
300
750
NPCL (μg mL -1)
Tumor cell line
Non tumor cell line

Figure 5. *Evaluation of cellular redox metabolism in tumor (HEp-2) and non-tumor (MRC5) cells treated with different concentrations of nanospheres of polycaprolactone (NPCL) for 24 h. Total thiol content (A); superoxide dismutase (B) and catalase (C) activities. Values are expressed as mean ± standard deviation (SD). The symbol # indicates a significant difference from the controls, and * indicates difference between the cell lines by analysis of variance (one-way ANOVA) and Tukey's post-hoc test.*

Pearson's correlation analysis between cell viability and redox status markers was performed and is shown in Table 2. In HEp-2 cells, viability was positively correlated with catalase activity ($r = 0.646$; $p = 0.009$); however, it was negatively correlated with oxidative damage to lipids ($r = -0.894$; $p = 0.001$) and proteins ($r = -0.768$; $p = 0.001$). On the other hand, in MRC5 cells, the viability was positively correlated with catalase activity ($r = 0.545$; $p= 0.036$) and with sulfhydryl content ($r = 0.868$; $p=0.001$). These data indicates that, at least in tumor cells, the reduction on viability induced by NPCL treatments is linked to oxidative damage. In addition, in normal cells, the maintenance of cell viability is associated with antioxidant defenses systems, which suggest a more efficient cellular defense mechanism by non-tumor cells. In the present study, the alterations found in the MTT assay, which measures mitochondrial dehydrogenases activities, along with SOD activity levels seems to suggest NPCL may alter mitochondrial dynamic and function, which, depending on cell type, can modify the cell energetic balance.

Cellular morphology

Microscopic analysis of MRC5 non-tumor and HEp-2 tumor cells treated with different concentrations of NPCL (3; 30; 300 and 750 μg mL^{-1}) is presented in the Figure 6. The images reveals NPCL treatments caused alterations on symmetry and cell adherence at highest doses in normal cells (Figure 6 G and I). In HEp-2 tumor cells it was also observed for the same NPCL doses (Figure 6 H and J).

Table 2. Pearson correlations between cell viability, lipid and protein oxidative damage, sulfhydryl content, and enzymatic antioxidant defenses in HEp-2 tumor (■) and MRC5 normal (□) cells treated with different concentrations of nanospheres of polycaprolactone (NPCL) for 24 h

	Viability	**Lipid damage**	**Protein damage**	**Sulfhydryl**	**Sod activity**	**Cat activity**
Viability	-	-0.894**	-0.768**	0.603	0.260	0.646**
	-	-0.440	-0.549	0.868**	0.482	0.545*
Lipid damage	-0.894**	-	0.811**	-0.772**	-0.537	-0.665**
	-0.440	-	0.757*	-0.370	-0.625	-0.386
Protein damage	-0.768**	0.811**	-	-0.661*	-0.461	-0.747**
	-0.549	0.757*	-	-0.454	-0.759**	-0.492
Sulfhydryl	0.603	-0.772**	-0.661*	-	-0.701*	0.370
	0.868**	-0.370	-0.454	-	0.139	-0.250
Sod activity	0.260	-0.537	-0.461	0.701*	-	0.471
	0.482	-0.625	-0.759**	0.139	-	0.682*
Cat activity	0.646**	-0.665**	-0.747**	0.370	0.471	-
	0.545*	-0.386	-0.492	-0.250	0.682*	-

Statistically significant * for $p \leq 0.05$ and ** for $p \leq 0.01$. Sod: superoxide dismutase; Cat: catalase.

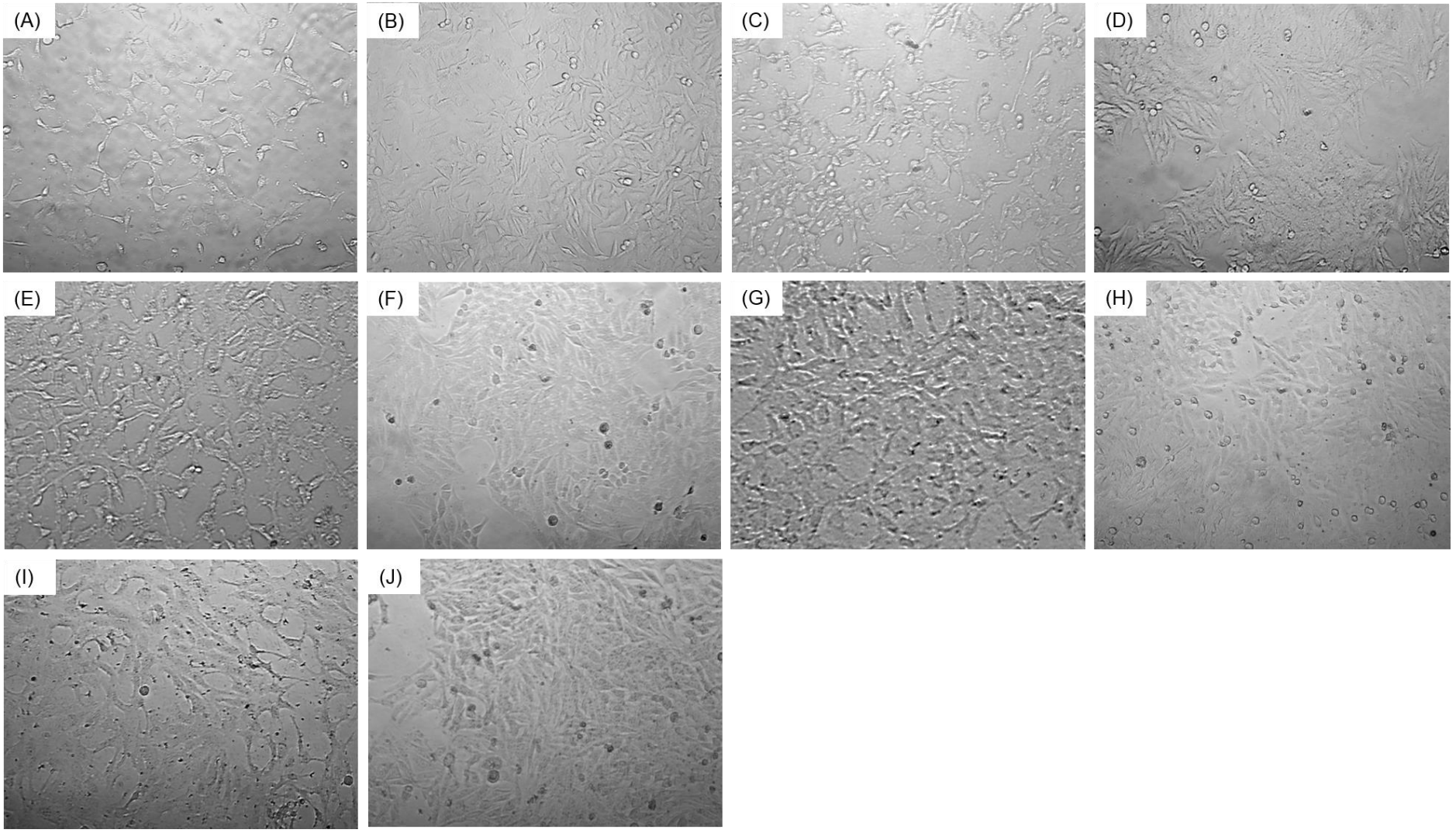

(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)

Figure 6. *Morphological alterations in MRC5 non-tumor and HEp-2 tumor cells treated with different concentrations of nanospheres of polycaprolactone (NPCL) for 24 h. Legend: (A) MRC5 control non-treated cells; (B) HEp-2 control non-treated cells; (C) MRC5 cells treated with NPCL 3 μg mL^{-1}; (D) HEp-2 cells treated with NPCL 3 μg mL^{-1}; (E) MRC5 cells treated with NPCL 30 μg mL^{-1}; (F) HEp-2 cells treated with NPCL 30 μg mL^{-1}; (G) MRC5 cells treated with NPCL 300 μg mL^{-1}; (H) HEp-2 cells treated with NPCL 300 μg mL^{-1}; (I) MRC5 cells treated with NPCL 750 μg mL^{-1} and (J) HEp-2 cells treated with NPCL 750 μg mL^{-1}. The photomicrographs show a more representative image for each treatment. Cells were grown in culture flasks as described in Section 2, and then photographed (magnification 20 ×).*

These data indicate that, at least on cellular morphology, both tumor and normal cells treated with NPCL show the similar response. Taking into account the interaction between nanoparticle surface and cellular membranes, more studies are necessary to better understand this data.

Anyway, taken together, our results show a possible link between NPCL toxicity and alterations on cellular redox metabolism of tumor and normal cells. Considering the few studies in scientific literature about the toxicity of polymeric systems, these data can contribute to understanding the effects of PCL nanospheres in mammalian cells.

CONCLUSIONS

The findings of our study demonstrated that the PCL spheres synthesized in the presence or absence of polyphenols-rich extract (PE) presented suitable physico-chemical parameters, including size, distribution and zeta potential. Nevertheless, a low association rate to the PE was found. The biological assays showed that the PCL spheres *per se* can induce cytotoxicity in concentrations higher than 3 and 30 μg mL^{-1} in tumor and normal cells, respectively. This effect was associated with oxidative damage to lipids and proteins along with alterations on antioxidant defense systems, mainly on sulfhydryl levels. Moreover, our findings showed that in contrast to non-tumor cells, tumor cells are more sensitive to the NPCL treatments. Although more studies are needed, these results suggest that nanoparticles made by using PCL could be a candidate for antitumor drug delivery. Considering the antiproliferative potential of PE from *Araucaria angustifolia* in cancer cells, retries encapsulation of this extract will be carried out.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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4 DISCUSSÃO GERAL

O câncer é, atualmente, a segunda causa de morte na população mundial, e sua incidência continua aumentando, especialmente nos países em desenvolvimento (GLOBOCAN 2012; WHO 2016). Trata-se de um grupo de doenças caracterizadas por modificações genéticas e epigenéticas que levam ao desequilíbrio entre sobrevivência e morte celular (Feinberg & Tycko 2004; Feinberg et al. 2006; Khan et al. 2007; Gerhäuser 2012; Fulda 2014). Dos mais de 100 tipos de carcinomas existentes (WHO 2016) destaca-se o tumor de laringe, o qual representa 25% dos tumores malignos que acometem a área de cabeça e pescoço e 2% de todas as doenças malignas (INCA 2016).

O microambiente e o metabolismo energético das células tumorais diferem significativamente das células normais, fornecendo uma série de vantagens às primeiras. Com respeito ao microambiente, células cancerosas são capazes de modificar os tecidos e estroma adjacentes a fim de construir um ambiente de apoio à progressão tumoral e metástase (Kalluri & Zeisberg 2006; Whipple 2015). Em relação à proliferação celular, estas adquirem a capacidade de modificar os sinais de controle e checagem do ciclo de divisão celular (Cairns et al. 2011), silenciar a expressão de genes supressores tumorais e ativar proto-oncogenes (Jones & Thompson 2009; Cairns et al. 2011; Chen et al. 2014), além de não responderem à ativação de apoptose, um processo crítico de controle para indução de morte celular programada (Cohen 1997; Susin et al. 1999; Spurgers et al. 2006; Fulda 2014). Além de alterar o microambiente, favorecendo o crescimento tumoral, as células de câncer possuem a capacidade de modificar o seu metabolismo, aumentando a efetividade de captação de glicose, glutamina e ácidos graxos para aumento de biomassa e geração de energia (Gatenby & Gillies 2004; Heiden et al. 2009; Heiden et al. 2012; Zhao et al. 2013). Como descrito por Otto Warburg (Warburg 1956), as células tumorais subutilizam a respiração mitocondrial e utilizam basicamente a via glicolítica para

formação de lactato, desviando a produção de piruvato, necessário para a ativação da fosforilação oxidativa mitocondrial. Este fenômeno é conhecido como "efeito Warburg" (Garcia-heredia & Carnero 2015; Liberti & Locasale 2015). Esta troca metabólica representa uma peculiar adaptação destas células para as condições de anaerobiose (Gatenby & Gillies 2004; Kim et al. 2006; Jones & Thompson 2009; Heiden et al. 2009), mesmo na presença de oxigênio. Tais características tornam o tratamento das neoplasias ainda mais desafiador, e indicam a necessidade do desenvolvimento de novos fármacos antitumorais com alvos moleculares específicos.

Considerando o papel da mitocôndria no metabolismo energético celular, estas organelas tornam-se alvos importantes para o estudo e desenvolvimento de novas terapias antineoplásicas (Bhat et al. 2015). Evidências demonstram que os polifenóis são compostos capazes de modular a função e dinâmica mitocondrial (Sandoval-Acuña et al. 2014; Gorlach et al. 2015) e estes são encontrados em grandes quantidades no extrato obtido a partir de brácteas, resíduos naturais da pinha de *Araucaria angustifolia*, tendo seus efeitos biológicos já estudados em leveduras (Michelon et al. 2012), linhagem de fibroblastos de pulmão humano (Souza et al. 2014) e em células eucarióticas de *Anticarsia gemmatalis* (Anexos I e II).

Tendo como finalidade identificar o possível efeito antiproliferativo do extrato de *A. angustifolia* (EAA) frente às células tumorais de laringe, este trabalho avaliou os efeitos de sua administração em células da linhagem HEp-2 por meio de análises moleculares da função e dinâmica mitocondrial, geração de EROs, danos ao DNA e alterações epigenéticas. Ainda, a possível incorporação do EAA ao sistema nanovetorizado baseado em nanoesferas (NE) também foi investigado.

Como se pode observar nas Figuras 1 e 2 e na Tabela 1 (Capítulo II), a análise química do EAA por meio de espectroscopia de alta resolução nos modos positivo (ESI-

MS (+)) e negativo (ESI-MS (-)) evidenciou a presença majoritária de compostos fenólicos, tais como isoflavonas e biflavonoides, alguns dos quais já tiveram seu efeito antitumoral e apoptótico estudado em cultura de células e em modelos animais (Hadi et al. 2007; Khan et al. 2012). Além disso, dois importantes ácidos graxos foram também identificados, o ácido dodecanóico (láurico) e o ácido hexadecanóico (palmítico), ambos apresentaram atividade antiproliferativa e apoptótica e capacidade de regulação do ciclo celular em células de câncer de cólon (Fauser et al. 2011; Matthews et al. 2012; Fauser et al. 2013). Em relação à atividade antiproliferativa do extrato, observou-se que o tratamento, com doses a partir de 100 μg/mL, inibe de maneira tempo-dependente o crescimento de células tumorais HEp-2 e não causam citotoxicidade significativa em células HEK-293 utilizadas como controle (Figuras 3 e 4; Capítulo II). Este efeito foi acompanhado por alterações morfológicas, indução de estresse oxidativo, danos ao DNA, aumento da expressão de proteínas apoptóticas, inibição do complexo I mitocondrial e depleção dos níveis de ATP. Todos estes fenômenos são mostrados no Esquema 1 do capítulo II. Neste capítulo fica evidenciado o interessante efeito seletivo do EAA, indicando que os compostos nele presentes possuem potencial para serem utilizados no desenvolvimento de fármacos antitumorais. Como mostrado no Capítulo III (Figura 1), a principal hipótese que foi explorada para tentar explicar o efeito diferencial do EAA em células tumorais e não tumorais seria a possível inibição da isoenzima chamada piruvato kinase M2 (PKM2), a qual regula o metabolismo da glicose e a consequente produção de piruvato, agindo como um fator transcricional do fator indutor de hipóxia (HIF-1α), o qual aumenta a atividade da enzima piruvato desidrogenase kinase 1 (PDK1) levando à inibição da piruvato desidrogenase (PDH) e consequentemente da formação de acetil Co-A, a qual é requerida para a continuação do processo oxidativo na mitocôndria (Hitosugi et al. 2010; Iqbal et al. 2014; Zhao et al. 2013; Ferriero et al. 2015). Nos capítulos II e III

foi mostrado que o EAA possui dois mecanismos principais de ação, primeiro revertendo o efeito Warburg das células tumorais, ativando a mitocôndria destas e então inibindo o complexo I, causando a morte da célula. Por outro lado, em células normais o extrato poderia estar atuando sobre uma classe de proteínas conhecidas como sirtuinas, especialmente a sirtuína mitocondrial (SIR 3) a qual é responsável por manter a atividade dos complexos, preservando a funcionalidade da mitocôndria e assim mantendo a viabilidade celular (Ahn et al. 2008; Chen et al. 2011; Li et al. 2013; Jeong et al. 2014).

A enzima PDH é frequentemente suprimida em vários tipos de câncer, impulsionando a glicólise (Sutendra & Michelakis 2013). Em pacientes com câncer de cabeça e pescoço foi demonstrado aumento da concentração de lactato, produto final resultante da glicólise, o qual foi clinicamente associado com progressão tumoral e metástase (Brizel et al. 2001). O aumento dos níveis de lactato em células tumorais sugere que o metabolismo oxidativo destas células é alterado em favorecimento da via de oxidação de glicose citosólica. Os resultados obtidos no Capítulo IV demonstram que as concentrações de 50 e 75 μg/mL de EAA aumentaram em 33 e 25%, respectivamente, os níveis de PDH nas células tumorais HEp-2 (Figura 2; Capítulo IV). Embora até o momento não existam estudos específicos avaliando o papel dos polifenóis sobre os níveis desta enzima em células de câncer, já foi previamente reportado que moduladores positivos de PDH apresentam efeitos antitumorais (Sutendra & Michelakis 2013). Um exemplo bem conhecido é o dicloroacetato, o qual tem sido apontado como um ativador de PDH, aumentando o influxo de piruvato na mitocôndria, ativando apoptose e subsequentemente suprimindo a proliferação tumoral (Michelakis et al. 2008).

A ativação mitocondrial é um alvo importante na terapia antineoplásica (Bhat et al. 2015), uma vez que a mitocôndria é responsável por importantes processos metabólicos, que vão além da geração de ATP, tais como controle da homeostasia redox,

influxo de cálcio e ativação de apoptose de via intrínseca (Wallace 2012; Smith et al. 2012; Zhao et al. 2013). A Figura 3 do Capítulo IV apresenta os resultados das modificações específicas nos níveis de cada complexo da CTE, e observou-se que o EAA foi capaz de reduzir os níveis de expressão dos complexos I e III. Este achado corrobora os dados apresentados no capítulo II, demonstrando a inibição da atividade do complexo I pelo extrato (Figura 8; Capítulo II). Como consequência desta inibição, ocorre acumulação de EROs na mitocôndria ocasionando perdas no potencial de membrana mitocondrial ($\Delta\Psi m$) e na manutenção do funcionamento da CTE, sinalizando para ativação de apoptose (Raam et al. 2008; Kroemer et al. 2007). De fato em nosso estudo verificamos alterações no $\Delta\Psi m$ (Figura 7; Capítulo IV) em células HEp-2 tratadas com o EAA, efeito que foi associado com a expressão de proteínas pró apoptóticas, como AIF, caspase-3 e Bax (Figura 7; Capítulo II). Nossos achados estão de acordo com dados prévios da literatura mostrando que polifenóis, tais como a curcumina, podem afetar o $\Delta\Psi m$, mediando a geração de EROs ativando apoptose em modelos de câncer (Teiten et al. 2010; Yang et al. 2012). Tomados em conjunto, os dados apresentados nos capítulos II e IV destacam a participação do estresse oxidativo como mediador dos eventos que causaram a morte das células HEp-2 após exposição ao EAA.

A epigenética está intimamente relacionada ao estresse oxidativo (Weitzman et al. 1994; Hitchler & Domann 2013). Os processos epigenéticos envolvem modificações em nível de DNA capazes de regular a expressão gênica (Jaenisch & Bird 2003). A metilação do DNA, um dos marcadores epigenéticos mais comuns, é caracterizada pela adição de um grupo metila (CH3) a uma citosina ou a uma adenina, levando ao silenciamento da expressão gênica (Smith & Meissner 2013). Evidências têm mostrado que um grande número de malignidades estão associados a altos níveis de 5-metilcitosina (5mC) através do silenciamento transcricional de genes supressores tumorais (Rhee et al. 2002; Ehrlich

2002). No capítulo IV (Figura 9) foi demonstrado uma redução nos níveis de 5mC em células HEp-2 tratadas com EAA, de maneira dose-dependente. Além disso foi encontrado redução da atividade de DNMT1 (Figura 11; capítulo IV), enzima responsável por manter os níveis de metilação, indicando que o EAA possa estar promovendo demetilação do DNA em células tumorais.

O processo de demetilação está associado com a conversão de 5mC para 5-hidroximetilcitosina (5hmC) (Hill et al. 2014). Tem sido previamente demonstrado que níveis baixos de 5hmC estão associados com malignidades mielóides (Ko et al. 2011). Os dados apresentados na Figura 10; capítulo IV demonstram aumento dos níveis de 5hmC nas células HEp-2 tratadas com o EAA. Diversos estudos têm mostrado que a geração de EROs pode diretamente oxidar o grupamento metila na 5mC levando à demetilação em modelos animais (Lim et al. 2008; Gong & Zhu 2011; Madugundu et al. 2014). Corroborando com nossos achados, um estudo mostrou inibição da atividade de DNMT1 e diminuição da metilação em genes específicos de regulação do ciclo celular, incluindo p16, RARb e MLH após tratamento de células tumorais com epigalocatequina-3-galato (Fang et al. 2003).

A Figura 5 sintetiza os principais efeitos do EAA em células tumorais de laringe HEp-2 abordados nos capítulos II-IV. O EAA altera a sinalização molecular destas células via modulação em nível mitocondrial (especialmente no CI, alterando a expressão gênica e protéica de duas importantes subunidades deste complexo) e epigenético (induzindo a redução nos níveis globais de metilação) causando uma série de eventos que levam à morte por apoptose.

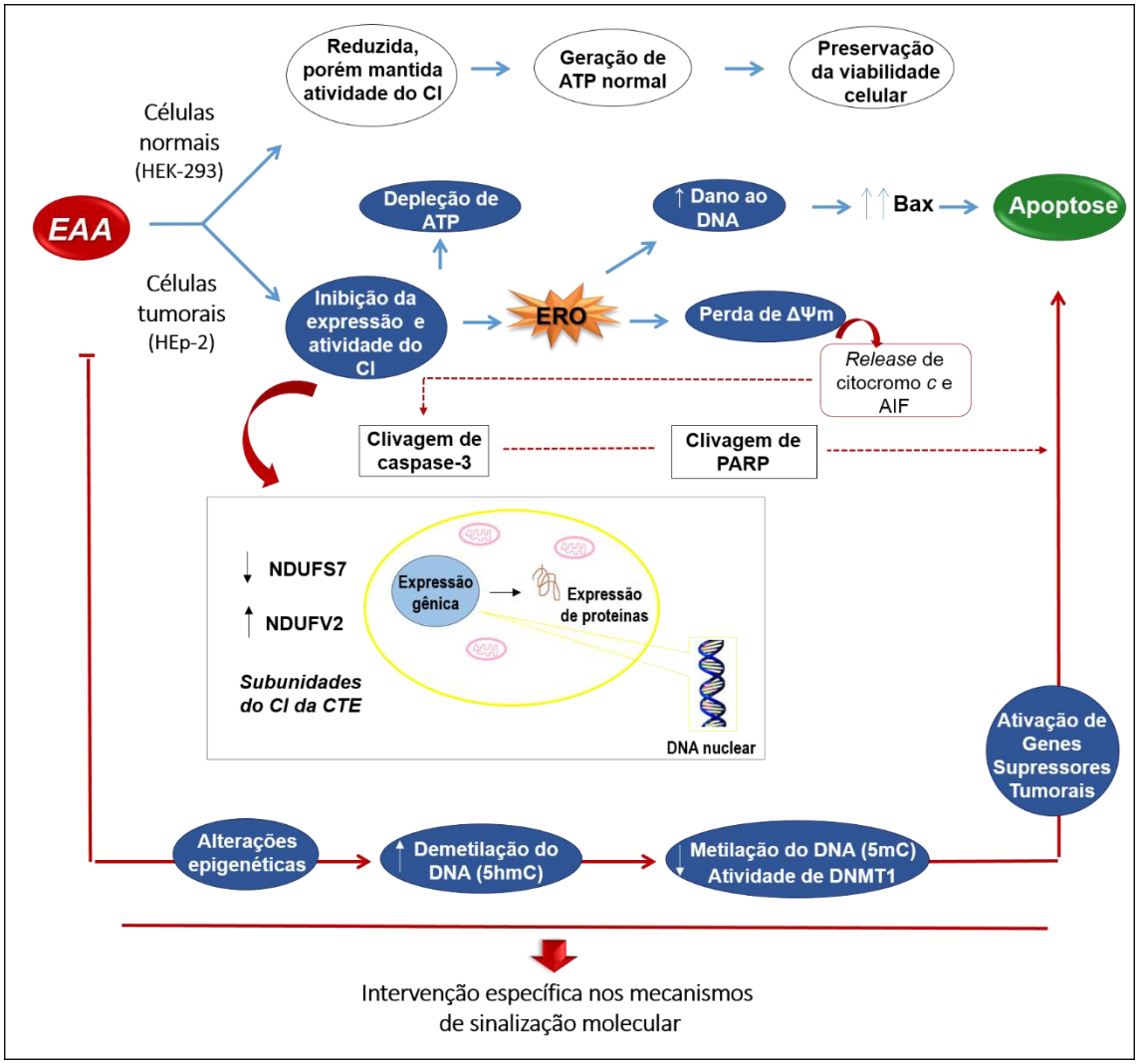

Figura 5. Resumo dos principais mecanismos de ação do extrato de *Araucaria angustifolia* (EAA) em células tumorais de laringe HEp-2. Legenda: AIF (fator indutor de apoptose); CI (complexo I mitocondrial); CTE (cadeia de transporte de elétrons); DNMT1 (DNA metil transferase 1); ERO (espécies reativas de oxigênio); PARP (poli ADP-ribose polimerase); ΔΨm (potencial de membrana mitocondrial); 5mC (5 metilcitosina); 5hmC (5 hidroximetilcitosina).

Devido ao número crescente de casos de câncer em todo o mundo e os altos índices de inefetividade dos tratamentos, os sistemas poliméricos para entrega de fármacos vem ganhando atenção. Atualmente já é possível veicular ativos utilizando sistemas poliméricos muito pequenos (50 nm) e dessa forma dirigir a substância a um alvo de ação específico, em nível subcelular. Um estudo recente demonstrou que nanopartículas de poli(ácido láctico-co-glicólico) são capazes de se acumular nos lisossomos, retículo endoplasmático e até nas mitocôndrias de células de epitélio pigmentar de retina humana (linhagem celular ARPE-19) (Lin et al. 2016), destacando a importância do emprego destes sistemas na farmacologia.

O Capítulo V teve como objetivo sintetizar formulações de nanoesferas utilizando poliepsoncaprolactona (NPCL) como polímero, associando o EAA ao sistema. Inicialmente, tentou-se adicionar o EAA na forma líquida, porém esta abordagem não foi satisfatória, uma vez que não houve interação entre o extrato líquido e as matérias-primas utilizadas na preparação, por meio da formação de grumos e precipitados. Dessa forma, não foi possível a obtenção desta amostra, bem como sua caracterização. Quanto à formulação utilizando o EAA na forma de pó liofilizado, esta foi obtida com êxito.

Os resultados sobre as principais características das formulações de NPCL na presença e ausência do EAA são apresentadas na Tabela 1 e Figuras 1-2 deste capítulo. Ambas as amostras avaliadas exibiram aspecto macroscopicamente homogêneo. A formulação de NPCL sem o extrato apresentou uma coloração branca opaca, enquanto que as NPCL contendo o EAA (NPCL + AAE) exibiram um aspecto ligeiramente acastanhado (característico da presença das proantocianidinas). O pH encontrado foi de cerca de 6,0 para ambas as formulações, e mostrou-se estável durante um período de 30 dias. De acordo com a técnica de dispersão dinâmica de luz, o diâmetro médio das partículas para NPCL foi de cerca de 190 nm, e para as NPCL + EAA foi em torno de

200 nm. Além disso, verificou-se baixos valores de índice de polidispersão (PDI) (máximo 0.19), o que está dentro dos parâmetros esperados para o sistema. Como previamente mostrado, valores de PDI de 0.1 à 1 são considerados adequados do ponto de vista da homogeneidade na distribuição de partículas (Gaumet et al. 2008). Em relação ao potencial zeta (PZ), foi encontrado valores de -9,6 a -10,6 mV em NPCL + EAA e NPCL, respectivamente. O PZ representa a potencialidade eletroquímica da superfície das nanopartículas e é um marcador muito importante de interação entre nanossistemas e membranas biológicas (Honary & Zahir 2013). Os valores negativos de PZ obtidos em nosso estudo estão de acordo com estudos anteriores, que também encontraram uma carga superficial negativa em nanoesferas usando PCL (Pilar et al. 1996; Campos et al. 2013) e em nanopartículas contendo polietilenoglicol (Wang et al. 2015).

Embora tenhamos encontrado características físico-químicas adequadas na formulação de NPCL + EAA, não houve uma associação significativa dos compostos ao sistema, tendo sido observado apenas 19% de taxa de incorporação. Estudos prévios têm relatado diferentes perfis de incorporação de compostos presentes em extratos aquosos de plantas nos nanosistemas (Zorzi et al. 2015). Um estudo utilizando extrato de *Phoenix dactylifera* alcançou uma eficiência de encapsulação entre 70 e 78% em nanocápsulas (Bagheri et al. 2013). Em trabalho recente, o extrato rico em antocianinas de *Hibiscus sabdariffa* incorporado à lipossomas apresentou uma eficiência em torno de 70% (Gibis et al. 2014). Por outro lado, a eficiência de encapsulação do extrato de chá verde (*Camellia sinensis*) encapsulado à nanopartículas foi de cerca de 30%, apenas (Sanna et al. 2015). Diversos fatores podem influenciar a eficiência de encapsulação, incluindo a natureza do polímero e as características químicas da substância ativa, e um dos fatores que se destaca nesse sentido é a diferença de polaridade entre o ativo e o polímero (Alonso et al. 1991; Rao & Geckeler 2011). Em estudo de Alonso et al. (1991), os autores relatam

o aumento da eficiência de associação de 4.76 para 60.47% por meio de modificações da massa do ativo associado e do tensioativo utilizado, aumentando, por conseguinte, a interação entre os constituintes da nanopartícula. Em nosso estudo, nós temos uma condição similar ao trabalho de Alonso e colaboradores, aonde o EAA é hidrofílico e o carreador (PCL) é dotado de natureza lipofílica. Sendo assim, os nossos dados indicam a necessidade de mais estudos empregando diferentes condições de polimerização a fim de melhorar a eficiência de associação do extrato ao sistema.

Em função da baixa taxa de associação obtida e considerando a lacuna de conhecimento existente sobre o potencial tóxico dos sistemas nanoparticulados *per se*, a formulação de NPCL sem o EAA foi testada em relação à sua possível toxicidade em cultura de células utilizando uma linhagem tumoral (HEp-2) e não tumoral (MRC5) humanas.

Na Figura 3; capítulo V é possível observar que baixas doses de NPCL (0,03 e 0,3 μg/mL) não causaram redução da viabilidade nas duas linhagens avaliadas. Por outro lado, observou-se que os tratamentos foram capazes de diminuir significativamente a proliferação a partir das concentrações de 3 e 30 μg/mL ($p<0,001$) nas células HEp-2 e MRC5, respectivamente. A toxicidade de PCL já foi discutida em poucos estudos. Em estudo recente, demonstrou citotoxicidade tempo-dependente (a partir de 2, 4 e 6 dias) em todas as doses testadas (25, 100 e 200 μg/mL) em células pigmentares e endoteliais vasculares da retina (Lin et al. 2016). Resultados semelhantes foram encontrados em hepatócitos após exposição à doses elevadas de PCL (300 e 1000 μg/mL) depois de 72 h de tratamento (Singh & Ramarao 2013). A dose capaz de inibir a taxa de proliferação celular em 50% (IC_{50}) encontrada em nosso estudo foi de 460±25 μg/mL para HEp-2 e 573±51 μg/mL para MRC5 ($p = 0,008$; *t*-teste), indicando que as células tumorais são mais sensíveis aos tratamentos do que as células não tumorais. A redução da viabilidade

celular foi associada com danos oxidativos e alterações redox nos sistemas de defesa antioxidante das células HEp-2 (Figuras 3-5 e Tabela 2; capítulo V). Ainda, observou-se que as doses mais elevadas de PCL são capazes de induzir alterações morfológicas nas células HEp-2 e MRC5 expostas (Figuras 6 e 7; capítulo V). O resultados deste capítulo demonstram uma possível ligação entre a toxicidade das NPCL e alterações no metabolismo redox das células tumorais, aspectos que devem ser levados em conta na escolha do melhor sistema polimérico. A partir dos resultados obtidos neste capítulo é possível considerar a PCL como um possível carreador para a vetorização de ativos antitumorais. Considerando o interessante potencial antitumoral do EAA via modulação da função mitocondrial e alterações epigenéticas, novas tentativas de encapsulação do extrato devem ser investigadas.

Por fim, os resultados obtidos nesta pesquisa mostram a potencialidade dos compostos químicos presentes no extrato de *A. angustifolia* para o desenvolvimento de novas terapias adjuvantes e/ou complementares para a terapêutica do câncer.

5 CONCLUSÕES

O conjunto de resultados desta tese permite concluir que o extrato proveniente das brácteas, resíduos naturais da pinha de *A. angustifolia*, apresenta importante e seletivo efeito antiproliferativo em células tumorais de laringe HEp-2, mediado por alterações na função e dinâmica mitocondrial, especialmente em nível de complexo I, além de alterações epigenéticas. Em conjunto, os dados aqui apresentados destacam o potencial terapêutico do EAA como antitumoral *in vitro*.

Este trabalho permitiu obter as seguintes conclusões específicas:

✓ A caracterização química do EAA identificou a presença de diferentes classes de polifenóis.

✓ O EAA foi citotóxico à linhagem tumoral HEp-2, no entanto, não foi capaz de induzir citotoxicidade significativa na linhagem não-tumoral HEK-293, nas mesmas condições testadas.

✓ Danos oxidativos à macromoléculas celulares, aumento da produção de óxido nítrico e depleção nas defesas antioxidantes enzimáticas (Sod e Cat) foram observados nas células tumorais tratadas com o EAA.

✓ O ensaio Cometa mostrou que o extrato apresenta atividade genotóxica nas células tumorais HEp-2 e a análise de microscopia revelou condensação da cromatina e fragmentação do DNA.

✓ O EAA foi capaz de inibir a atividade do complexo I da CTE de células tumorais e afetar os níveis de produção de ATP por estas células.

✓ A exposição das células tumorais ao extrato aumentou a expressão de proteínas apoptóticas de via intrínseca mitocondrial, mediada pela ativação da proteína Bax, liberação de AIF e foi independente da ativação de p53.

✓ O EAA aumentou os níveis de PDH nas células tumorais, alterando o metabolismo de obtenção de energia via glicólise pela fosforilação oxidativa mitocondrial.

✓ Falhas no potencial de membrana mitocondrial ($\Delta\Psi m$) foram observadas, juntamente com diminuição dos níveis de proteínas dos complexos I e III da CTE e produção de ERO após o tratamento das células tumorais com o EAA.

✓ Alterações nos marcadores epigenéticos foram encontradas nas células tumorais tratadas com o EAA, como hipometilação do DNA e redução da atividade de DNMT1.

✓ A taxa de associação do EAA à NE não foi satisfatória, obtendo-se apenas 19% de taxa de incorporação.

✓ As NE contendo ou não o EAA apresentaram um pH estável, tamanho de partícula adequado, índice de polidispersibilidade inferior a 1 e potencial zeta negativo.

✓ As NE *per se* demonstraram capacidade de alterar o metabolismo de células tumorais e não tumorais, reduzindo a viabilidade celular e induzindo alterações redox.

6 PERSPECTIVAS

Para uma melhor compreensão dos efeitos gerados pela exposição das células tumorais ao EAA, assim como para a efetiva encapsulação do mesmo, seria de grande relevância a avaliação dos seguintes aspectos:

- ✓ Analisar os compostos fenólicos presentes no EAA individualmente, a fim de tentar identificar qual(is) o(s) responsável(is) pelo efeito antitumoral.
- ✓ Avaliar as principais enzimas regulatórias do metabolismo de carboidratos, em especial a isoenzima piruvato kinase M2 (PKM2) e a piruvato desidrogenase kinase 1 (PDK1) nas células tumorais, visando identificar os alvos do EAA e/ou seus constituintes.
- ✓ Analisar os níveis de expressão da sirtuína 3 (SIR 3) em células tumorais e não tumorais tratadas com EAA.
- ✓ Verificar a possível modulação nos níveis de expressão da DNA metiltransferase 1 (DNMT1) a fim de correlacionar com a sua atividade.
- ✓ Testar novas alternativas de encapsulação do EAA às NE, bem como alterar os parâmetros de carga e afinidade polimérica, visando à otimização da taxa de associação.
- ✓ Avaliar o efeito de NE contendo EAA sobre a viabilidade e parâmetros de estresse oxidativo em células tumorais.

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ANEXOS

Anexo I

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Araucaria angustifolia (Bert.) O. Kuntze induces oxidative and genotoxic damage in larvae of *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae)

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Plant-derived products have been considered as an important alternative to common pesticides. *Araucaria angustifolia* extract (AAE) contains high levels of polyphenols, which are correlated with mortality in defoliating insects. Therefore, this study aimed to evaluate the effects of AAE treatments on *A. gemmatalis* larvae. Results showed that AAE was able to induce lipid, protein, and DNA damage in the larvae via oxidative stress. AAE treatment did not induce larvae mortality; however, there was an increase in the number of malformed pupae, along with a decrease in the emergence of the insects. These biological effects may be correlated with the polyphenol content of the AAE.

Keywords: *Araucaria angustifolia*; velvetbean caterpillar; oxidative damage; polyphenols

1. Introduction

The continued need for pest management in agriculture is self-evident, with pressure increasing on farmers to boost production (Birch et al. 2011; Corley and Jervis 2012). In Brazil, the velvetbean caterpillar *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Erebidae) is a major defoliating pest of soybean and one of the largest defoliating pests in the world, causing major losses to agricultural production, especially in tropical America. Moreover, soybean is one of the most cultivated grains in the world and contributes to about two-thirds of global agricultural calories (Ray et al. 2013).

The emergence of insects resistant to chemical insecticides and the growth of organic agriculture are prompting an increase in studies on entomotoxic plant-derived compounds, which are considered to be a valuable alternative to the usual synthetic pesticides widely used in agriculture. The great diversity of vegetal species in Brazilian ecosystems constitutes an important source for the discovery of new agents with potential entomotoxic activity. The conifer *Araucaria angustifolia* (Bert.) O. Kuntze (Araucariaceae) is an endemic tree of Southern Brazil, known popularly as "pinheiro-do-paraná" or "pinheiro-brasileiro". It is a dioecious species, which means it features male and female specimens that have their own distinct strobilus. The female strobilus consists of seeds (the edible part of *A. angustifolia*) and bracts, which are undeveloped seeds commonly discarded into the environment. Our group has previously studied the aqueous extract obtained from the bracts of *A. angustifolia,* showing that it is rich in polyphenols (Michelon et al. 2012; Souza et al. 2014). Phenolic compounds are produced by the plant as secondary metabolites to provide self-defense against pests with some of them being associated with pupal and/or larvae mortality in defoliating insects, including *A. gemmatalis* (Gazzoni et al. 1997; Piubelli et al. 2005; Salvador et al. 2010).

In this context, the objective of this study was to investigate oxidative and genotoxic damage induced by *A. angustifolia* extract (AAE) on the velvetbean caterpillar *A. gemmatalis*. In addition, weight gain, viability, malformation of the pupae, and emergence of the adults were evaluated.

2. Materials and methods

2.1. *Araucaria angustifolia extract (AAE)*

Female strobiluses of *A. angustifolia* were collected in Caxias do Sul, Rio Grande do Sul (29°10'05''S, 51°10'46''W), Brazil. Voucher specimens were identified by the Herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (HUCS 40710/40711). Bracts were manually separated from the pine, and the extract was obtained using 5 g of bracts in 100 mL of distilled water, under reflux (100 °C) for 15 min, filtered in Millipore equipment (pore size, 0.45 μm; SFGS 047LS, Millipore Corp.) and then lyophilized (LIOBRAS model L-101) under vacuum pressure to yield a powder. This extract had a total polyphenolic content of 1586 ± 14.53 mg gallic acid equivalents per 100 g of bracts. The main phenolic compounds of AAE (Figure 1) are catechin (140.6 ± 2.86 mg/100 g bracts), epicatechin (41.3 ± 2.73 mg/100 g bracts), quercetin (23.2 ± 0.06 mg/100 g bracts), and apigenin (0.6 ± 0.06 mg/100 g bracts), as already described by our group (Souza et al. 2014). To perform the assays, AAE was solubilized in distilled water immediately before its use.

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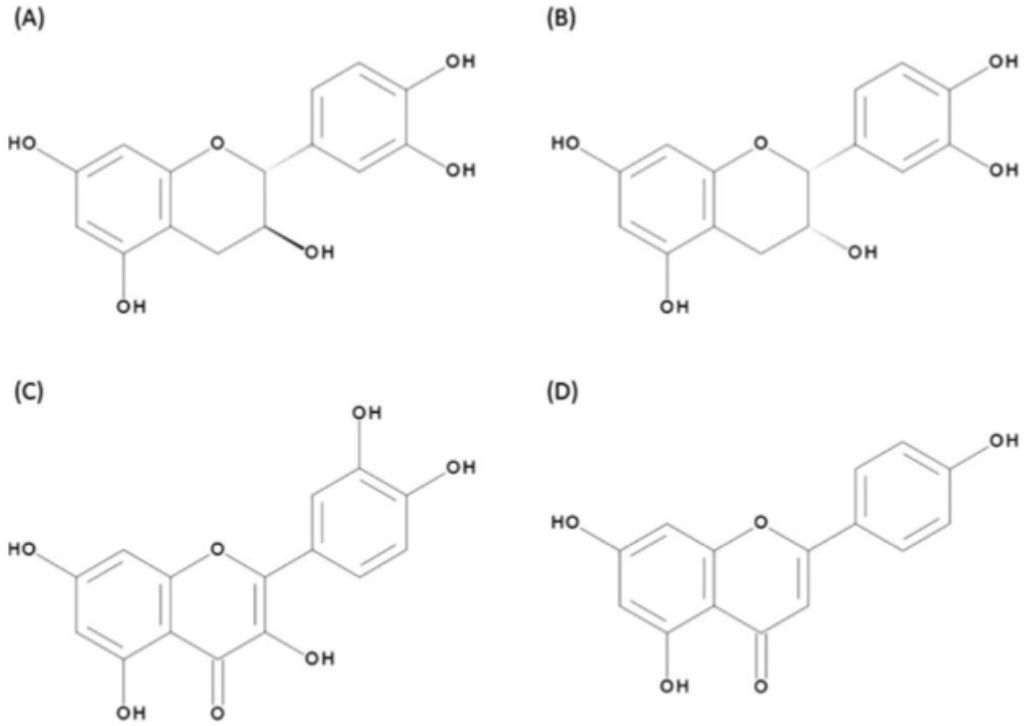

Figure 1. Phenolic compounds found in *Araucaria angustifolia* extract. Identification and quantification were performed through by HPLC analysis, using an HP 1100 system equipped with a UV/VIS detector (Santa Clara, California, USA). Compound separation was performed with a 5 μm Li-Chrospher RP18 column (250 mm × 4 mm) at a flow rate of 1 mL/min. (A) catechin, (B) epicatechin, (C) quercetin, and (D) apigenin. Images obtained using Symyx Draw 4.0.0 software (Symyx Technologies Inc., Santa Clara, California, USA).

2.2. Insects

The insects used in this study were from colonies kept at the Laboratory of Control of Pests, Institute of Biotechnology, University of Caxias do Sul, Brazil. The insects were reared on a soy-based diet, according to Greene et al. (1976). They were maintained in an acclimatized chamber at 27 ± 1 °C; 50 ± 10% relative humidity, and a 14 : 10 h light/dark photoperiod.

2.3. Treatments

Thirty larvae (third instars) of *A. gemmatalis* were used in each group, which included three AAE treatments (1, 10, and 50 mg/mL of AAE) and one control group. The larvae were transferred to individual cups, and every two days the insects were fed on *Glycine max* foliar discs (1.0 cm diameter) with an air-dried 25 μL drop of a solution containing 1, 10, and 50 mg/mL of AAE for 10 days. AAE concentrations were defined from previous tests. Control insects were fed on foliar discs containing air-dried 25-μL drops of distilled water. Leaf discs were consumed by the insects within 2 days and were replaced until the insect reach the pre-pupal stage. All the insect groups were evaluated every 2 days for weight gain. Rates of mortality, malformation of pupae (morphological alterations), and emergence of adults were also evaluated. Oxidative damage to lipids and proteins as well as the genotoxic effects were evaluated on the fourth day of the treatment, before the larvae reached the pre-pupal stage.

2.4. Oxidative damage to lipids and proteins

Larvae of each treatment were homogenized in 1 mL of ice-cold 50 mM potassium phosphate buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and centrifuged at 1500 × *g* at 4 °C for 5 min. The supernatant was used for the assays. To determine the extent of lipid damage, the assay described by Hermes-Lima and Storey (1995) was used. Briefly, aliquots (100 μL) of the supernatant were mixed with 100 μL of the color reagent (1% thiobarbituric acid [TBA], 50 mM sodium hydroxide (NaOH), 0.1 mM butylated hydroxytoluene [BHT]) and 50 μL 7% (v/v) phosphoric acid. The mixture was placed in a boiling water bath for 15 min. After cooling, 1.5 mL of *n*-buthanol was added to the mixture followed by centrifugation for 5 min at 1600 × *g*. The absorbance of supernatant was measured at 532 nm, and the results were expressed in nanomoles per milligram of protein. Oxidative damage to proteins was measured by quantifying the

carbonyl group based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al. 1990). An aliquot of 200 µL of DNPH (10 mM) or 200 µL of hydrochloric acid (HCl) (2 M) for the blank was added to 50 µL of the samples. The reaction mixture was incubated in the dark for 30 min and vortexed every 10 min. Next, 250 µL of 20% trichloroacetic acid (TCA) was added and the mixture centrifuged at 1500 × *g* for 8 min. The supernatant was discarded and the pellet was washed three times with ethanol−ethyl acetate (1 : 1) to remove free reagent. Samples were centrifuged and pellets were redissolved in 1000 µL of urea solution (8 M) at 37 °C for 15 min. Absorbance was read at 365 nm, and results were expressed as nmol DNPH / mg protein. Total protein content was assayed by the method of Bradford (1976) using the bovine serum albumin as standard.

2.5. *DNA damage*

The genotoxicity of the AAE was evaluated through the alkaline single-cell gel test (Comet assay) according to the method of Singh et al. (1988), with modifications (Bertholdo-Vargas et al. 2009). Twenty µL of the hemolymph from the larvae, collected by capillary, was mixed with 80 µL of low-melting-point agarose (0.75%). The mixture was poured onto a frosted microscope slide coated with normal-melting-point agarose (1%) and covered with a cover slip. After solidification, the cover slip was removed and the slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris [pH 10.0], 10% dimethyl sulfoxide [DMSO], and 1% Triton X-100) at 4 °C for 1 h. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA [pH 12.6]) for 20 min to allow DNA unwinding. The DNA was electrophoresed for 30 min at 25 V (0.9 V/cm) and 300 mA. After the run, the agarose-fixed cells were neutralized with 0.4 M Tris (pH 7.5). Finally, DNA was stained with silver nitrate, and the slides were coded for blind analysis. Images of 100 randomly selected cells (of four replicated slides) were analyzed from each sample. The integrity of cell nuclei was assessed visually and the DNA damage was scored according to tail size into five classes, from no tail (0) to maximal long tail (4). Therefore, the damage index (DI) could range from 0 (all cells with no tail, 100 cells ×0), which means no damage, to 400 (all cells with maximally long tails, 100 cells ×4), which means the highest damage. The frequency (%) of the different classes (0 to 4) of DNA damage was also evaluated.

2.6. *Statistical analysis*

Results were expressed as mean ± standard deviation (SD) obtained from two independent experiments. The Kolmogorov−Smirnov test was used to assess the parametric distribution of data. Statistical significance was evaluated using one-way analysis of variance (ANOVA) and Duncan's post-hoc test. The relationships between the variables were assessed with Pearson's product-moment correlation coefficient. Significance was accepted at $P \leq 0.05$. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 21 (Illinois, USA) for Windows.

3. Results

In order to examine the effects of AAE on *A. gemmatalis* development, third instars larvae received six doses of *G. max* foliar discs containing 25 µL of 1, 10, and 50 mg/mL of AAE for 10 days, resulting in a total intake of 0.15, 1.5, and 7.5 mg of AAE. To evaluate the possible cytotoxic mechanism of the extract, AAE concentrations were administered for 4 days, resulting in a total intake of 0.05, 0.5, and 2.5 mg of AAE.

No statistical differences were observed between weight gain of the control group and the larvae treated with AAE (Figure 2). Moreover, there was no alteration

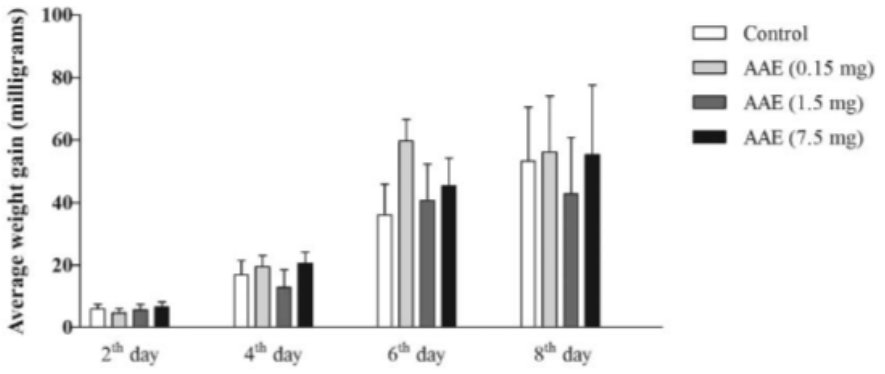

Figure 2. Average weight gain (mg) of insects treated with *Araucaria angustifolia* extract (AAE) for 10 days. Two groups of 30 insects (third instars) in individual cups were fed *Glycine max* foliar discs containing increasing concentrations of extract (0.15, 1.5, and 7.5 mg of AAE) and were weighed every 2 days. Control insects were fed on foliar discs containing distilled water. The bars represent mean ± SD of two independent experiments.

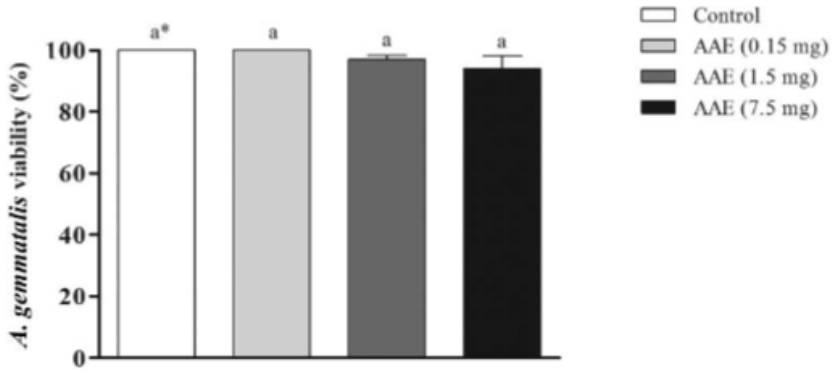

Figure 3. Percentage of viability (%) of the insects in the control and larvae treated with *Araucaria angustifolia* extract (AAE) after 10 days. Two groups of 30 insects (third instars) in individual cups were fed with *Glycine max* foliar discs (0.15, 1.5, and 7.5 mg of AAE). Control insects were fed on foliar discs containing distilled water. The bars represent mean ± SD of two independent experiments.

in the number of surviving larvae until the 10th day of the treatment (Figure 3). However, AAE induced an increase in the number of malformed pupae (Figure 4A), along with a decrease in the emergence of the insects (1.5 and 7.5 mg of AAE treatments) (Figure 4B). Together, these data indicate that AAE causes structural and physiological changes in *A. gemmatalis* larvae.

AAE treatments induced an increase in oxidative damage to both lipids and proteins. The increase in the TBARS (thiobarbituric acid reactive substances) levels was around 1.7 and 1.8 times higher than the control at doses of 0.5 mg and 2.5 mg AAE, respectively. Similar effects were observed in the carbonyl protein levels, being around 1.8 and 2 times higher than the control at doses of

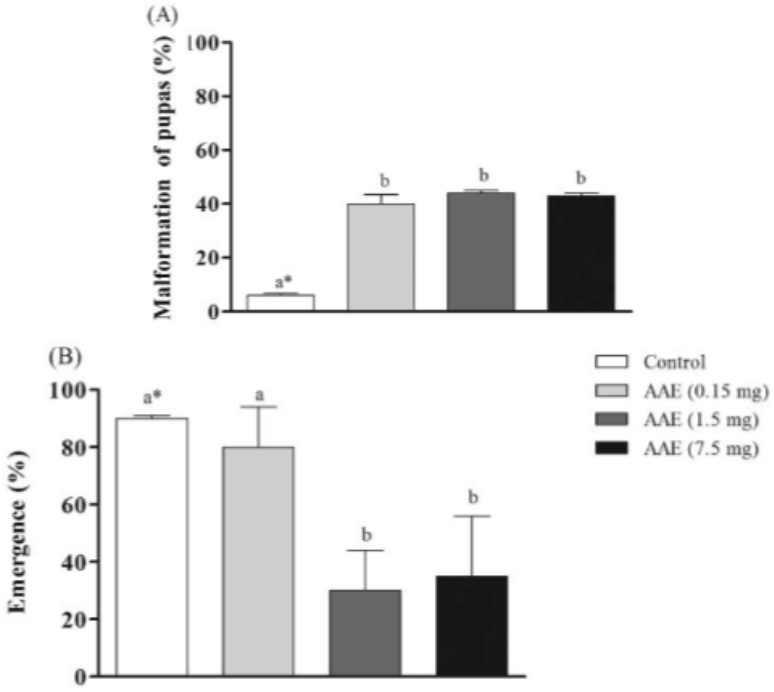

Figure 4. Morphological and structural alterations induced by *Araucaria angustifolia* extract treatment in *Anticarsia gemmatalis*. (A) Percentage of malformation pupae in the control and larvae-treated groups. (B) Percentage of emergence of adults. The bars represent mean ± SD of two independent experiments. *Different letters correspond to values significantly different using analysis of variance (ANOVA) and Duncan's post-hoc test for $P \leq 0.05$.

Table 1. Oxidative damage to lipids and proteins, and DNA damage in *Anticarsia gemmatalis* induced by treatment with *Araucaria angustifolia* extract (AAE).

AAE (mg/mL)	TBARS (nmol/mg protein)	Carbonyl protein (nmol DNPH / mg protein)	DNA damage index (DI)
0.0 (control)	19.11 ± 3.53^{a}*	14.53 ± 0.10^{a}	4.67 ± 0.57^{a}
0.05	23.24 ± 2.35^{a}	16.93 ± 3.42^{a}	15.67 ± 3.22^{b}
0.5	32.87 ± 2.58^{b}	26.60 ± 0.20^{b}	78.00 ± 12.73^{c}
2.5	35.41 ± 1.43^{b}	29.48 ± 0.30^{b}	96.50 ± 14.85^{c}

Third instar *A. gemmatalis* fed for two days on foliar discs containing 0 or 0.05−2.50 mg /mL of AAE. On the fourth day, larvae of each treatment were homogenized (five insects / 1 mL) in order to obtain cellular extracts. Hemolymph was used for DNA damage evaluation (Comet assay).
*Different letters correspond to values significantly different using analysis of variance (ANOVA) and Duncan's post-hoc test for $P \leq 0.05$ for each treatment evaluated.
TBARS = thiobarbituric acid reactive substances.

0.5 mg and 2.5 mg AAE, respectively. In addition, DNA damage was significantly increased at all the AAE concentrations tested when compared with the control, which showed a basal damage index (Table 1). The DNA damage found for 0.05 mg of AAE treatment was approximately 4 times higher than the control, whereas for 0.5 mg and 2.5 mg of AAE, it reached 17 and 20 times higher than the control, respectively. Furthermore, AAE administration (0.05, 0.5, and 2.5 mg) decreased the frequency of undamaged cells and increased the frequency of classes 2, 3, and 4 of DNA damage, mainly at higher doses. In the treatment with 2.5 mg of AAE, a significant increase in the frequency of cells with maximal DNA damage (class 4) demonstrated a higher level of genotoxicity (Table 2).

Correlation analysis (Pearson test) between bioassays data, oxidative stress markers, DNA damage index, and polyphenols content from AAE are shown in Table 3. No emergence was positively correlated with damage to any of the biomolecules and the contents of catechin, epicatechin, and apigenin found in AAE. In relation to the malformation of pupae, only oxidative damage to proteins was positively correlated with this parameter. There was a significant correlation between oxidative damage to lipids, proteins, and DNA with all the polyphenols present in the AAE.

4. Discussion

Phenolic compounds represent a valuable biological control alternative, because they play important functions in plant defense against microorganisms and insects (Dixon and Steele 1999; Samoilova et al. 2014). Taking into account the high polyphenol content of AAE, this extract

Table 2. Frequency (%) of the different classes of DNA damage in control and *Araucaria angustifolia* extract (AAE)-treated groups in *Anticarsia gemmatalis* hemolymph.

AAE treatments (mg/mL)	Undamaged cells	Damage class 1	Damage class 2	Damage class 3	Damage class 4
0.0 (control)	96.67 ± 0.57^{a}	2.00 ± 0.02^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0.5	86.67 ± 3.06^{b}	11.00 ± 3.00^{b}	2.33 ± 0.58^{a}	1.00 ± 0.01^{b}	0.00 ± 0.00^{a}
2.5	40.50 ± 0.71^{c}	44.00 ± 9.19^{c}	14.50 ± 4.95^{b}	2.00 ± 0.07^{c}	2.50 ± 0.12^{b}
	40.00 ± 5.66^{c}	34.00 ± 2.66^{d}	19.00 ± 2.73^{b}	3.50 ± 0.71^{d}	4.00 ± 0.05^{c}

Cells were assessed visually and received scores from 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail. Results represent average values ± SD.
*Superscript letters indicate significant differences according to ANOVA and Duncan's post-hoc test for $P \leq 0.05$ in each treatment evaluated.

Table 3. Pearson correlations between bioassays data, oxidative damage to lipids and proteins, DNA damage, and polyphenols found in *Araucaria angustifolia* extract (AAE).

	No emergence	Malformation	Lipid damage	Protein damage	DNA damage
No emergence	–	0.620 ($P = 0.101$)	0.895 ($P = 0.003$)	0.858 ($P = 0.006$)	0.879 ($P = 0.004$)
Malformation	0.620 ($P = 0.101$)	–	0.699 ($P = 0.054$)	0.735 ($P = 0.038$)	0.675 ($P = 0.067$)
Lipid damage	0.895 ($P = 0.003$)	0.699 ($P = 0.054$)	–	0.921($P = 0.001$)	0.960 ($P = 0.000$)
Protein damage	0.858 ($P = 0.006$)	0.735 ($P = 0.038$)	0.921($P = 0.001$)	–	0.977 ($P = 0.000$)
DNA damage	0.879 ($P = 0.004$)	0.675 ($P = 0.067$)	0.960 ($P = 0.000$)	0.977 ($P = 0.000$)	–
Catechin	0.624 ($P = 0.049$)	0.407 ($P = 0.158$)	0.766 ($P = 0.027$)	0.805 ($P = 0.016$)	0.820 ($P = 0.013$)
Epicatechin	0.621 ($P = 0.050$)	0.404 ($P = 0.160$)	0.764 ($P = 0.027$)	0.802 ($P = 0.017$)	0.818 ($P = 0.013$)
Quercetin	0.601 ($P = 0.058$)	0.389 ($P = 0.171$)	0.747 ($P = 0.033$)	0.786 ($P = 0.021$)	0.801 ($P = 0.017$)
Apigenin	0.624 ($P = 0.049$)	0.407 ($P = 0.158$)	0.766 ($P = 0.027$)	0.805 ($P = 0.016$)	0.820 ($P = 0.013$)

was assayed in *A. gemmatalis*, a caterpillar known to be a major defoliating pest of soybean (Piubelli et al. 2005; Salvador et al. 2010). Assays were conducted using leaf discs of *G. max,* because results obtained by other authors (Duffey and Stout 1996) have shown that ingredients used in artificial diets can increase the impact of chemical compounds related to plant defense. The concentrations of AAE tested were 1, 10, and 50 mg/mL for 10 days (larvae viability, malformation of pupae, and emergence rate determination) or 4 days (oxidative damage to lipids and proteins and DNA damage assay), resulting in a total intake of 0.15, 1.5, and 7.5 mg (10 days treatments) or 0.05, 0.5, and 2.5 mg (4 days treatments). Higher concentrations of AAE were not tolerated by the insects (data not shown).

Although AAE did not induce mortality in the larvae, important secondary effects were observed, such as malformation of pupae and a reduction in the rate of adult emergence. Similar results were found by Hoffmann-Campo et al. (2006), showing detrimental effects on the larval growth without any significant effects on mortality in *A. gemmatalis* fed on a rutin-amended diet.

The AAE was able to induce oxidative damage to lipids and proteins on *A. gemmatalis* larvae. Similar results were found by Summers and Felton (1994), which demonstrated increased levels of oxidative damage to lipids and proteins in *Helicoverpa zea* larvae treated with phenolic acids (caffeic and chlorogenic acids). Lipid peroxidation caused by oxidative stress can lead to a progressive decrease in the fluidity of the membrane, reducing the potential increased the ion permeability and generating a cascade of damaging events within the cell (Barber and Bernheim 1967). Oxidative damage to proteins is involved in several events, such as a loss in specific protein function, disruption of the cellular redox-balance, and interference with the cell cycle (Halliwell and Gutteridge 2007). Although other more studies are needed, it is possible that the oxidative stress induced by AAE could be associated with the malformations and lack of emergence observed in the *A. gemmatalis* larvae.

Measurement of DNA damage is a very important tool to evaluate the toxicity of different compounds. The Comet assay is one of the most versatile techniques for the evaluation of genotoxicity. Moreover, this is a sensitive method to assess both quantitative and qualitative levels of DNA damage in any nucleated cell (Fairbairn et al. 1995; Collins et al. 2008). In the present study, we employed the standard alkaline comet assay, which is able to detect single- and double-strand breaks as well as alkali-labile sites (Singh et al. 1988; Collins et al. 2008). Our results showed that AAE is able to induce high levels of DNA damage in *A. gemmatalis* larvae. However, it is important to mention that the DNA damage detected by the Comet assay could be repaired. Therefore, it would be interesting to assess how much of this damage could be fixed, by employing mutagenicity experiments, such as a micronucleus assay (Kier and Kirkland 2013), which evaluates the frequency of chromosomal alterations in the genome.

Oxidative damage to lipids, proteins, and DNA along with the non-emergence of the insects were positively correlated with the polyphenol content of the AAE. These compounds are secondary metabolites produced by the plants for the purpose of self-defense. Polyphenols can be structurally classified into flavonoids (flavan-3-ol, flavonol, flavone, isoflavone, flavanone, and anthocyanidin subclasses) and non-flavonoids (Del Rio et al. 2013). Catechin and epicatechin, the major compounds found in the AAE, are flavan-3-ols, the most complex subclass of flavonoids. Quercetin belongs to the flavonol subclass. Flavones, such as apigenin, also found in AAE, have similar structure to flavonols, except they lack a hydroxyl group at carbon 3 (Del Rio et al. 2013). The three classes (flavan-3-ols, flavonols, and flavones) of polyphenols presented in AAE could be linked to the biological effects observed in *A. gemmatalis.* Polyphenols have been shown to increase reactive oxygen species (ROS) levels in cell culture and multicellular organisms (Tang and Halliwell 2010). Moreover, phenolic compounds can induce oxidative stress in herbivores (for review, see Appel 1993), including lepidopteran insects (Felton et al. 1989; Felton and Duffey 1991, 1992; Bi et al. 1994). This is detrimental to the organism since high levels of ROS affect the cellular redox balance, and will lead to lipid and protein oxidation, as well as DNA damage (Halliwell and Gutteridge 2007).

In summary, the findings of our study show that AAE can induce oxidative stress and DNA damage in *A. gemmatalis*, an important defoliating pest of soybeans. In addition, AAE causes significant alterations during the stages of insect development, leading to pupal malformation and reduced adult emergence. Although further studies are needed to fully examine the mechanism of action of AAE in the context of insect biology, our results open new perspectives regarding biological pest management in breeding programs.

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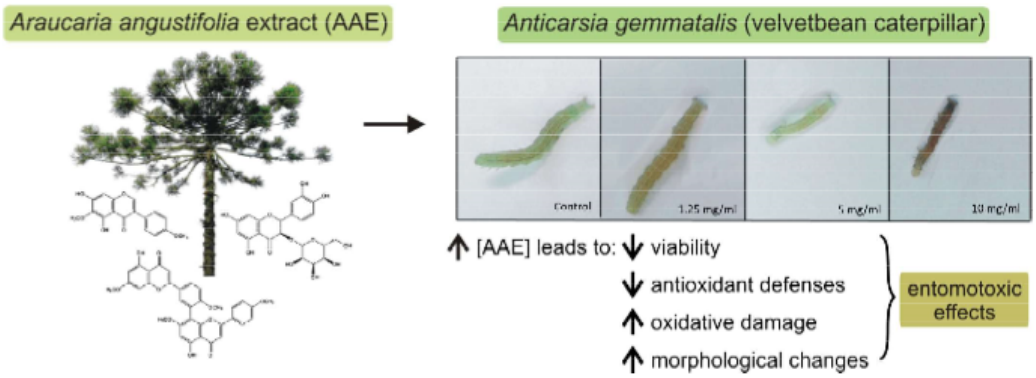
Anexo II



FOOD SCIENCE & TECHNOLOGY | RESEARCH ARTICLE

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Redox imbalance mediates entomotoxic effects of the conifer *Araucaria angustifolia* in *Anticarsia gemmatalis* velvetbean caterpillar

Cátia dos Santos Branco[1], Tiago Selau Rodrigues[1], Émilin Dreher de Lima[1], Lúcia Rosane Bertholdo-Vargas[2], Neiva Monteiro Barros[2] and Mirian Salvador[1]*

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Abstract: The velvetbean caterpillar, *Anticarsia gemmatalis* is one of the most important pests of soybean crops in tropical America. By feeding on leaves, significant defoliation occurs resulting in reduced photosynthetic capacity required for plants' maintenance and growth, which subsequently can lead to crop losses and reduced agricultural productivity. Many studies have sought to look for compounds that have insecticidal effects. One class of compounds is phenolics, which are produced by plants and have been found to influence the behavior and development of defoliators, representing an important alternative approach to many synthetic insecticides. Particularly, *Araucaria angustifolia* is a plant rich in polyphenols, which are compounds able to alter cellular dynamics through modulating redox status. In this study, *A. angustifolia* extract (AAE) was added to the artificial diet of *A. gemmatalis*. The results demonstrated that AAE was able to reduce larval viability by inducing morphological changes and a delay in the insect's development. In addition, AAE was found to induce oxidative damage to lipids and proteins, as well as increased nitric oxide levels in *A. gemmatalis* larvae. AAE treatments also decreased the antioxidant defense systems, leading to a redox imbalance. The reduction in viability in *A. gemmatalis* was positively correlated with oxidative markers, suggesting that redox imbalance can lead to larvae's death. These results suggest that AAE possess

Mirian Salvador

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Mirian Salvador is a full professor at the University of Caxias do Sul. She received her PhD in Chemistry from the Universidad de La Republic del Uruguay (1995), and Specialist in Biotechnology from University of Caxias do Sul (1987). She works in the area of Biochemistry, with an emphasis on oxidative damage and antioxidants. Her research focuses on understanding of the role of natural products and polyphenols on redox metabolism and cellular signaling. She is currently exploring the possible effects of natural compounds on mitochondrial modulation leading to apoptosis or cell survival in different models of study. The study, redox imbalance mediates entomotoxic effects of *Araucaria angustifolia* in *Anticarsia gemmatalis*, covers a broad research about the antioxidant/oxidant potential of natural extracts from Brazilian plants in eukaryotic cells, aiming the development of new active compounds from natural sources.

PUBLIC INTEREST STATEMENT

Soybean is an important agricultural crop that is often sought for its high nutritional content. Soybean accounts for a significant proportion of the world's production and is continuing to expand due to increasing global consumption. However, the velvetbean caterpillar, *Anticarsia gemmatalis* represents an increasing problem as they can impede the growth, quality, and yield of soybean crops. The proper management of these pests through the search of new active compounds is therefore crucial. In this study, we evaluated the entomotoxic effects of a natural extract, *Araucaria angustifolia* extract (AAE) on *A. gemmatalis*. AAE induced redox imbalance, significant morphological alterations, and decreased larval and pupal viability in *A. gemmatalis*. These findings suggest that AAE has entomotoxic potential, which may contribute to novel development of biological control in soybean crops.

insecticidal potential through the mechanisms of action of altering cellular redox state. Though further studies are required to confirm this, our study nevertheless contributes to a better understanding of AAE's mechanisms of action as potential biopesticides in pest management, opening new perspectives on the development of compounds with insecticidal action.

Subjects: Agriculture & Environmental Sciences; Biochemistry; Biotechnology; Environmental Studies & Management

Keywords: biological control; lepidopteran; velvetbean caterpillar; polyphenols

1. Introduction

The Lepidoptera order covers a broad and diverse group of insects around the world, including species that are crop pests. Among them, the velvetbean caterpillar, *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Erebidae) is the main insect pest that requires control measures in soybeans crops, especially in Brazil, which is the world's second-largest producer of soybeans, behind the United States of America (USDA, 2015). Attack of *A. gemmatalis* on soybean crops causes defoliation, which in turn compromises the quality and yields by altering the filling of the grains and pods of the plant (Levy, Falleiros, Moscardi, & Gregório, 2011). Considering the importance of soybean as one of the most cultivated grains in the world, (Ray, Mueller, West, & Foley, 2013) pest control actions and management are therefore crucial.

Currently, the most common intervention for *A. gemmatalis* control in soybean agriculture is the employment of synthetic insecticides (Bernardi et al., 2012). However, the continuous intensive use of chemical pesticides may contribute to an increase in pest resistance leading to failure to pest control (Sarfraz, Keddie, & Dosdall, 2005). Considering the diverse problems associated with the massive use of these chemicals in agriculture and in environment, the biotechnology field can contribute to the discovery and development of new alternative pest control strategies (Penman, 1994). Some natural opponents including viruses (*Baculovirus anticarsia*) (Braconi et al., 2014; Piubelli, Hoffmann-Campo, Moscardi, Miyakubo, & de Oliveira, 2006; Piubelli, Moscardi, & Hoffmann-Campo, 2009), entomopathogenic fungi (Bertholdo-Vargas et al., 2009), and bacteria (Fiuza, Schünemann, Pinto, & Zanettini, 2012; Gobatto et al., 2010) have been used for biological control of pest populations of *A. gemmatalis*; however, they are not completely effective, prompting more studies to investigate better pest management strategies for agronomical applications.

The great diversity in plant kingdom represents an important source of molecules with potential entomotoxic value. *Araucaria angustifolia* (Bert. O. Kuntze) belongs to the Araucariaceae family and is found in South America. It is a dioecious species that contains male and female reproductive organs carried on distinct strobilus. The female strobilus consists of seeds (the edible part of *A. angustifolia*) and bracts, which are undeveloped seeds naturally discarded into the environment that contains high levels of bioactive compounds, particularly polyphenols (Branco et al., 2015; Michelon et al., 2012; Souza et al., 2014). Plants naturally produce phenolic compounds in order to provide self-defense against pests. Some of these phenolic compounds have been shown to present entomotoxic effects on defoliating insects, including *A. gemmatalis* (Batista Pereira et al., 2002; Gazzoni, Hülsmeyer, & Hoffmann-Campo, 1997; Hoffmann-Campo, Ramos Neto, Oliveira, & Oliveira, 2006; Piubelli et al., 2006; Salvador et al., 2010) and *S. frugiperda* (Batista Pereira et al., 2002).

The potential of natural compounds must be taken into account when defining the best pest control approach. In this context, the aim of this study was to investigate possible entomotoxic effects induced by *A. angustifolia* extract (AAE) when administered in an artificial diet to soybean caterpillar, *A. gemmatalis*. In order to understand the possible entomotoxic mechanisms induced by AAE, oxidative damage to lipids and proteins, nitric oxide levels, and antioxidant defenses systems were evaluated.

2. Materials and methods

2.1. Araucaria angustifolia extract

Mature female strobili of *A. angustifolia* were collected in Caxias do Sul, Rio Grande do Sul (29°9′34.90″ S, 51°8′45.34″ W); Ibama nº 02001.001127/2013-94, Brazil. Voucher specimens were identified by the Herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (HUCS 40710/40711). Bracts were manually separated from the pine, and the extract was obtained using 5 g of bracts in 100 mL of distilled water, under reflux (100°C) for 15 min, filtered in Millipore equipment (pore size, 0.45 µm; SFGS 047LS, Millipore Corp.) and then lyophilized (LIOBRAS model L-101) under vacuum pressure to yield a powder. To perform the assays, AAE was solubilized in distilled water immediately before added to artificial diet. The chemical characterization of AAE was already described by our research group (Branco et al., 2015; Michelon et al., 2012; Souza et al., 2014) and the presence of several polyphenols, including isoflavones and bioflavonoids was confirmed (Figure 1).

2.2. Insects

The insects (*A. gemmatalis*) used in this study were from colonies kept at the Laboratory of Control of Pests, Institute of Biotechnology, University of Caxias do Sul, Brazil. The insects were reared on artificial diet (Greene, Leppla, & Dickerson, 1976), maintained in an acclimatized chamber at 27 ± 1°C; 50 ± 10% relative humidity; and a 14:10 light/dark photoperiod.

2.3. Treatments and experimental design

Thirty larvae (third instars) of *A. gemmatalis* were used in each group, which included three AAE treatments (1.25, 5, and 10 mg/mL AAE) and one control group. The larvae were transferred to individual cups, and every two days, the insects were fed on artificial diet of 1.25, 5, and 10 mg/mL AAE. The AAE concentrations were defined from previous tests. Insects served as control were fed with artificial diet without AAE. The diet was cut with a stainless steel spatula, previously cleaned with 70% alcohol, and individually offered to each caterpillar, in cubes around 1 cm^3, during the daily maintenance activities. Diets were consumed by the insects within two days and were replaced until the insects reach the pre-pupal stage. All the insect groups were evaluated every two days for weight gain. Rates of larvae and pupae mortality and malformation of larvae and pupae (morphological alterations) were evaluated. Oxidative and nitrosative stress, as well as enzymatic and non-enzymatic antioxidant cellular defenses were evaluated on the fifth day of the treatment.

2.4. Lipid and protein oxidative damage

Larvae of each treatment were homogenized (five insects per mL) in ice-cold 50 mM phosphate potassium buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and centrifuged at 1,500 × *g* at 4°C for 5 min. The supernatant was used for the assays. To determine lipid damage, aliquots (100 µL) of the supernatant were mixed with 100 µL of the color reagent (1% thiobarbituric acid (TBA), 50 mM sodium hydroxide (NaOH), 0.1 mM butylated hydroxytoluene (BHT)) and 50 µL 7% (v/v) phosphoric acid. The mixture was placed in a boiling water bath for 15 min. After cooling, 1.5 mL of *n*-buthanol was added to the mixture followed by centrifugation for 5 min at 1,600 × *g*. The absorbance of supernatant was measured at 532 nm, and the results were expressed in nmol/mg of protein (Hermes-Lima & Storey, 1995). Oxidative damage to proteins was measured by quantifying the carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). Two hundred µL of DNPH (10 mM) or 200 µL of hydrochloric acid (HCl) (2 M) for blank was added to 50 µL of the samples. The reaction mixture was incubated in the dark for 30 min and vortexed every 10 min. Next, 250 µL of 20% trichloroacetic acid (TCA) was added and centrifuged at 1,500 × *g* for 8 min. The supernatant was discarded and the pellet was washed three times with ethanol-ethyl acetate (1:1) to remove the free reagent. Samples were centrifuged and pellets were redissolved in 1,000 µL of urea solution (8 M) at 37°C for 15 min. Absorbance was read at 365 nm, and results were expressed as nmol DNPH/mg protein.

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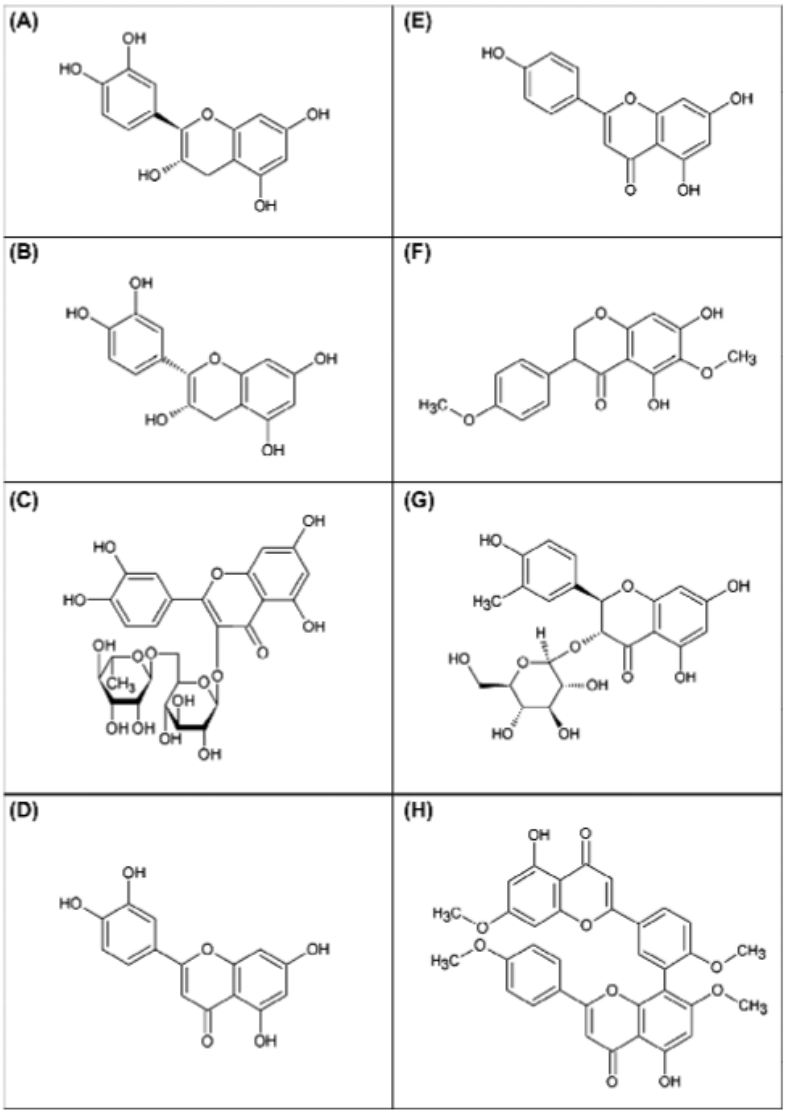

Figure 1. Polyphenolic compounds identified in *Araucaria angustifolia* extract (AAE). (A) Catechin; (B) Epicatechin; (C) Rutin; (D) Quercetin; (E) Apigenin (F) 4'-Methoxytectorigenin; (G) 3-Glucoside-dihydroquercetin; (H) Amentoflavone 4',4''',7,7''-tetramethyl ether.

Note: Images obtained using Symyx Draw 4.0.0 software (Symyx Technologies Inc., Santa Clara, California, USA).

2.5. Nitric oxide levels

To evaluate the possible nitrosative stress induced by AAE in *A. gemmatalis* larvae, nitric oxide (•NO) levels were determined as nitrite (NO_2^-) production, using the Griess reaction-based method (Green, Tannenbaum, & Goldman, 1981). Larvae of each treatment were homogenized (five insects per mL) in ice-cold 50 mM phosphate potassium buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and centrifuged at 1,500 × *g* at 4°C for 5 min. For the assay, 50 µL of supernatants was reacted with an equal volume of Griess reagent (0.1% naphthylethylenediamine and 1%

sulfanilamide in 5% H_3PO_4) for 10 min at room temperature, and the absorbance was read at 550 nm. Sodium nitroprusside was used as the standard. The results were expressed as nmol of nitrite per mg of protein.

2.6. Superoxide dismutase and catalase activities

After treatments, third instar *A. gemmatalis* were homogenized (five insects per mL) in ice-cold 50 mM phosphate potassium buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (protease inhibitor). The homogenate was centrifuged at 1,600 × g at 4°C for 30 min, and the supernatants were used for both enzymatic assays. Superoxide dismutase (Sod) activity was measured by the inhibition of self-catalytic adrenochrome formation rate at 480 nm, in a reaction medium containing 1 mmol/L adrenaline (pH 2.0) and 50 mmol/L glycine (pH 10.2) at 30°C for 3 min (Bannister & Calabrese, 1987). Results were expressed as USod (units of enzyme activity)/mg protein. One unit is defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50%. Catalase (Cat) activity was measured by rate of H_2O_2 decomposition at 30°C for 1 min in 240 nm (Aebi, 1984). Results were expressed as UCat/mg of protein. One unit is defined as the amount of enzyme that decomposes 1 mmol of H_2O_2 in 1 min at pH 7.4. All absorbance were measured in a microplate reader (Victor-X3, multilabel counter, Perkin Elmer, Finland).

2.7. Protein sulfhydryl content

This assay is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB), whose absorption is determined spectrophotometrically at 412 nm (Aksenov & Markesbery, 2001). The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were expressed as mmol of DTNB/mg of protein.

2.8. Protein content determination

Larvae total protein content was quantified by the method of Bradford, using the bovine serum albumin as standard (Bradford, 1976).

2.9. Statistical analysis

Results were expressed as mean ± standard deviation (SD) obtained from two independent experiments. The Kolmogorov–Smirnov test was used to assess the parametric distribution of data. Statistical significance was evaluated using a one-way analysis of variance (ANOVA) and Duncan's *post hoc* test. Relationships between the continuous variables were assessed using Pearson's correlation coefficient. Significance was accepted at $p \leq 0.05$. All statistical analyses were performed using the SPSS 21.0 software (SPSS Inc., Chicago, IL).

3. Results

In this study, we started the experiments with third instars *A. gemmatalis*, which received an artificial diet containing 1.25, 5, and 10 mg/mL AAE for 10 days. The diet was offered until the insects reached the pre-pupation stage, which occurred between 8th and 10th days. In view of this, the parameter weight gain was evaluated until the 6th day. It was observed that larvae fed with AAE had a reduced weight in all tested concentrations when compared to control group, even before the 4th day (Figure 2). Moreover, higher concentrations of AAE (5 and 10 mg/mL) were able to induce a significant decrease in weight compared to those fed with 1.25 mg/mL AAE on the 4th and 6th days.

In addition to weight changes, AAE-treated *A. gemmatalis* showed significant altered morphology, mainly with 5 and 10 mg/mL treatments (Figure 3), along with malformation of pupae and low emergence of adults (data not shown). Among AAE-treated larvae, mortality at the larval stage reached to 10.0, 96.7, and 99.0% with 1.25, 5, and 10 mg/mL AAE, respectively (Figure 4(A)). In comparison to pupal mortality, 26.6% of mortality with 1.25 mg/mL AAE was observed, while 99% of pupae mortality was observed at both concentrations of 5 and 10 mg/mL AAE (Figure 4(B)).

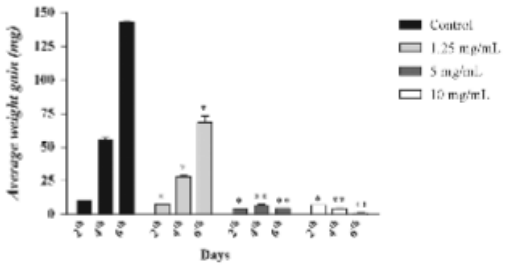

Figure 2. Average weight gain (milligrams) of insects treated with *Araucaria angustifolia* extract (AAE) for 10 days.

Notes: Groups of 30 insects (third instars) in individual cups were fed with artificial diet containing increasing concentrations of extract (1.25; 5 and 10 mg/mL of AAE) and were weighed every 2 days. Control insects were fed with artificial diet containing distilled water. Values are expressed as mean ± standard deviation. The symbol * indicates a significant difference ($p \leq 0.05$) from the control group. The symbol ** indicates a significant difference ($p \leq 0.05$) from larvae treated with AAE. Analysis of variance (ANOVA) and Duncan's post-hoc test for $p \leq 0.05$.

For evaluation of redox status, we analyzed cellular extracts of larvae fed with artificial diet, in the presence or absence of AAE on the fifth day of treatment. AAE treatments (5 and 10 mg/mL) induced an increase in oxidative damage to both lipids and proteins. In addition, nitric oxide levels were significantly increased in all AAE concentrations tested when compared to the control (Table 1), indicating that AAE is able to induce oxidative and nitrosative stress. This condition, along with low antioxidant defenses systems, is very dangerous to cells. For this reason, we next investigated the activity of the antioxidant enzymes, superoxide dismutase, and catalase, as well as the non-enzymatic cellular defense, sulfhydryl protein content in *A. gemmatalis* larvae. Our results showed that the activities of Sod and Cat enzymes, and the protein sulfhydryl levels were significantly decreased at all treatment concentrations of AAE (Table 2), indicating a depletion in antioxidant defenses.

Pearson's correlation analysis between *A. gemmatalis* viability (at the larval stage or pupal) and redox status markers was performed and is shown in Table 3. Viability was positively correlated with both superoxide dismutase ($r = 0.933$; $p = 0.001$) and catalase activities ($r = 0.756$; $p = 0.030$), and with sulfhydryl content ($r = 0.904$; $p = 0.002$); however, it was negatively correlated with oxidative damage to lipids ($r = -0.940$; $p = 0.001$) and proteins ($r = -0.966$; $p = 0.001$); and nitric oxide

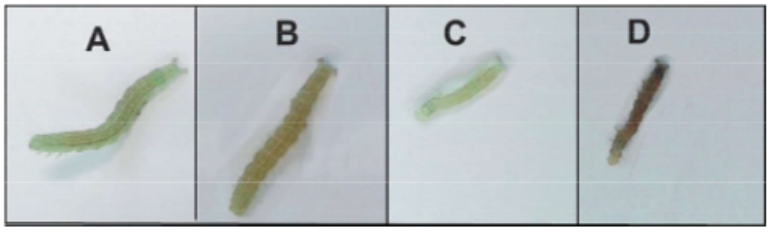

Figure 3. Evaluation of larvae morphology of *Anticarsia gemmatalis* treated with increasing concentrations of *Araucaria angustifolia* extract (AAE).

Notes: Photographs of *Anticarsia gemmatalis* on the five day of treatment with *Araucaria angustifolia* extract (AAE). (A) exemplary of the control group showing normal morphology; (B) exemplary of the group treated with 1.25 mg/mL of AAE showing normal morphology; (C) exemplary of the group treated with 5 mg/mL of AAE showing altered morphology; (D) exemplary of the group treated with the higher concentration of AAE (10 mg/mL) showing significantly altered morphology.

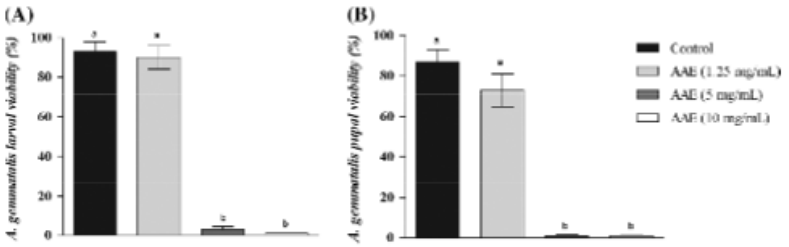

Figure 4. Percentage of viability (%) of *Anticarsia gemmatalis* larval and pupal in the control and treated groups with *Araucaria angustifolia* extract (AAE)

Note: Groups of 30 insects (third instars) in individual cups were fed with artificial diet containing increasing concentrations of extract (1.25; 5 and 10 mg/mL of AAE). Control insects were fed with artificial diet containing distilled water. Different letters correspond to values with significant effects using analysis of variance (ANOVA) and Duncan's post-hoc test for $p \leq 0.05$.

Table 1. Oxidative and nitrosative stress parameters evaluated in *A. gemmatalis* treated with *A. angustifolia* extract (AAE)

Groups	TBARS (nmol/mg protein)	Carbonyl protein (nmol/mg protein)	Nitrite concentration (nmol/mg protein)
Control	0.127 ± 0.014^a	1.674 ± 0.108^a	0.720 ± 0.004^a
AAE 1.25 mg/mL	0.173 ± 0.035^a	2.366 ± 0.304^a	0.958 ± 0.136^b
AAE 5 mg/mL	0.446 ± 0.062^b	6.743 ± 0.059^b	1.057 ± 0.087^b
AAE 10 mg/mL	0.632 ± 0.040^c	8.028 ± 1.537^b	1.009 ± 0.044^b

Notes: Third instar *A. gemmatalis* larvae fed for four days with artificial diet containing 0 or 1.25–10 mg/mL of AAE. Different superscript letters correspond to values statistically different using analysis of variance (ANOVA) and Duncan's *post hoc* test for $p \leq 0.05$ for each parameter evaluated. TBARS: thiobarbituric acid reactive substances.

Table 2. Enzymatic and non-enzymatic antioxidant defense systems evaluated in *A. gemmatalis* treated with *A. angustifolia* extract (AAE)

Groups	Superoxide dismutase (USod/mg protein)	Catalase (UCat/mg protein)	Sulfhydryl content (mmol/mg protein)
Control	0.704 ± 0.064^a	0.973 ± 0.044^a	0.030 ± 0.004^a
AAE 1.25 mg/mL	0.487 ± 0.056^b	0.651 ± 0.084^b	0.016 ± 0.005^b
AAE 5 mg/mL	0.243 ± 0.029^c	0.553 ± 0.098^b	0.004 ± 0.002^c
AAE 10 mg/mL	0.280 ± 0.005^c	0.586 ± 0.049^b	0.003 ± 0.001^c

Notes: Third instar *A. gemmatalis* fed for four days with artificial diet containing 0 or 1.25–10 mg/mL of AAE. Different superscript letters correspond to values statistically different using analysis of variance (ANOVA) and Duncan's *post hoc* test for $p \leq 0.05$ for each parameter evaluated. USod: amount of enzyme that inhibits the rate of adrenochrome formation in 50%. UCat: amount of enzyme that decomposes 1 mmol of H_2O_2 in 1 min at pH 7.4.

production ($r = -0.746$; $p = 0.034$). Moreover, negative correlations were found significant between oxidative damage to biomolecules and nitric oxide levels with antioxidant defenses (Table 3).

4. Discussion

Natural products constitute an important source of phytochemical agents with several biological activities, including entomotoxic activity (Céspedes, Calderón, Lina, & Aranda, 2000; Macedo, Oliveira, & Oliveira, 2015; Miyazawa, Wada, & Kameoka, 2001). The use of these compounds offers numerous advantages over conventional synthetic pesticides used in crops, and therefore could

Table 3. Pearson correlations between *A. gemmatalis* viability, lipid and protein oxidative damage, nitric oxide levels, and antioxidant defense systems

	Viability	Lipid damage	Protein damage	Nitric oxide	Sod activity	Cat activity	Sulfhydryl
Viability	–	−0.940**	−0.966**	−0.746*	0.933**	0.756*	0.904**
Lipid damage	−0.940**	–	0.970**	0.702	−0.849**	−0.699	−0.871**
Protein damage	−0.966**	0.970**	–	0.706	−0.885**	−0.744*	−0.875**
Nitric oxide	−0.746*	0.702	0.706	–	−0.889**	−0.865**	−0.921**
Sod activity	0.933**	−0.849**	−0.885**	−0.889**	–	0.853**	0.986**
Cat activity	0.756*	−0.699	−0.744*	−0.865**	0.853**	–	0.854**
Sulfhydryl	0.904**	−0.871**	−0.875**	−0.921**	0.986**	0.854**	–

Notes: Sod: superoxide dismutase; Cat: catalase.

*Statistically significant for $p \leq 0.05$.

**Statistically significant for $p \leq 0.01$.

potentially prevent problems associated with insecticidal resistance, reduction in predatory and parasitoid insects, and also toxicity to other animals and to environment (Duke et al., 2003).

Phenolic compounds found in plants cover a chemically diverse and widespread group of defensive molecules that ranges from simple phenolics to complex polymers (Del Rio et al., 2012; Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011). These secondary metabolites participate in defense mechanisms against ultraviolet radiation and plant pathogens (Appel, 1993; Whitehill, Rigsby, Cipollini, Herms, & Bonello, 2014). Although polyphenols are well known by its antioxidant effects, they can become pro-oxidant at high doses, leading to the formation of reactive oxygen species (ROS) and inducing oxidative stress (Halliwell, 2007, 2008; Procházková, Boušová, & Wilhelmová, 2011). Thus, phenolic compounds can be toxic to herbivorous insects (Appel, 1993; War, Paulraj, War, & Ignacimuthu, 2011a, 2011b).

The endemic conifer, *A. angustifolia* (Bert. O. Kuntze), contains phenolic compounds of different classes including flavonoids and non-flavonoids. Previously, we investigated the entomotoxic effect of an aqueous extract obtained from the sterile seeds of AAE on velvetbean caterpillar *A. gemmatalis* cellular state. We found that this extract was able to induce oxidative and genotoxic damage in larvae of *A. gemmatalis*; however, it was unable to induce larval mortality (Branco et al., 2014). Considering that phenolic compounds are molecules that may be oxidized by different conditions, such as luminosity exposition, heating, and humidity level, we believe that the absence of significant effect on the viability of *A. gemmatalis* could be attributed to the experimental design performed by us. Therefore, in the present study, we added the *Araucaria* extract powder on the insect's artificial diet in order to maintain its stability, while avoiding possible food interferences. We observed that AAE was able to induce a significant reduction in weight of *A. gemmatalis*, as well as alter the larval morphology, deform the structure of pupas, and decrease emergence of adults. Besides these actions, we found that AAE administration caused significant reduction in *A. gemmatalis* viability, both at larval and pupal stages. At larval stage, the lethal concentration (LC_{50}) found was at 3.5 mg/mL AAE, i.e. the lethal concentration that is needed to reduce 50% of the individuals of a population. On the other hand, the LC_{50} was 2.3 mg/mL during the pupal stage, indicating that these insects are more sensitive to AAE at this particular stage of development. These results are consistent with a study performed by Batista Pereira et al. (2002) who demonstrated that flavonoid astilbin was able to induce significant toxicity during the pupal stage of *A. gemmatalis* and *S. frugiperda*. The data found in our study indicate that concentrations around 3.0 mg/mL are most promising for future tests aimed at evaluating the toxic effects of AAE on the biology and development of *A. gemmatalis*.

In order to evaluate the entomotoxic mechanisms of action of AAE, we decided to investigate levels of oxidative stress, as well as the possible modulation of the antioxidant defense systems in third instars *A. gemmatalis*. The AAE treatments induced an increase in oxidative damage to both lipids and proteins, as well as an elevated production of nitric oxide, indicating high index of oxidative stress and cellular damage. Oxidative damage to biomolecules such as lipid peroxidation and protein modifications is involved in several events that subsequently may lead to cell death (Cobb & Cole, 2015). Cellular nitrosative stress may also occur through nitrosylation of proteins and lipids through the incorporation of nitric oxide and its derivatives (O'Donnell et al., 1999). Nitric oxide (NO) is a ubiquitous and water-soluble molecule, which plays key role in various physiological and pathological processes in mammals (Korde Choudhari, Chaudhary, Bagde, Gadbail, & Joshi, 2013). In invertebrates, including insects, NO production has been shown to play a key role in inducing cellular response of these organisms against pathogens and other stress conditions (Faraldo, Sá-Nunes, Del Bel, Faccioli, & Lello, 2005; Foley & O'Farrell, 2003; Gourdon, Guérin, Torreilles, & Roch, 2001; Nappi & Ottaviani, 2000). Moreover, NO plays a critical role in the initiation of insect metamorphosis, as observed during Drosophila development (Caceres et al., 2011), indicating that this molecule is associated with important biological processes regarding metabolism and behavior (Yamanaka & O'Connor, 2011). In our study, the increment in NO levels induced by AAE may be understood as the insect's response to toxicity as a result of polyphenol metabolism.

To counteract oxidative stress, eukaryotic cells possess an antioxidant defense system that controls ROS generation and levels. However, when ROS levels exceed the capacity of antioxidant defense systems, cellular oxidative stress occurs (Halliwell, 2007, 2008; Halliwell & Gutteridge, 1995). In our study, we demonstrated increased oxidative stress, as the activities of Sod and Cat antioxidant enzymes were found significantly depleted after treatments. This suggests that AAE is inducing the generation of high levels of superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), contributing to cellular redox imbalance in *A. gemmatalis*. Sod enzyme is important for the detoxification of $O_2^{\cdot-}$ into H_2O_2, which is subsequently dismutated by Cat enzyme. In addition to the reduction of Sod and Cat activity, we demonstrated for the first time that AAE was able to reduce the levels of protein sulfhydryl, an important non-enzymatic antioxidant defense marker, in *A. gemmatalis* larvae. Superoxide anion and hydrogen peroxide are thought to be byproducts of aerobic respiration generated by electron transport chain of the mitochondria (Bae, Oh, Rhee, & Yoo, 2011). Though mitochondria are traditionally known for producing energy for cellular survival, they also play an important role in regulating other cellular mechanisms such as apoptosis, an essential dynamic process that maintains the stability of the internal environment and controls the development of multicellular organisms, including insects (Huang, Lv, Hu, & Zhong, 2013). Taking into account the link between redox imbalance and apoptosis, our results suggests that AAE-induced ROS generation, mainly by $O_2^{\cdot-}$ formation observed in this study, may be responsible for the reduction in insect viability, leading to cell death possibly via mitochondrial regulation of apoptosis signaling. However, further studies are needed in order to confirm this hypothesis.

In summary, our data demonstrated that the entomotoxic mechanisms presented by AAE are associated with redox imbalance in *A. gemmatalis* larvae, leading to reduction in viability and deleterious changes in its development. As well, our data suggest that the natural extract of *A. angustifolia* presents a potential to be used as a biological control agent for the management of velvetbean caterpillar *A. gemmatalis* in soybean crops.

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Competing interests
The authors declare no competing interests.

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