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**ATIVIDADE CITOTÓXICA DE AMOSTRAS DE PRÓPOLIS
BRASILEIRA E CUBANA CONTRA CÉLULAS TUMORAIS
HUMANAS**

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de Botucatu, para obtenção do título de Doutora em Patologia

Orientador: Prof. Dr. José Maurício Sforcin
Coorientador: Prof. Dr. Alexis Diaz Garcia

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RESUMO

A própolis é um produto elaborado por abelhas a partir da matéria vegetal coletada ao redor da colmeia. Recentes pesquisas têm identificado o potencial terapêutico da própolis, tais como: antimicrobiano, antiviral, anti-inflamatório, antitumoral, cicatrizante, imunoestimulante, hepatoprotetor, entre muitos outros. Atualmente, muitos estudos que envolvem amostras de própolis de diferentes regiões têm focalizado a descoberta de candidatos promissores para o tratamento do câncer. Nesse sentido, o efeito anti-proliferativo e citotóxico de amostras de própolis verde do Brasil (PB) e vermelha de Cuba (PC) foi avaliado em linhagens de células tumorais humanas de origem epitelial (A549, Hep-2, MDA-MB-231) e em células não tumorais (Vero) pelo ensaio com brometo de 3-(4,5-dimetil-2-tiazolil)-2, 5-difenil-2H-tetrazólio (MTT) e lactato desidrogenase (LDH). O mecanismo molecular envolvido na morte celular das linhagens tumorais como apoptose/necrose foi determinado por coloração fluorescente com laranja de acridina/brometo de etídio (LA/BE), diamidinofenilindol (DAPI), permeabilização da mitocôndria mediante alteração do potencial de membrana mitocondrial, e pela expressão dos genes pro-apoptóticos (*TP53*, *CASP3*, *BAX*, *NOXA*, *PUMA*, *P21* e anti-apoptóticos (*BCL2*, *BCL-XL*) mediante transcrição reversa e reação em cadeia da polimerase (RT-PCR). As células tumorais A549 e HEp-2 apresentaram redução significativa da viabilidade celular pela ação citotóxica de PB (IC_{50} : $69,17 \pm 11,28 \mu\text{g/ml}$, IC_{50} : $64,1 \pm 14,6 \mu\text{g/ml}$ respectivamente). Esta amostra induziu morte celular por apoptose nas duas linhagens tumorais, evidenciado pela condensação da cromatina, alteração do potencial de membrana mitocondrial, aumento da expressão dos genes pro/apoptóticos e redução da expressão dos genes anti-apoptóticos. Referente à viabilidade da linhagem MDA MB-231, esta não foi afetada pelo tratamento com PB. Os efeitos citotóxicos induzidos pela amostra PC nas linhagens HEp-2 e MDA MB-231 foram diferentes (IC_{50} : $56,2 \pm 9,2 \mu\text{g/ml}$, IC_{50} : $67,27 \pm 12,8 \mu\text{g/ml}$ respectivamente). A morte celular por apoptose foi evidenciada na linhagem HEp-2 pela condensação da cromatina, aumento da expressão dos genes pro-apoptóticos, redução dos genes anti-apoptóticos, geração de espécies reativas do oxigênio e alteração do potencial de membrana mitocondrial. Porém, na linhagem MDA MB-231, foi evidenciada a morte celular por necrose com liberação da enzima LDH,

geração de espécies reativas do oxigênio, afetação do potencial de membrana mitocondrial, interferência na migração celular, e redução da expressão dos genes pro-apoptóticos sem alteração das vias de sinalização PI3K/Akt ERK1/2. Nossos dados permitiram concluir que o efeito citotóxico e tipo de morte celular induzido pela própolis dependem da composição química, origem geográfica da amostra e fontes vegetais. Ainda são necessários estudos correlacionando a composição química com a atividade biológica, definindo cada tipo de própolis com a sua aplicação terapêutica para o tratamento de câncer.

PALAVRAS-CHAVES: própolis, citotoxicidade, apoptose, necrose

ABSTRACT

Propolis is produced by bees from plant material collected around the hive. The efficacy of propolis in some treatments has been associated to its important activities such as antimicrobial, antiviral, anti-inflammatory, antitumor, healing, immunostimulant, hepatoprotective, and many others. Currently, many studies involving propolis samples from different regions have focused on the discovery of promising candidates for the treatment of cancer. The antiproliferative and cytotoxic activities of Brazilian green propolis (BP) and Cuban red propolis (CP) against a panel of epithelial human tumor cell lines (A549, Hep-2, MDA-MB-231) and non-tumor cells (Vero) were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase assay (LDH). Additionally, the effect of both samples of propolis on tumor cell death by apoptosis/necrosis was assayed by fluorescence microscopy and the expression pro-apoptotic genes (*TP53*, *CASP3*, *BAX*, *NOXA*, *PUMA*, *P21*) and antiapoptotic genes (*BCL-2*, *BCL-XL*) by reverse transcription polymerase chain reaction (RT-PCR). Brazilian propolis displayed antiproliferative and cytotoxic effects on A549 cells and HEp-2 in a dose-dependent manner (IC_{50} : $69.17 \pm 11.28 \mu\text{g/ml}$, IC_{50} : $64.1 \pm 14.6 \mu\text{g/ml}$, respectively). Apoptotic cell death was evidenced in both tumor cells by chromatin condensation, overexpression of pro-apoptotic genes, down-expression of antiapoptotic gene and reduction of mitochondrial membrane potential. In contrast, BP did not affect the viability of MDA MB-231 cells. Cuban propolis revealed a dose-dependent cytotoxic effect on HEp-2 and MDA MB-231 cells (IC_{50} : $56.2 \pm 9.2 \mu\text{g/ml}$, IC_{50} : $67.27 \pm 12.8 \mu\text{g/ml}$ respectively). The effects of CP on cell death were different in HEp-2 and MDA MB-231 cells, what indicated that CP exerted its cytotoxic effects in HEp-2 cells by inducing apoptosis, regulating the over expression of pro-apoptotic genes, down-expression of antiapoptotic gene and decreasing mitochondrial membrane potential with possible association of reactive oxygen species production. In MDA MB-231 cells, CP induced necrosis associated with reactive oxygen species production, LDH release and reduction of mitochondrial membrane potential and down-expression of apoptosis-related genes. CP effects on breast cancer cells were associated to a decreased cell migration but not to modification of PI3K/Akt and

ERK1/2 pathways. Our results indicated that propolis samples displayed a differential cytotoxicity due to their chemical compositions, which depend on the geographical region and vegetal source, what may influence its biological activity. Further investigation is needed to correlate the chemical composition with the biological activity, defining the therapeutic application of each type of propolis regarding cancer treatment.

Keywords: propolis, cytotoxicity, apoptosis, necrosis

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1.- INTRODUÇÃO

Câncer é o nome genérico dado a uma série de doenças, cuja principal característica é o crescimento e divisão descontrolados de células anormais. O processo se inicia quando células de algum tecido ou órgão do corpo começam a crescer de forma desordenada, gerando células anômalas, que podem se multiplicar adquirindo capacidade invasiva (1-4).

Cerca de 10% dos casos de câncer são hereditários. A maioria dos causadores desta enfermidade tem relação direta com fatores ambientais e hábitos de vida, tais como tabagismo, consumo excessivo de álcool, sedentarismo, alimentação inadequada e exposição exagerada ao sol ou à alguns microrganismos (5, 6).

O número de casos de câncer na população mundial cresceu nas últimas décadas. Segundo estimativas mundiais surgem a cada ano nove milhões de novos casos e cinco milhões de pessoas vão a óbito em sua decorrência. Em 2030, a estimativa global será de 21,4 milhões de novos casos, seguidos de 13,2 milhões de mortes em consequência ao aumento da expectativa de vida da população. Em ambos os sexos, a taxa de incidência está aumentando. Observa-se entre os homens um aumento principalmente no desenvolvimento de câncer de pulmão, próstata e coloretal. Entre as mulheres, as principais neoplasias malignas com maior incidência são: câncer de mama, pulmão e coloretal (5-10).

Ao longo dos anos, as pesquisas na área oncológica apresentaram avanços consideráveis. O desenvolvimento de novos medicamentos, tecnologias e o melhor entendimento da biologia tumoral favoreceram marcadores para a detecção, aumentando o sucesso do tratamento e melhorando a qualidade de vida dos pacientes (11-13).

A descoberta de anticancerígenos de origem natural tem fornecido vários recursos e subsídios para o desenvolvimento de pesquisas nesta área (14-18). A maioria dos fármacos antineoplásicos (60%) introduzidos na terapêutica moderna são de origem natural (14-21). Os fitoquímicos possuem boas perspectivas como agentes antitumorais por apresentarem grande diversidade química e ilimitada possibilidade nas modificações racionais, oferecendo as melhores possibilidades de encontrar substâncias de interesse terapêutico (14-17). Dentre os produtos naturais com potencial anti-neoplásico que vêm sendo extensivamente estudados, destaca-se a própolis (22-31). Este produto é uma

resina balsâmica produzida por abelhas do gênero *Apis* e utilizada pelas mesmas na proteção das colmeias contra invasão de insetos e microrganismos (32).

A própolis tem conquistado o status de apiterápico por demonstrar amplas ações biológicas como efeitos antimicrobiano, antimicótico, imunomodulador, cicatrizante e antioxidante (23, 33-38). Estudos adicionais têm demonstrado que os efeitos atribuídos a este produto natural dependem da sua composição química, a qual varia de acordo com sua origem geográfica (23, 26, 39-46).

Diante do exposto, este trabalho propõe-se a avaliar o efeito citotóxico de amostras de própolis cubana e brasileira sobre células tumorais.

2.- OBJETIVOS

Objetivo geral

- ✓ Investigar o efeito citotóxico de amostras de própolis cubana e brasileira sobre linhagens celulares de origem epitelial: carcinoma humano de pulmão (A549), carcinoma humano de laringe (HEp-2), carcinoma humano de mama (MDA MB-321) e células renais de macaco verde africano (Vero).

Objetivos específicos

- ✓ Avaliar a ação anti-proliferativa e citotóxica da própolis cubana e brasileira sobre as linhagens celulares mediante os ensaios de MTT (3-(4,5- dimetiltiazol-2-il)-2,5-difeniltetrazólio brometo) e LDH (lactato desidrogenase);
- ✓ Identificar o evento de morte celular nas linhagens tumorais sensíveis mediante microscopia de fluorescência (laranja de acridina/brometo de etídio e diamidinofenilindol (LA/BE, DAPI)) após incubação com as amostras de própolis;
- ✓ Determinar os níveis de expressão dos genes pró/anti-apoptóticos (*TP53*, *CASP3*, *BAX*, *BCL2*, *BCL-XL*, *PUMA*, *NOXA*, *P21*) por RT-PCR nas linhagens tumorais;
- ✓ Determinar o efeito das amostras sobre a permeabilização da mitocôndria mediante alteração do potencial de membrana mitocondrial nas linhagens HEp-2 e MDA MB-231;
- ✓ Avaliar o efeito da possível geração de espécies reativas do oxigênio pelas amostras sobre a viabilidade celular nas linhagens HEp-2 e MDA MB-231;
- ✓ Determinar o efeito sobre a migração celular após incubação das amostras de própolis na linhagem MDA MB-231;
- ✓ Determinar o possível efeito inibidor das amostras de própolis sobre as vias de sinalização PI3K/Akt e ERK1/2 na linhagem MDA MB-231.

3. - REVISÃO DA LITERATURA

3.1.- Câncer: Visão geral

O câncer é uma doença que resulta do crescimento autônomo e desordenado de células que, ao se dividirem, desencadeiam o surgimento de tumores ou neoplasias malignas. Este processo constitui diversos estágios que estão associados aos diferentes eventos moleculares e celulares envolvidos na transformação de uma célula normal em maligna. Desta maneira, por ser uma enfermidade crônica e degenerativa, o crescimento celular desordenado pode espalhar-se para outras regiões do corpo (1-4).

Essa doença tem se apresentado em uma das patologias de maior impacto nos países desenvolvidos e em desenvolvimento, além de estar entre as primeiras causas de morte no mundo. A cada ano, cerca de 12,7 milhões de novos casos de câncer são diagnosticados no mundo; se não houver uma melhora considerável no controle da doença, a previsão é de que esse valor anual alcance 21,3 milhões de novos casos de câncer e 13,1 milhões de mortes até 2030. As estatísticas mostram que, nos países desenvolvidos, cerca de uma a cada cinco pessoas poderia ter algum tipo de câncer. Para a região da América Latina e do Caribe, estima-se que 1,7 milhões de casos de câncer serão diagnosticados até 2030, e que mais de 1 milhão de mortes por câncer ocorrerão anualmente. Nesse contexto, a América Latina corre o risco de enfrentar um aumento substancial no número de mortes por câncer, caso não ocorra uma melhoria no diagnóstico precoce da doença e no acesso a tratamentos pelas populações mais pobres (5-10).

Atualmente, o câncer é a segunda maior causa de morte no Brasil, somando 600 mil casos por ano. O Instituto Nacional de Câncer no Brasil (INCA) prevê que em 2020 a doença ocupe a primeira posição superando as doenças cardíacas. De acordo com estimativas, neste país a incidência de câncer aponta a 300.870 mulheres e 295.200 homens acometidos ao ano, reforçando a magnitude do problema. Os tipos de cânceres mais frequentes são os de pele não melanoma, mama, colorectal, colo do útero e de pulmão para o sexo feminino. Já para o sexo masculino, as neoplasias malignas de maior incidência são: pele não melanoma, próstata, pulmão, colorectal e estômago.

Dentre dos fatores que contribuem para a incidência da doença no Brasil, encontra-se: o envelhecimento da população, assim como a qualidade das informações e da assistência prestada, o excesso de gordura corporal, o tabagismo e outros fatores relacionados. Entretanto, o INCA chama a atenção para o fato de que 60% dos casos de câncer no Brasil são diagnosticados em estágios avançados (47).

Em Cuba, o câncer ocupa a primeira causa de morte no país, seguido pelas doenças cardíacas. Entre os fatores que parecem estar relacionados com a alta tendência de morte por câncer em Cuba está o envelhecimento da população. A taxa de mortalidade por tipos de cânceres corresponde a tumores de traqueia, brônquios e pulmão seguido pelo o câncer colorretal e outros tumores do tecido linfático. Para o sexo masculino os tipos de cânceres mais incidentes são de próstata, colorretal, cabeça e pescoço e do trato urinário. Os cânceres de mama, intestino e colo do útero são os de maior prevalência nas mulheres. Porém, tem-se centrado esforços pelo Programa Nacional de Controle do Câncer Cubano para minimizar este problema de saúde através da prevenção, diagnóstico e tratamento precoce e cuidados paliativos (48).

Infelizmente, muitos tumores ainda apresentam respostas modestas aos protocolos clínicos, limitando a indicação e a eficácia do tratamento adjuvante tanto para os tumores primários quanto para as metástases. Portanto, torna-se imperativa a necessidade de encontrar, desenvolver e introduzir no arsenal médico modalidades terapêuticas mais eficientes que possam oferecer mais oportunidades reais de controle ao número crescente de pacientes com doenças malignas disseminadas. Sendo assim, as pesquisas para encontrar tratamentos e formas de eliminação da doença combinam avanços na área da biotecnologia, farmacologia, toxicologia, química, farmacognosia, entre outras (49-52).

3.1.1.- Câncer de pulmão

O câncer de pulmão transformou-se na doença neoplásica mais comum e mortal em todo o mundo. É, atualmente, a principal causa de morte por câncer entre os homens da América do Norte e da Europa; sua mortalidade vem aumentando significativamente entre as populações da Ásia, América Latina e da África. Essa modificação no comportamento da doença foi observada a partir da década de 1920, quando o número de

casos começou a crescer progressivamente, transformando-se em verdadeira epidemia mundial no início do século XXI (53).

O principal fator implicado na causa do câncer de pulmão é o tabagismo. Alguns estudos epidemiológicos e laboratoriais, assim como estudos *in vitro*, ligaram a presente pandemia de câncer de pulmão com os efeitos carcinogênicos da fumaça do tabaco. O tabagismo é responsável por aproximadamente 85% a 90% de todos os casos de câncer de pulmão (54). Outros riscos podem ser considerados tais como riscos ocupacionais de exposição ambiental (como arsênico, cromo, níquel, radônio, poluição atmosférica, exposição à radiação e exposição passiva à fumaça do tabaco) (53).

Mais de 95% de todos os casos de câncer de pulmão apresentam quatro principais tipos histológicos: espinocelular ou escamoso, adenocarcinoma, carcinoma de células grandes e de células pequenas. Os três primeiros são conhecidos como carcinoma de pulmão de células não-pequenas (CPCNP) devido às suas semelhanças na disseminação, sensibilidade à quimioterapia e a radioterapia quando são comparados com o carcinoma de pulmão de células pequenas (CPCP) (55).

As linhagens celulares derivadas de tumores de pulmão humano têm sido amplamente utilizadas para investigar a maioria dos aspectos da biologia do câncer do pulmão (56, 57). Para caracterizar as linhagens celulares de CPCNP e CPCP, mais de 200 células de câncer do pulmão foram avaliadas. Atualmente, o catálogo da ATCC (*American Type Culture Collection*) lista um total de 173 linhagens de células de câncer de pulmão (58, 59). Dentro delas, encontra-se a linhagem A549 criada em 1976 (60).

A linhagem A549 pertence ao grupo das CPCNP por sua morfologia escamosa. Esta linhagem faz parte de nosso estudo porque tem sido amplamente utilizada como modelo *in vitro* na investigação e descoberta biomédica do câncer de pulmão (58, 60-63).

3.1.2.- Câncer de laringe

O câncer de laringe é um dos tumores malignos de cabeça e pescoço mais frequentes, excluindo carcinomas originários da pele. Representa o 2% dos tumores do corpo, e 25% dos tumores de cabeça e pescoço, dos quais 98% são carcinomas de células escamosas ou epidermóide (geralmente bem diferenciado). Aproximadamente 2/3

desses tumores surgem na corda vocal verdadeira e 1/3 acomete a laringe supraglótica (ou seja, localizam-se acima das cordas vocais) (64).

Esta doença é mais comum em indivíduos com idade entre 50 e 70 anos, no entanto, a incidência tem aumentado nas últimas décadas dentre os indivíduos jovens. A proporção entre homens e mulheres é de 4:1, porém nas mulheres esta patologia está aumentando devido ao aumento do consumo de tabaco e álcool pelo sexo feminino nas últimas décadas (64-66).

A incidência de câncer de laringe varia no mundo e os países industrializados são os mais afetados. A cada ano cerca de 12.000 novos casos de câncer de laringe são diagnosticados nos Estados Unidos da América (EUA), com cerca de 4.000 mortes ao ano. Espanha apresenta uma incidência de 25 casos a cada 100.000 habitantes, e França, Uruguai, Itália, Cuba, Argentina, Brasil, Colômbia e Grécia se apresentam como os países com maior mortalidade por câncer de laringe (64).

O tratamento do câncer de laringe pode causar problemas na dentição, fala e deglutição. Quanto mais precoce for o diagnóstico, melhores são as possibilidades de que o tratamento possa evitar deformidades físicas e problemas psicossociais. A associação da quimio e radioterapia é utilizada em protocolos de preservação de órgãos, os quais são desenvolvidos para tumores em estágio mais avançados. Os resultados na preservação da laringe têm sido positivos. Da mesma forma, novas técnicas cirúrgicas e pesquisas de novos fármacos foram desenvolvidas permitindo a preservação da função da laringe, mesmo em tumores moderadamente avançados (65-69).

Nas pesquisas para o tratamento do câncer de laringe, vários estudos *in vitro* utilizam um painel de linhagens celulares. Entre as linhagens descritas e derivadas de tumores primários de células escamosas encontram-se: SNU-46, SNU-585, SNU-899, SNU-1066, SNU-1076, y SNU- 1214 (70). A linhagem HEP-2, utilizada em várias pesquisas (68, 69, 71, 72) e também em nosso estudo, tem sido descrita como derivada de carcinoma epitelial da laringe.

3.1.3.- Câncer de mama

O câncer de mama contempla uma das maiores causas de morbimortalidade em todo o mundo. Esta neoplasia maligna apresenta-se como a mais incidente e mais mortal

entre mulheres. Fatores ambientais, estilos de vida e status socioeconômico têm relevante importância no desenvolvimento do câncer de mama. Populações com maior nível socioeconômico, países do ocidente e mulheres residentes em áreas urbanas apresentam maiores taxas de incidência e mortalidade (6, 73-77).

Grande parte desses tumores se inicia no epitélio de revestimento dos ductos ou dos lóbulos, glândulas individualizadas separadas por tecido conjuntivo denso e constituídas por inúmeros ductos. Por sua vez, o tumor de mama invasivo espalha-se no tecido adjacente e o grau de comprometimento está relacionado ao estágio da doença. Do ponto de vista clínico, podem ser identificadas diversas subclasses de câncer de mama com diferenças nas características histopatológicas, no padrão de organização celular e na expressão de receptores hormonais (73, 74, 77-79). Esses fenótipos tumorais estão associados a fatores genéticos e epigenéticos e há evidências de que também podem ser determinados pelo tipo celular do qual o tumor deriva (79).

Apesar de ser considerado um câncer de bom prognóstico, a mortalidade por câncer de mama continua elevada e a sobrevida após cinco anos se mantém reduzida. Nas últimas décadas diversos esforços vêm sendo realizados a fim de identificar os determinantes do câncer de mama, assim como os fatores protetores, e a abordagem terapêutica adequada (80, 81).

Um problema frequente no tratamento do câncer de mama é que, embora muitas terapias antineoplásicas exerçam importante papel no controle do aumento da massa tumoral, na maioria dos casos este efeito não é muito duradouro (80, 81). Portanto, o direcionamento de uma terapia alvo contra os fatores que estão envolvidos nos processos de proliferação, angiogênese, invasão e metástase, se faz necessário na tentativa de melhorar a eficácia do tratamento.

Uma parte considerável das pesquisas para o tratamento do câncer de mama baseia-se em estudos *in vivo* e *in vitro* realizados com linhagens celulares oriundas do câncer de mama (CCM). As linhagens CCM atualmente utilizadas foram estabelecidas no final de 1970 e a maioria delas foram obtidas a partir de tumores metastáticos. Entre as linhagens CCM descritas encontram-se: BT-474, SK-BR-3, Hs578T, MDA-MB-231, MCF-7, T-47D, sendo as três últimas as mais utilizadas (82).

A linhagem MDA-MB-231 é alvo de nossa pesquisa porque se destaca de forma relevante na literatura e têm contribuído grandemente com a investigação e desenvolvimento de medicamentos para o tratamento do câncer de mama triple negativo (83-87). Estas células caracterizam-se pela ausência de receptores de estrógeno (ER-), de progesterona (PR-) e para o fator de crescimento epidérmico humano tipo 2 (HER2-) (78, 82, 88).

3.2- Ação citotóxica sobre linhagens tumorais

As estratégias para a descoberta de novos fármacos têm mudado ao longo dos anos. Os programas de prospecção no *National Cancer Institute* (NCI) dos EUA começaram no início dos anos 1950 e consistiam em testes de novos compostos em modelos experimentais de leucemias murinas (L1210 e P388). Este modelo foi bastante questionado, uma vez que não era considerado representativo dos tumores humanos, em sua maioria sólidos (89). Sendo assim, o modelo foi reconsiderado e, com o aprimoramento da metodologia de cultivo de células, foi possível o desenvolvimento de diversas linhagens celulares oriundas de tumores humanos. Estas linhagens celulares possibilitaram o desenvolvimento da metodologia para triagem *in vitro*. Com esse objetivo, o NCI desenvolveu um painel de células cancerígenas que atualmente conta com 60 linhagens oriundas de oito tipos de tumores sólidos (pulmão, melanoma, mama, rim, cólon, próstata, ovário e cérebro) e do sistema hematopoiético (leucemia). Desta maneira, a realização de estudos de prospecção inclui uma etapa inicial de testes *in vitro* em diversos tipos de células neoplásicas e possibilita a descoberta de moléculas e compostos promissores para o tratamento do câncer com maior especificidade (15, 89-92).

A seleção subsequente destes compostos e moléculas é feita com base em seu efeito citotóxico ou à sua capacidade de induzir alterações imunológicas contra o tumor. Portanto a administração em pacientes com câncer é realizada quando uns dos compostos ou molécula tem uma atividade pré-clínica *in vitro* e *in vivo* demonstrada e uma toxicidade aceitável ou reversível (93, 94).

Neste contexto os testes *in vitro* têm um amplo uso para o estudo da atividade citotóxica de produtos de origem naturais e/ou de síntese dos mesmos (90, 91). Assim, o sistema permite a análise de impacto de diferentes produtos em apenas quatro dias, além

do estudo dos mecanismos moleculares envolvidos na disfunção celular. Simultaneamente possibilita a seleção de drogas “antitumorais”, reduzindo o uso de animais nos experimentos, além de permitir o estudo de seu potencial mutagênico em células de mamíferos (90).

3.2.1 - Testes indicados na avaliação citotóxica

Nos primeiros testes de avaliação da inibição do crescimento celular por fármacos, foram testadas linhagens de células tumorais durante um período de 2 a 5 horas. O número de células foi medido utilizando um hemacitômetro ou um contador de células eletrônico. O progresso nesta metodologia surgiu com a produção comercial de placas multipoços de plástico que permitem a adesão celular e o crescimento das mesmas, além da utilização de leitores de placas (92).

Os testes para a detecção da citotoxicidade ou viabilidade celular após a exposição a certos compostos ou fármacos são capazes de detectar por vários mecanismos celulares conhecidos, os efeitos adversos de interferência nas estruturas ou propriedades essenciais para a sobrevivência e proliferação da célula (95). Vários autores têm desenvolvido diferentes ensaios *in vitro* para prever os efeitos tóxicos de drogas e produtos químicos. Atualmente, estes testes foram divididos em três grupos:

- a) Testes primários utilizados para indicar alterações na viabilidade das células pela ação de um agente tóxico. Dentro deste grupo de ensaios encontram-se: a coloração com azul de tripano, vermelho neutro (95, 96) e a determinação da atividade da lactato desidrogenase (LDH). Esta enzima está normalmente presente no citoplasma de células vivas, sendo liberada para o meio extracelular quando a membrana celular é afetada por um agente tóxico (95).
- b) Grupo de testes que permitem conhecer os efeitos tóxicos de um composto ou fármaco sobre o crescimento e proliferação das células. Estes testes são geralmente mais sensíveis às baixas concentrações de fármaco do que os ensaios que determinam a viabilidade celular. Dentro deste grupo de ensaios encontram-se: a utilização de timidina tritiada (3H) e análise do ciclo celular (97).
- c) Grupo de testes que indicam se o composto ou fármaco afeta os processos metabólicos celulares. Estes testes podem determinar o efeito de qualquer tóxico

envolvido em processos bioquímicos ou de danos da membrana celular ou organela, e também podem ser utilizados em conjunto com os testes primários, a fim de determinar a concentração citotóxica e tempo de susceptibilidade. Este grupo inclui: a coloração com alamar azul, a utilização de fluoróforos, e o ensaio de redução de brometo de 3- (4,5-dimetil-2-tiazolil) -2,5-difeniltetrazólio (MTT). Este último é determinado pela formação de um composto colorido (formazan). A reação ocorre na mitocôndria pela enzima succinato desidrogenase das células vivas. A capacidade das células em reduzir o MTT é um indicador da integridade mitocondrial e a sua atividade funcional é interpretada como uma medida da viabilidade celular (95, 97-99). Este ensaio tem sido usado pelo NCI para estabelecer um painel de 60 linhagens tumorais atualmente utilizadas na pesquisa de fármacos antitumorais (98, 100).

Todos estes ensaios são utilizados para determinar a concentração máxima não tóxica (CMNT) de um determinado fármaco, que será a concentração mais alta do composto, e compatível com a viabilidade e sobrevivência celular. Neste contexto, para expressar a citotoxicidade de um composto conhecido podem ser usados os índices de citotoxicidade IC10 e IC50, os quais se referem às concentrações que causam a morte das células em 10% e 50% (96, 101).

Além dos ensaios mencionados anteriormente, atualmente novos métodos tais como a determinação da síntese de ácido desoxirribonucleico (DNA), o funcionamento das organelas, a integridade da membrana celular ou a síntese proteica são utilizados para identificar compostos citotóxicos. Os resultados destes ensaios podem ser considerados como critério de viabilidade celular (102, 103).

A avaliação da indução da morte celular por apoptose ou necrose também é considerada um sinal de citotoxicidade e uma ferramenta na procura de medicamentos para o tratamento do câncer. A partir dos métodos utilizados são obtidas informações úteis para o desenvolvimento de novas terapias. Assim, esses estudos beneficiam a identificação de novos alvos moleculares, em sua maioria proteínas cuja expressão apresenta-se alterada em células tumorais em relação a células de tecidos saudáveis (102, 103).

Vários autores apontaram que a determinação dos métodos apropriados para avaliar a morte celular por apoptose ou necrose em linhagens celulares depende principalmente do tipo de células, das possíveis características de indução, das restrições técnicas, entre outras condições (104).

A apoptose pode ser reconhecida por características morfológicas muito marcantes e coordenadas. De um modo geral, a apoptose é um fenômeno bastante rápido, originando-se a partir de uma retração da célula que causa a perda da aderência com a matriz extracelular e células vizinhas. As organelas celulares mantêm a sua morfologia, com exceção, em alguns casos das mitocôndrias, que podem apresentar ruptura da membrana externa. A cromatina sofre condensação e se concentra junto à carioteca, que se mantém intacta. A seguir, a membrana celular forma prolongamentos (blebs) e o núcleo se desintegra em fragmentos envoltos pela membrana nuclear. Os prolongamentos da membrana celular aumentam de número e tamanho e se rompem, originando estruturas compostas de conteúdo celular. Estas porções celulares envoltas pela membrana celular são denominadas corpos apoptóticos (105, 106).

A ativação da apoptose pode ser iniciada pela via extrínseca (citoplasmática) ou pela via intrínseca (mitocondrial). A via extrínseca é desencadeada pela ligação de ligantes específicos a um grupo de receptores de membrana da superfamília dos receptores de fatores de necrose tumoral (rTNF). Esta ligação é capaz de ativar uma cascata de proteínas adaptadoras, que culminará na ativação das caspases. A via intrínseca é ativada por estresse intracelular ou extracelular como danos no DNA, hipóxia ou ativação de oncogenes. Os sinais que são transduzidos em resposta a estes insultos convergem principalmente para a mitocôndria. Essa organela integra os estímulos de morte celular, induzindo a permeabilização mitocondrial e conseqüente liberação de moléculas pró-apoptóticas nela presentes (106-109).

Os métodos de detecção da apoptose incluem alterações citomorfológicas (105); fragmentação internucleossômica do DNA, a qual possui um padrão característico que pode ser detectado por extração do DNA e separação por eletroforese em gel de agarose (110). A utilização de técnicas fluorimétricas também pode detectar este evento, um exemplo são os marcadores de DNA, tais como: diamidinofenilindol (DAPI), Tunel, a combinação dos corantes laranja de acridina (LA) e brometo de etídio (BE) e a dupla

coloração com anexina V-FITC e PI (iodeto de propídio). Outros testes envolvem a detecção de alterações na membrana, atividade das caspases, fragmentação do substrato e detecção de reguladores e inibidores apoptóticos. Além destes, estão a detecção do potencial da membrana mitocondrial ($\Delta\psi$) (105, 111), a realização de uma eletroforese alcalina em células individuais (teste do cometa) (112) e a análise genética do fenômeno apoptótico através da expressão simultânea de genes pro/anti-apoptóticos envolvidos na inibição da proliferação de celular, o que é conseguido através do uso de *microarrays*, reação em cadeia da polimerase (PCR) e Western blot, etc. (111).

A demonstração de que a apoptose é um mecanismo inato de defesa antineoplásica e que vários agentes quimioterápicos agem através da indução desse tipo de morte celular (59, 113-116), levou a uma intensa investigação dos mecanismos moleculares da apoptose e sua aplicação no tratamento do câncer. Existem evidências claras que mostram que o mecanismo pelo qual a apoptose é regulada fornece vias de detecção *in vitro* que são distinguíveis da morte celular por necrose (106, 117).

A necrose é um tipo de morte na qual as células sofrem um insulto resultando no aumento do volume celular, agregação da cromatina, desorganização do citoplasma, perda da integridade da membrana plasmática e consequente ruptura celular. Durante a necrose, o conteúdo celular é liberado causando danos às células vizinhas e uma reação inflamatória local (118). A necrose é considerada uma resposta passiva à injúria celular. Entretanto, estudos recentes sugerem que a necrose também pode ser regulada geneticamente (106, 119, 120).

Entre os testes que avaliam o mecanismo de morte celular por necrose, encontram-se a combinação dos corantes LA/BE e anexina V-FITC/PI. O emprego de marcadores moleculares como as proteínas que interagem com o receptor de morte (sigla em inglês: RIP) ou fator 2 associado ao receptor de TNF (121, 122), a avaliação da ativação da poli-ADP-ribose polimerase, a qual depleta o cofator da glicólise NAD^+ (123), a determinação do aumento do conteúdo intracelular de cálcio e de espécies reativas de oxigênio que promovem a ativação de calpaínas, proteases citoplasmáticas e de fosfolipase A2 que levam à permeabilização da célula e à morte por necrose (124).

3.3.- Produtos naturais e câncer

Os produtos naturais são considerados importantes fontes na descoberta de novas drogas (125, 126). Estima-se que compostos naturais e seus derivados contribuíram para produção de cerca de um terço dos medicamentos presentes atualmente no mercado (127).

Muitos estudos têm sido dirigidos à identificação de produtos naturais com propriedades terapêuticas no tratamento do câncer (49-51, 128, 129). Cerca de 50% das drogas anticâncer aprovadas nos EUA desde 1960 tiveram origem a partir de fontes naturais, especialmente de plantas. No período de 1996-2000 aproximadamente 400 compostos foram isolados a partir de plantas, organismos marinhos e de microrganismos com base em estudos de citotoxicidade em células tumorais (130).

Nas últimas décadas houve um avanço no campo da oncologia propiciando a descoberta de diversas substâncias bioativas utilizadas atualmente na terapêutica antineoplásica. A maioria dos fármacos utilizados no tratamento do câncer atualmente tem sua origem a partir de fontes naturais ou são sintetizados e obtidos industrialmente a partir de princípios ativos purificados de fontes naturais (49-51, 128, 129). Neste contexto, os produtos naturais desempenham um papel importante como efetivos agentes anticâncer por serem fonte inesgotável de novos medicamentos antineoplásicos (14, 16, 129, 131). Atribui-se que a atividade anticâncer de tais agentes seja decorrente da presença de diversas substâncias químicas tais como, lignanos, flavonoides (132, 133), alcaloides (134, 135), cumarinas (136), triterpenoides (137), etc., os quais aparentam retardar e inibir o processo carcinogénico (138).

Vários autores observaram que os produtos elaborados pelas abelhas e os compostos fenólicos neles contidos são candidatos promissores por apresentarem efeito imunomodulador (36, 139-145), radioprotetor (33, 34, 144, 146, 147) e antitumoral (36, 148-150), contribuindo na expansão do arsenal existente de drogas eficazes contra o câncer (144).

3.3.1.- Própolis: visão geral

A palavra própolis é derivada do grego (*pró-*, em defesa, e *polis-*, cidade ou comunidade) tendo seu significado relacionado à defesa da colmeia. Este produto natural é uma mistura complexa formada por material resinoso e balsâmico coletado pelas abelhas do gênero *Apis* de ramos, flores, pólen, brotos e exsudatos de árvores. Além destes, na colmeia, as abelhas adicionam secreções salivares e enzimas (26, 151-153). De modo geral, a própolis contém 50-60% de resinas e bálsamos, 30-40% de ceras, 5-10% de óleos essenciais, 5% de grão de pólen, além de microelementos como alumínio, cálcio, estrôncio, ferro, cobre, manganês e pequenas quantidades de vitaminas B1, B2, B6, C e E (26, 154).

As abelhas utilizam a própolis para a assepsia dos locais destinados à manutenção da rainha, para vedação de frestas e para a mumificação de insetos invasores na colmeia. (155). Esse produto apiterápico pode ser produzido pelas abelhas ao longo do ano, e sua produção pode variar de acordo com as condições climáticas e material vegetal disponível na área próxima a colmeia (156).

Geralmente, a própolis é coletada antes do início do inverno em regiões temperadas e em climas tropicais, e também no início da estação chuvosa porque é considerado o momento mais ativo da propolização (153). A produção anual da própolis é de 10-300 g/colmeia, no entanto, isso varia de acordo com a variedade de abelhas, clima, flora e dos dispositivos de coleta utilizados pelo homem. O consumo de própolis no mundo é estimado entre 700-800 toneladas / ano (153, 157, 158).

- **Usos tradicionais e médicos**

O homem tem usado a própolis ao longo dos anos de forma empírica, a fim de aliviar a dor e curar doenças. A sua utilização para combater doenças provocadas por fungos e bactérias data de 1700 (a.C.). É conhecido que, no papiro de Eber, o primeiro livro médico intitulado "Livro de preparação de medicamentos para todas as partes do corpo humano", são descritos os primeiros usos da própolis na medicina (159, 160).

As civilizações antigas, como a egípcia e a grega já tinham conhecimento das propriedades curativas da própolis. Nas múmias do Egito foram encontrados resíduos de

própolis usados para conservar os cadáveres. Outras civilizações, como os incas, também fizeram uso da própolis para curar a febre.

Na Europa e Norte de África a própolis tem sido usada para curar feridas, para o tratamento de cáries, infecções da boca e da garganta. Em outras regiões muitas vezes foi usado conjuntamente com o mel para curar diversas doenças de diferente etiologia como lesões ulcerosas, lepra e gangrena (153, 159-161).

Devido ao crescente interesse de dezenas de pesquisadores em compreender melhor a utilidade da própolis, o estudo dos mesmos tem se expandido. Em diferentes partes do mundo têm sido realizados estudos que demonstram sua aplicação na medicina. Além disso, o progresso nas técnicas de análise também permitiu conhecer sua composição e assim poder estudar sua atividade biológica (153, 162-164).

Atualmente a própolis é considerada um produto de extraordinário interesse para a indústria médica e farmacêutica devido ao seu efeito antioxidante, anti-inflamatório, imunestimulante, hepatoprotetor, carcinoestático, antimicrobiano, antiviral, antifúngico, antiprotozoário, anestésico e de regeneração tecidual (29, 153, 165-172).

- **Origem botânica e composição química**

Graças ao conteúdo de óleos essenciais, as amostras de própolis costumam ser aromáticas, e de acordo com sua origem botânica e tempo de coleta, distinguem-se pela sua cor (de amarelo claro ao marrom escuro), sabor (amargo, levemente picante ou suave) e consistência (151, 153, 163).

A classificação de própolis também pode ser de acordo com sua origem geográfica. Em zonas temperadas a maioria das amostras de própolis advêm dos exudatos de árvores pertencentes ao gênero *Populus spp.* Entretanto, no norte da Rússia derivam dos botões dos vidoeiros (*Betulaverrucosa*) e das espécies do gênero *P. tremula* (165, 173, 174). Nas regiões mediterrânicas, provêm dos botões e folhas do gênero *Cistus spp* (175). Nos países latino-americanos como o Brasil, provêm das folhas das espécies *Baccharis dracunculifolia* (151-153, 156-158, 162, 163, 165, 173, 174). No entanto, na Venezuela e Cuba derivam geralmente da resina floral do gênero *Clusia* (176).

Os principais compostos químicos descritos da própolis são os flavonóides, ácidos fenólicos e ésteres. No entanto, os métodos de análise atualmente disponíveis permitem

detectar um número crescente de compostos nas amostras, verificando que a variabilidade na composição das mesmas é muito alta (153, 177).

Alguns componentes estão presentes em todas as amostras de própolis, enquanto outros ocorrem somente em própolis derivadas de espécies particulares de plantas. Por exemplo, na própolis europeia o conteúdo total de fenóis responsável pela maioria das suas propriedades farmacológicas representa mais da metade dos 160 compostos identificados neles (165, 178). Em algumas amostras da Suíça e Itália a composição da própolis carece de compostos fenólicos. No caso de amostras de própolis turcas (179), egípcias (180-182), brasileiras (176, 177, 183), do Chile (184, 185) e de Cuba (176) há uma composição variada, e em alguns casos, as amostras contêm compostos característicos, os quais são úteis para identificar a sua origem. Em geral, a composição da própolis é muito complexa e variada dependendo da diversidade fitogeográfica da zona de colheita (186).

3.3.2.- Própolis brasileira

No Brasil, o interesse pela própolis aconteceu somente na década de 1980 com um estudo pioneiro de Ernesto Ulrich Breyer, demonstrando em seu livro, “Abelhas e saúde”, as propriedades terapêuticas da própolis e sua utilização como antibiótico natural (187). Comercialmente, a própolis brasileira tem ocupado lugar de destaque no mercado nacional e internacional de produtos apícolas. Tal inserção deve-se essencialmente à constatação das diferentes atividades biológicas atribuídas aos constituintes químicos desse produto (188). O Brasil é um dos principais produtores mundiais de própolis, com uma produção anual estimada em torno de 50 a 150 toneladas, sendo que aproximadamente 75% desse total é exportado, especialmente para o Japão (97% das exportações) (189).

No Brasil, uma enorme quantidade de marcas e produtos feitos à base de própolis encontra-se disponível em lojas de produtos naturais, tais como balas, chocolates, doces, chá, protetor solar, gel pós-barba, xampus, cremes para pele, soluções antissépticas, creme dental, sabonetes, batom, cápsulas, extratos, spray bucal, pastilhas, xaropes, comprimidos, etc. (190).

Dadas às dimensões continentais do Brasil e a grande diversidade da flora e variedade de ecossistemas, sua composição química é extremamente rica e complexa, sendo vários os tipos de própolis produzidos no país (191). A própolis pode ser produzida em todo o Brasil, porém a qualidade varia de uma região para outra em função de fatores ambientais. As amostras de própolis coletadas em todas as regiões do Brasil (exceto região Norte) foram classificadas em 12 grupos de acordo com a aparência e coloração dos extratos (Tabela 1) (192). Posteriormente, foi encontrada uma nova própolis em colmeias localizadas ao longo do litoral e manguezais no nordeste brasileiro, classificada como própolis do grupo 13, denominada própolis vermelha (193, 194).

A própolis mais conhecida e estudada no Brasil é a própolis verde, produzida a partir de uma planta conhecida popularmente por alecrim do campo (*Baccharis dracunculifolia*). A própolis verde é composta fundamentalmente por compostos fenólicos, como a artepillina C e o ácido hidroxicinâmico (195). A fonte natural da própolis verde é uma espécie não adaptada às condições naturais existentes em outros países (196), o que confere a este tipo de própolis características químicas e biológicas diferenciadas.

Neste contexto, destaca-se a própolis verde produzida em Botucatu, alvo de nosso estudo e composta por ácido benzóico, ácido di-hidroxicinâmico, ácido cumárico, prenil-p-cumárico, artepillin C, di-hidroxi flavonol, di-hidroxi metoxi flavonol, e triterpenos (197).

Tabela 1 Agrupamento da própolis brasileira quanto à cor. Local de coleta: RS- Rio Grande do Sul; PR- Paraná; BA- Bahia; PE-Pernambuco; CE- Ceará; PI- Piauí; SP- São Paulo) (192).

Grupos	Cor	Origem da própolis
Grupo 1 (RS)	Amarelo	Região Sul
Grupo 2 (RS)	Castanho Claro	Região Sul
Grupo 3 (PR)	Castanho Escuro	Região Sul
Grupo 4 (PR)	Castanho Claro	Região Sul
Grupo 5 (PR)	Marrom esverdeado	Região Sul
Grupo 6 (BA)	Marrom avermelhado	Região Nordeste
Grupo 7 (BA)	Marrom esverdeado	Região Nordeste
Grupo 8 (PE)	Castanho Escuro	Região Nordeste
Grupo 9 (PE)	Amarelo	Região Nordeste
Grupo 10 (CE)	Amarelo Escuro	Região Nordeste
Grupo 11 (PI)	Amarelo	Região Nordeste
Grupo 12 (SP)	Verde ou marrom esverdeado	Região Sudeste

3.3.3.- Própolis cubana

Em 1979, foram realizados os primeiros estudos sobre as propriedades da própolis cubana. Vários estudos analíticos demonstraram a existência de diferenças na composição das amostras, devido à diversidade que apresenta a flora cubana (159). Os veterinários foram os primeiros a demonstrar as propriedades terapêuticas das mesmas (198).

Os extratos de própolis utilizados na medicina popular cubana ficaram conhecidos como “bálsamos milagrosos” (199). Nas últimas décadas a própolis cubana vem sendo utilizada por suas propriedades medicinais constatadas. Dentro do amplo espectro de atividades biológicas destacadas encontram-se: sua ação antipsoriática, anti-inflamatória, analgésica, antiparasitária, antioxidante (200-202) e para o tratamento da estomatite aftosa (203).

As propriedades farmacológicas da própolis cubana estão associadas com a composição química complexa e variada, a qual está intimamente relacionada com a ecologia da flora de cada região visitada pelas abelhas. Levando em consideração as

diferenças significantes na composição química e cor, as amostras de própolis cubana foram classificadas em marrom, amarela e vermelha (176, 204-206). A mais estudada é a própolis de cor marrom (176). Esta variedade tem sua origem botânica da *Clusiarosea* (Copey) pertencente à família *Guttiferae* (*Clusiaceae*) e apresenta em sua composição química benzofenonas poli-isopreniladas tais como: xanthochimol, guttiferona E, propanolona A, pluketione A e nemorosona (176, 207). Além de outros compostos como mucronulatol, escrobiculatona A e escrobiculatona B (208).

A própolis amarela tem sido menos estudada em termos de sua fonte vegetal. Os constituintes químicos de maior destaque que apresentam os extratos desta amostra são os compostos alifáticos, tais como alcoóis triterpênicos e acetatos (209).

Nos últimos anos a própolis vermelha, alvo de nosso estudo, ganhou popularidade por suas propriedades biológicas, tais como: hepatoprotectora, antiparasitas, antimicrobiana e imunomoduladora (37, 171, 210). Devido a cor da resina, a fonte vegetal identificada desta amostra é a espécie *Dalbergia ecastaphyllum* da família *Leguminosae* (211).

Estudos realizados em Cuba determinaram a presença de flavonóides e isoflavonoides como principais metabólitos desta amostra de própolis. Compostos tais como: isoflavonas, isoflavanos, Pterocarpanos, o (3S) -vestitol, o (6a, 11aS) -medicarpina, os (3S) -neovestitol e (3S) -isosativan foram isolados. É interessante que os últimos quatro compostos anteriormente mencionados, embora representem os principais metabolitos, não são responsáveis pela pigmentação vermelha destas amostras de própolis, que é devida realmente ao composto retusapurpurina A (211).

3.3.4.- Atividade antitumoral da própolis

A atividade antitumoral da própolis, incluindo a sua citotoxicidade, foi publicada antes de 1995 e melhor documentada em 1998 por Burdock (162). Nos últimos anos, vários estudos têm demonstrado a atividade citostática (212, 213), anticancerígena e antitumoral da própolis em modelos *in vivo* e *in vitro* (144, 146). Estes efeitos são principalmente atribuídos à presença de diferentes componentes presentes nas amostras avaliadas, tais como o ácido cafeíco (CA), cafeato de fenilo (CAPE), artepilina C, quercetina, naringenina, resveratrol, galangina, genisteína, pluketiona A, entre outros (139, 141, 214-216).

Estudos têm evidenciado que o tratamento de células tumorais com flavonóides isolados da própolis inibe a proliferação celular através da indução da citotoxicidade e apoptose (214, 217). Um exemplo baseado nisso são experimentos *in vitro* realizados com a própolis coreana, a qual demonstrou ser capaz de induzir apoptose em células de hepatocarcinoma humano (SNU449) (168). A própolis chinesa também mostrou-se citotóxica sobre 4 linhagens de carcinoma de colón (CaCo2, HCT116, HT29 y SW480), a partir do aumento dos níveis de expressão genes p53 e p21 (218).

Em outros experimentos conduzidos por Najafi e colaboradores, extratos aquosos de própolis inibiram o crescimento e a proliferação de diferentes linhagens neoplásicas (McCoy, HeLa, SP20, HEp-2 e BHK21) sem afetar o crescimento de células humanas normais (219). Própolis proveniente da Tunísia também apresentou efeito citotóxico frente a diferentes linhagens de células neoplásicas (HEp-2, HT-29, A549, Raw 264.7 e Vero), comprovando seu potencial inibidor sobre o crescimento de todas as linhagens testadas (38). No caso de quatro tipos de própolis da Turquia avaliadas sobre carcinoma da mama (MCF-7), houve variabilidade na ação antiproliferativa devido à diversidade na composição dos mesmos (220).

A própolis do Brasil e de Cuba também têm sido objetos de pesquisa para o tratamento do câncer. Experimentos desenvolvidos por Banskota e outros colaboradores avaliaram a citotoxicidade de nove amostras de própolis coletadas em diferentes regiões, as quais demonstraram propriedades anti-metastásicas sobre as linhagens de carcinoma murino de cólon 26-L5 e HT-1080 fibrossarcoma humano (165). A própolis cubana, dependendo da sua classificação, também apresentou efeito citotóxico para uma variedade de linhagens tumorais (221-223).

Considerando todos esses aspectos, verifica-se o potencial dos produtos naturais na indústria farmacêutica, sendo evidente o avanço das pesquisas sobre própolis para quimioprevenção ou tratamento do câncer (224).

- **Possíveis mecanismos antitumorais da própolis**

A apoptose é um dos mecanismos propostos para os efeitos terapêuticos da própolis (22, 225). Estudos *in vitro* demonstram a sensibilidade das células tumorais aos extratos de própolis. Neste contexto (46, 172, 226, 227), o mecanismo de apoptose

induzida pela própolis depende dos componentes, da concentração do extrato da própolis e parece ser independente do tipo de células tumorais estudadas (226).

A literatura indica que a própolis induz apoptose em células tumorais pela liberação do citocromo c da mitocôndria para o citosol, através da cascata de caspase e ligante indutor de apoptose relacionado ao fator de necrose tumoral (TRAIL) (Figura 1) (226). Apesar dos resultados sobre a atividade pró-apoptótica da própolis, também há evidências de que algumas amostras de própolis vermelha cubana induzam necrose em linhagem tumoral de carcinoma de pulmão A549 (228).

Outros estudos demonstram que a própolis pode inibir o ciclo celular pela supressão da ciclina A, ciclina B, expressão de CDK2, aumento do nível de expressão de p21, p27 e inibição do efeito de hTERT (*human telomerase reverse transcriptase*) nas células tumorais. Além destes, a própolis também pode suprimir a proliferação de células cancerosas através de dois mecanismos: parada do ciclo celular na fase G2 e diminuição da atividade da telomerase (Figura 2) (226).

Em geral, os efeitos citotóxicos e antitumorais da própolis e de seus compostos estão relacionados principalmente com os processos de apoptose, parada do ciclo celular e interferência sobre vias metabólicas de células neoplásicas e pré-neoplásicas. O bloqueio específico das vias de sinalização oncogênicas, efeitos antiangiogênicos e modulação do microambiente tumoral, sugerem a utilização da própolis como possível adjuvante no tratamento complementar com outras terapias antitumorais (226).

Todos estes aspectos incitam a ideia de que mais estudos são necessários para definir com precisão o mecanismo de ação da própolis, para assim compreender exatamente sua influência sobre as vias que desencadeiam a morte celular.

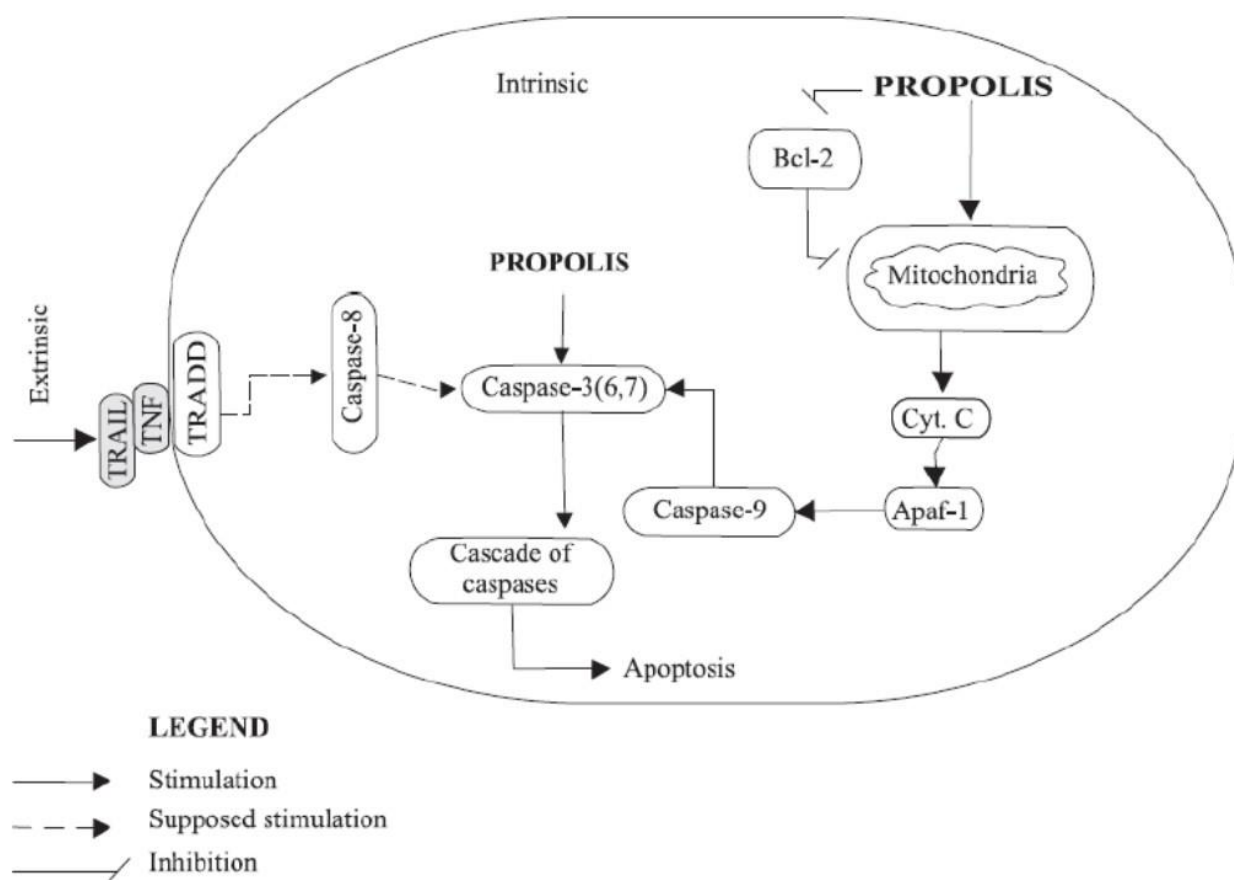


Figura 1: Efeito da própolis nas vias apoptóticas de células de tumorais. Bcl-2 - linfoma de células B 2; Cit. C - citocromo C; Apaf-1 - fator ativador protease apoptótica 1; TNF - fator de necrose tumoral; TRAIL - TNF ligante indutor de apoptose relacionado ao TNF; TRADD - proteína associada ao domínio de morte (226).

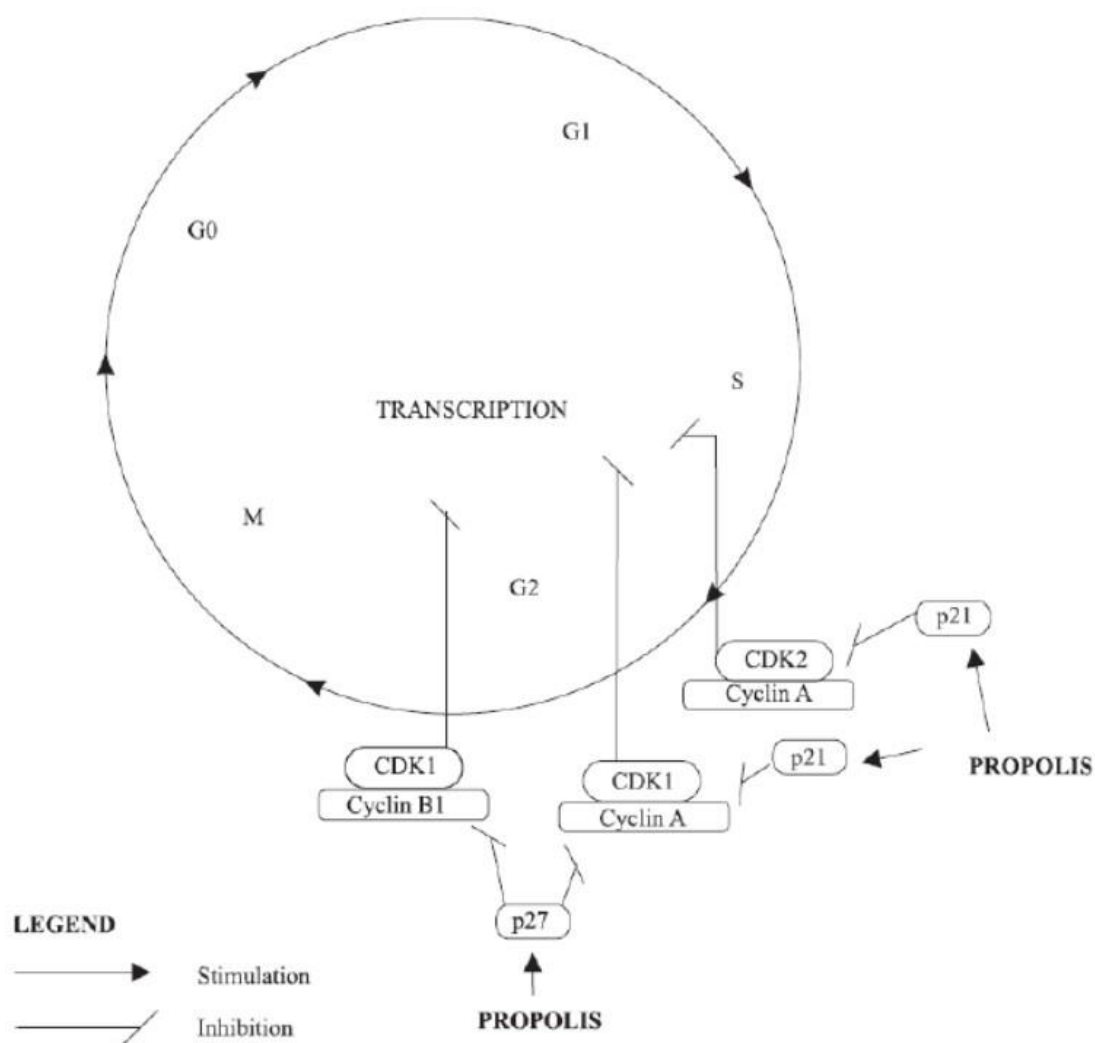


Figura 2: Efeito da própolis sobre as células cancerosas. Fase G1 (Gap1) - crescimento da célula; fase S (síntese) - replicação do DNA; fase G2 (GAP2) - crescimento celular; fase M (mitose) - divisão celular; fase G0 (Gap0) - final da divisão celular, CDK - cinase dependente de ciclina (226).

No intuito de aprofundar o conhecimento sobre a ação citotóxica da própolis sobre células tumorais, investigamos amostras provenientes de Cuba e do Brasil in vitro. Esta tese encontra-se apresentada sobre a forma de três artigos a serem publicados.

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5.- ARTIGOS CIENTÍFICOS

5.1.- Brazilian propolis induced apoptosis in human lung cancer A549 cells through mitochondrial-mediated pathway

Yahima Frión-Herrera, Alexis Díaz-García, Jenny Ruiz-Fuentes, Hermis Rodríguez-Sánchez and José Maurício Sforcin. Journal of Pharmacy and Pharmacology. 2015 Oct; 67(10):1448-56 /doi: 10.1111/jphp.12449

Objetivo

Visto que a própolis cubana foi previamente avaliada na linhagem de adenocarcinoma de pulmão humano (A549), induzindo necrose na mesma (228), o objetivo deste artigo foi: Avaliar o efeito da própolis brasileira sobre a proliferação, morte celular e mecanismo molecular envolvido.

Materiais e Métodos

- Incubação das células com a amostra da própolis (6,25-100 µg/ml) durante 72 h
- Determinação da viabilidade e citotoxicidade celular (MTT e LDH)
- Identificação do evento de morte celular por coloração fluorescente
- Determinação do potencial de membrana mitocondrial
- Determinação da expressão de genes pró e anti-apoptóticos

Resultado e conclusões

A própolis brasileira exibiu efeito anti-proliferativo e citotóxico de modo concentração dependente. O evento apoptótico foi observado nas células tratadas, concluindo-se que houve interferência sobre o potencial de membrana mitocondrial, aumentando a expressão dos genes *BAX*, *NOXA* e *P21* e reduzindo a expressão de *BCL-XL*. A própolis não apresentou ação sobre os genes *TP53*, *CASP3* e *PUMA*. Estes resultados sugerem que a

própolis brasileira induz a apoptose através da via mitocondrial independente de *CASP3* e de *TP53*, assim como a parada do ciclo celular pela expressão de *P21*.

Brazilian green propolis induced apoptosis in human lung cancer A549 cells through mitochondrial-mediated pathway

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Keywords

apoptosis; lung cancer; mitochondria pathway; propolis

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Abstract

Objectives Propolis effect on the growth and apoptosis of human lung adenocarcinoma (A549 cells) was investigated as well as its mechanisms.

Methods Cells were incubated with propolis for 72 h, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase assays were employed to assess cell viability and the inhibitory concentration (IC). Apoptosis was detected by Acridine Orange/Ethidium Bromide and 4',6-diamidino-2-phenylindole staining after 24 and 48 h of incubation with $\frac{1}{4}$ IC₅₀ of propolis by testing the mitochondrial membrane potential ($\Delta\Psi_m$) and the expression of apoptosis-related genes (*p53*, *Caspase-3*, *Bax*, *Bcl-2*, *Bcl-X_L*, *Noxa*, *Puma* and *p21*) by reverse transcription polymerase chain reaction.

Key findings Propolis displayed antiproliferative and cytotoxic effects on A549 cells in a dose- and time-dependent manner, but it did not suppress the growth of normal Vero cells. An enhanced apoptosis was seen in A549 propolis-treated cells after 48 h compared with the control cells. Propolis decreased mitochondrial membrane potential by overexpression of pro-apoptotic genes (*Bax* and *Noxa*) and reduction of the antiapoptotic gene *Bcl-X_L*. The expression level of other genes remained unchanged (*p53*, *Caspase-3* and *Bax*), whereas *p21* expression was increased. Propolis induced caspase-independent apoptosis through a *p53*-independent mitochondrial pathway, and cell cycle arrest by upregulation of *p21*.

Conclusions Although propolis induces apoptosis mainly by *p53*-independent manner, it may be induced by another pathway, and new insights may arise for preventing or treating lung cancer.

Introduction

Lung cancer has high morbidity and mortality rates and has drawn significant attention in prevention, diagnosis and therapy.^[1] Despite improvements in surgical treatment and radiotherapy, the 5-year survival rate for all patients diagnosed with lung cancer remains between 15% and 20%.^[2] It is therefore necessary to develop more effective and less harmful therapies to reduce lung cancer mortality.

In recent years, several groups have reported the efficacies of different natural products for the treatment of lung cancer with few side effects.^[3] The therapeutic potential of propolis to treat several diseases, such as cancer, is well recognized.^[4–6] The chemical composition of this resinous

material collected by bees defines its pharmacological properties and varies in accordance with the phytogeographical diversity of the area from which it is collected. In the apiary of our university (UNESP, Campus of Botucatu, Brazil), the main vegetal sources of propolis produced by Africanized honeybees are *Baccharis dracunculifolia*, *Eucalyptus citriodora* and *Araucaria angustifolia*.^[7]

Brazil has great diversity and rich flora, and due to this, propolis collected from diverse regions of this country show distinct colours and chemical compositions depending on the local flora at the site of collection.^[8] According to different reports, the Brazilian propolis characterized as 'green'

has a wide range of biological properties such as antibacterial,^[9] anti-inflammatory,^[10] anti-hypertensive,^[11–13] antihyperlipidemic,^[14] antioxidant^[15] and antitumour^[16–18] activities.

Propolis from southeastern Brazil has previously been found to exert a cytotoxic action against human laryngeal epidermoid carcinoma, leukaemia and prostate cancer cells.^[16,18,19] Lung cancer is one of the most common cancers in the world, and the objective of this study was to investigate the mechanisms by which propolis may induce the death of human lung carcinoma cells. This paper assays for apoptosis, mitochondrial transmembrane potential disruption and gene expression of pro- and anti-apoptotic proteins (*p53*, *Caspase-3*, *Bax*, *Bcl-2*, *Bcl-X_L*, *Puma*, *Noxa* and *p21*).

Materials and Methods

Cell culture

Human lung carcinoma cell line A549 (ATCC CCL-185) and Vero cells (normal African green monkey kidney ATCC CRL-1586) were grown in Dulbecco's Modified Eagle Medium (Gibco Laboratories, Grand Island, NY, USA) (90% v/v) and heat inactivated fetal bovine serum (10% v/v) containing penicillin (100 U/ml), and streptomycin (100 µg/ml). Culture cells were maintained at 37°C under an atmosphere of 5% CO₂ for a maximum of 12 passages.

Propolis sample

Propolis was produced by Africanized honeybees and provided by UNESP, Campus of Botucatu (Brazil) and obtained as described previously.^[16] Propolis was ground and 30% ethanolic extracts of propolis were prepared in the absence of bright light, at room temperature and with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated. Concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml of propolis crude hydroethanolic extract were made by diluting the stock solution (130 mg/ml) with the medium prepared as described above.

Cell viability by MTT assay

A549 and Vero cells (2×10^5 cells/ml) were seeded into 96-well plates and treated with different concentrations of propolis (6.25, 12.5, 25, 50 and 100 µg/ml). Untreated cells were used as a negative control. The cells were then cultured for 72 h before the addition of 10 µl of 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) into each well. The incubation was continued for another 3 h before the medium was removed. Dimethyl sulfoxide

(150 µl) was added to each well and mixed to ensure cell lysis and dissolution of the formazan crystals.

Absorbance (A) was read in a ultraviolet-visible spectrophotometer multiplate reader (MRX Revelation Dynex Technologies; Denkendorf, Germany) at 570 nm. Absorbance from untreated cells was considered as 100% of growth and used for viability calculation. The effect of propolis extract on the viability for cell lines panel was expressed as the % viability, using the formula: % viability = A_{570} of treated cells / A_{570} of control cells $\times 100\%$. The median inhibitory concentration (IC₅₀) values from line cells were determined.

Cytotoxicity assay

The cytotoxic effects of propolis on cells were measured by lactate dehydrogenase (LDH) assay after incubation with various concentrations of propolis (6.25, 12.5, 25, 50 and 100 µg/ml) for 72 h. The assays were carried out in triplicate, and untreated cells were used as a negative control. Briefly, 100 µl/well of the supernatant was transferred to the corresponding well of an optically clear 96-well flat-bottom microtitre plate and analyzed using a Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, St Louis, MO, USA). The absorbance of each sample was measured at 490 nm in a microplate reader (MRX Revelation Dynex Technologies). LDH release was expressed according to the formula: % LDH release = (LDH activity in the medium / total LDH activity) $\times 100$.

Morphological changes and apoptosis assessment

Apoptosis was analyzed by Acridine Orange/Ethidium Bromide (AO/EB) and 4',6-diamidino-2-phenylindole (DAPI) staining. A549 cells (2×10^5 cell/ml) were grown in 24-well culture plates and treated with propolis for 24 and 48 h using $\frac{1}{4}$ IC₅₀ of propolis, because cells died using the IC₅₀ or $\frac{1}{2}$ IC₅₀. After these periods, cells were incubated with a mixture of AO/EB in phosphate-buffered saline (PBS) (100 µg/ml AO/100 µg/ml EB). The nuclei of viable cells were stained by the membrane-permeable dye AO, whereas necrotic cells were stained by the highly fluorescent EB. To evaluate the changes in the chromatin morphology of cells by DAPI staining, cells were fixed with 500 µl of acetic acid fixing solution (acetic acid (Quimipur, Madrid, Spain)/methanol (Merck, Darmstadt, Germany) – 1:2 v/v). After 5 min, cells were washed with PBS and stained with DAPI (1 µg/ml) for 10 min. Apoptotic nuclei were identified by the condensed chromatin gathered at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. In both stains, untreated cells were used as a negative control. A total of 200 cells were analyzed and counted in triplicate in three independent experiments

using an Olympus IX-71 fluorescent microscope equipped with a Color View camera Olympus DP72, filters with emission shift from red (480 nm) to green (520 nm) and a magnification of 400× (Melville, NY, USA).

Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$) disruption

The changes in the mitochondrial potential were detected using a JC-1 mitochondrial membrane potential assay (Mitochondria Staining Kit, Sigma-Aldrich). A549 cells were cultured in a 24-well plate at a density of 2×10^5 cells/ml and treated with $\frac{1}{4}$ IC₅₀ of propolis for 24 and 48 h (triplicate wells per condition). Untreated cells were used as a negative control and valinomycin-treated cells were considered as positive control. All cells were imaged with Olympus IX-71 fluorescent microscope equipped with a Color View camera Olympus DP72 using filters with emission shift from red (590 nm) to green (525 nm) and a magnification of 400×.

Isolation of total RNA and reverse transcription polymerase chain reaction (RT-PCR) for pro- and anti-apoptotic genes (*p53*, *Caspase-3*, *Bax*, *Bcl-2*, *Bcl-X_L*, *Puma*, *Noxa* and *p21*) evaluation

A549 cells (2×10^5 cell/ml) were seeded on 24-well plate and treated with $\frac{1}{4}$ IC₅₀ of propolis for 24 and 48 h (triplicate wells per condition). Total RNA was isolated from untreated or treated A549 cells with TRIzol reagent (LS Reagent, Sigma-Aldrich, St Louis, MO, USA). The RNA final concentration was determined using a Biophotometer plus (Eppendorf, Hauppauge, NY, USA). Each sample of isolated RNA (1 µg) was reverse transcribed in a thermal cycler (AUXILAB, Navarra, Spain) by Moloney Murine Leukemia Virus reverse transcriptase system (Promega Inc., Madison, WI, USA) in a 50-µl volume reaction.

Each PCR was carried out in a master mix containing 1X Green Go Taq Flexi Buffer, MgCl₂, (2 mM), dNTPs (10 mM) and 1.25 U GoTaq DNA polymerase (Promega

Inc.) containing 0.2 mM of respective forward and reverse primers, and 5 µl of complementary DNA. The reaction mix was made up to 25 µl with water molecular biology reagent (Sigma-Aldrich, EUA).

The PCR amplification was carried out in a Thermal cycler (AUXILAB) using specific primers (Table 1). Each reverse-transcribed mRNA product was internally controlled by β -actin gene.

PCR conditions for every one of genes were 35 cycles at 94°C for 1.5 min, at 59°C for 1 min, at 72°C for 1 min and at 72°C for 7 min. Amplified PCR products were subjected to electrophoresis at 70 V in 1.5% (w/v) agarose gel for 1.5 h. A 50-bp DNA ladder marker was used as a molecular marker. Gels were visualized with EB in 1× TBE (Tris-borate-EDTA) buffer. The gels were examined and the intensity of each band was measured by using Gel Doc imaging system UVIsave D-55/20M version 15.08 (UVItect, Cambridge, England). Size of the amplified PCR products were 191 pb for β -actin, 100 pb for *p53*, 100 pb for *Caspase-3*, 283 pb for *Bax*, 100 pb for *Bcl-2*, 492 pb for *Bcl-X_L*, 209 pb for *Puma*, 295 pb for *Noxa* and 257 pb for *p21*.

Statistical analysis

The IC₅₀ values were determined by interpolation of the graph of propolis concentration vs cell viability. Band intensity of each gene from propolis-treated and non-treated cells was compared using the Kruskal–Wallis test for multiple comparisons and Dunn's post-hoc test. For all analysis, we used the GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Significant differences were considered for $P < 0.05$.

Results

MTT assay

The A549 cell viability after 72-h treatment with propolis was determined by MTT reduction assay. A dose–response curve for the percentage of viable cells was obtained against the concentration displayed a significant reduction of cell

Table 1 Primers used in polymerase chain reaction reactions

Primers	Forward (5'-3')	Reverse (5'-3')
<i>β-actin</i>	CCTTCCTGGGCATGGAGTCCTG	GGAGCAATGATCTTGATCTTC
<i>p53</i>	GGGTTAGTTTACAATCAGCCACATT	GGCCTTGAAGTTAGAGAAAATTCA
<i>Caspase-3</i>	AGAACTTAGGCATCTGTGGGC	ATCCAGGGGCATTGTAGCAC
<i>Bcl-2</i>	ATGGGCTGGACATTGGACTTC	GGGAGGTCAGCAGGGTAGAT
<i>Bax</i>	ATGGGCTGGACATTGGACTTC	CCATAAACAGCTCTGGGGCA
<i>Bcl-X_L</i>	ACCCCTTAGCCTCCCTGAAA	CCATAAACAGCTCTGGGGCA
<i>Puma</i>	GTCCTCAGCCCTCGCTCT	CTGCTGCTCTCTTGCTCC
<i>Noxa</i>	TTCAATGTGTTCTGTTGGGC	GTGACAAGGAGCATTTCCGA
<i>p21</i>	CAGGGGACAGCAGAGGAAGA	GGCAGAAGATGTAGAGCGGG

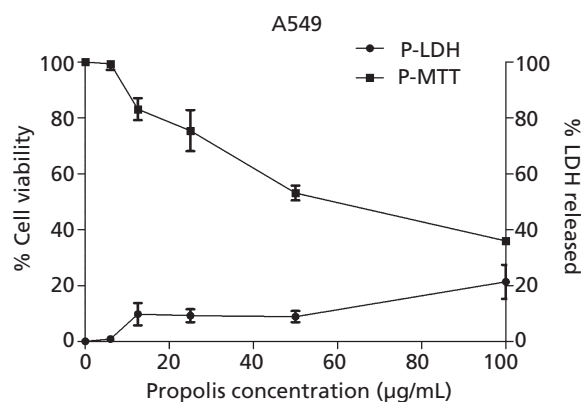


Figure 1 Viability and cytotoxicity of propolis (P) against A549 cell lines. The cells were incubated for 72 h with propolis at concentrations of 6.25–100 µg/ml. The % of cell viability was measured by MTT and % of cytotoxicity was measured by lactate dehydrogenase released. Data represent the mean of three replicates in three independent experiments.

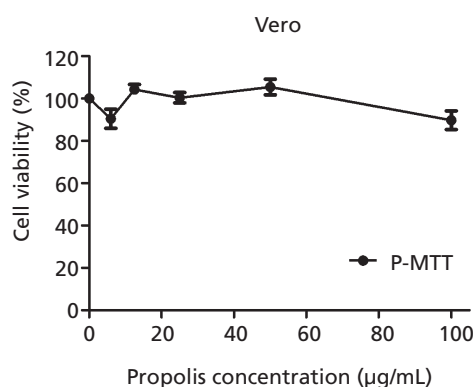


Figure 2 Viability of Vero cell line after incubation with propolis (P) at concentrations of 6.25–100 µg/ml for 72 h. The % of cell viability was measured by MTT. Data represent the mean of three replicates in three independent experiments.

viability (Figure 1). The IC_{50} value obtained for the mean of the three independent experiments was 69.17 ± 11.28 µg/ml ($P < 0.05$).

We have also determined the effects of propolis on the growth of Vero cells (Figure 2). The IC_{50} obtained from three independent experiments was >100 µg/ml, showing that propolis exhibited a high selectivity for tumour cells over normal Vero cells.

Lactate dehydrogenase leakage assay

To determine the cytotoxic effects of propolis on A549 using LDH assay, cells were incubated with increasing concentrations of propolis for 72 h. LDH release increased slightly with increasing propolis concentrations to a

maximum of 21.35% for the highest concentration of propolis assayed (100 µg/ml) (Figure 1), suggesting the loss of membrane integrity.

Analysis of apoptosis induction by fluorescence staining

Analysis of changes in cell morphology was evaluated using AO/EB and DAPI fluorescence staining. AO/EB staining allows separate enumeration of populations of viable non-apoptotic, necrotic and non-viable apoptotic cells. DAPI is known to form blue fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for A-T rich regions of DNA. When DAPI binds to DNA, the blue fluorescence is enhanced greatly. Direct observation of the morphological changes of A549 cells revealed induction of cell death after treatment with propolis. Apoptotic cells were clearly observed with the AO/EB staining (Figure 3a). Control cells were seen to be uniformly green with normal nuclear morphology and cells treated with propolis at 48 h showed an increase of non-viable apoptotic cells with a green fluorescent coloration relative to the cells treated with propolis at 24 h and negative control. DAPI staining also exhibited significant changes (Figure 3b). Cells incubated with propolis at 48 h showed nuclear fragmentation, chromatin condensation and apoptotic bodies compared with the control group and the cells treated during 24 h.

JC-1 staining to measure mitochondrial membrane potential

The effect of propolis on mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated by JC-1 mitochondrial membrane potential assay. Fluorescent microscopic observation revealed that incubation of A549 cells with $\frac{1}{4}$ IC_{50} of propolis during 24 and 48 h markedly increased the green fluorescence, similar to the positive control (valinomycin), indicating a significant disruption of mitochondrial potential and onset of cell death (Figure 4).

Alteration in gene expression profile by propolis

In order to identify the pathways responsible in induced apoptosis by propolis, we have analyzed the altered gene expression profile in A549 cells treated with this extract ($\frac{1}{4}$ IC_{50} of propolis for 24 and 48 h). The genes *p53*, *Caspase-3*, *Bax*, *Bcl-2*, *Bcl-X_L*, *Noxa*, *Puma* and *p21* were detected by RT-PCR. As shown in Figure 5a and 5b, propolis treatment of A549 cells resulted in downregulation of the expression of *Bcl-X_L* and upregulation of the expression of *Bax*, *Noxa* and *p21* in a time-dependent manner. Propolis did not affect the level of *p53*, *Caspase-3*, *Bcl-2*, *Puma* and β -actin when it was employed as housekeeping gene.

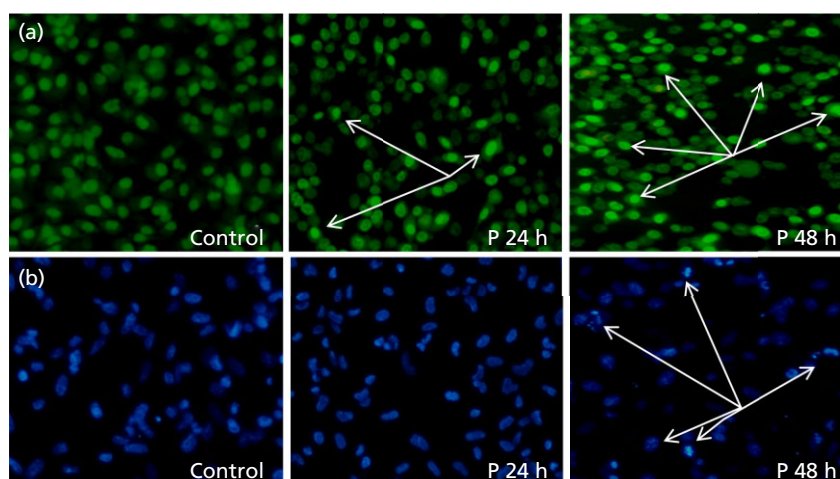


Figure 3 Morphological changes in A549 cells treated with propolis (P) followed by Acridine Orange/Ethidium Bromide and 4',6-diamidino-2-phenylindole staining. The cells were incubated for 24 and 48 h with $\frac{1}{4}$ IC₅₀ of propolis. A total of 200 cells were analyzed and counted in triplicate, in three independent experiments. (a) Acridine Orange/Ethidium Bromide stain, white arrows indicate apoptotic cells. (b) 4',6-Diamidino-2-phenylindole stain, white arrows indicate chromatin condensation and apoptotic bodies. Images are magnified at 400x.

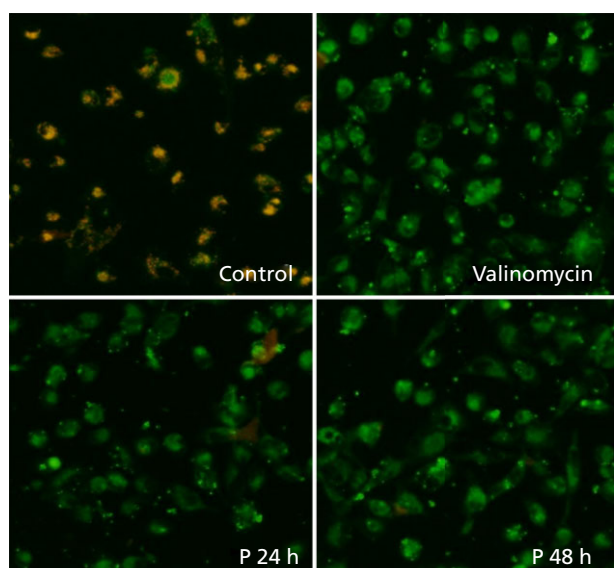


Figure 4 Effect of propolis (P) on mitochondrial membrane potential in A549 cells. The cells were incubated for 24 and 48 h with $\frac{1}{4}$ IC₅₀ of propolis. Red fluorescence represents JC-1 aggregates accumulated in mitochondria due to high $\Delta\Psi_m$, whereas green fluorescence represents monomeric JC-1 in the cytoplasm indicating a lowering of $\Delta\Psi_m$. Images are magnified at 400x.

Discussion

Globally, lung cancer is the second most common cancer in both men and women and is by far the leading cause of cancer death.^[2] Current therapies for lung cancer are inefficient and several studies have demonstrated the effective

use of herbal medicines for the treatment of this disease.^[3,20] Propolis has long been used in the treatment of different diseases including cancer and has the advantages of being inexpensive and possessing low toxicity.^[4–6] This study demonstrated that Brazilian propolis decreased cell viability by exerting a cytotoxic effect on cells of human lung adenocarcinoma (A549 cells) associated with the induction of apoptosis.

The study of antiproliferative and cytotoxic activities of Brazilian green propolis against A549 cells showed that it could inhibit the growth of such cells in a dose-dependent manner, showing a selectivity toward tumour cells compared with normal Vero cells. To further confirm the cytotoxic effect of propolis on A549 cells, LDH leakage assay was also performed as another indicator of A549 cytotoxicity. LDH leakage of A549 cells was slightly increased with the presence of propolis. Orsolic *et al.* showed that several water-soluble compounds of propolis could be extremely useful for controlling tumour growth in experimental models^[21,22] and could inhibit cell growth, depending on the geographical source and composition of propolis.^[23] The main constituents of the propolis used in this study were phenolic compounds (flavonoids, aromatic acids) including artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) and triterpenes.^[24] Artepillin C has demonstrated a cytotoxic activity against other tumour cells,^[25–29] and one may speculate that artepillin C may be one of propolis constituents related to its antiproliferative and cytotoxic against A549 cells.

The inhibitory effect of propolis on A549 proliferation is of great value for exploring the related signalling pathways. Using AO/EB and DAPI fluorescence staining, our results

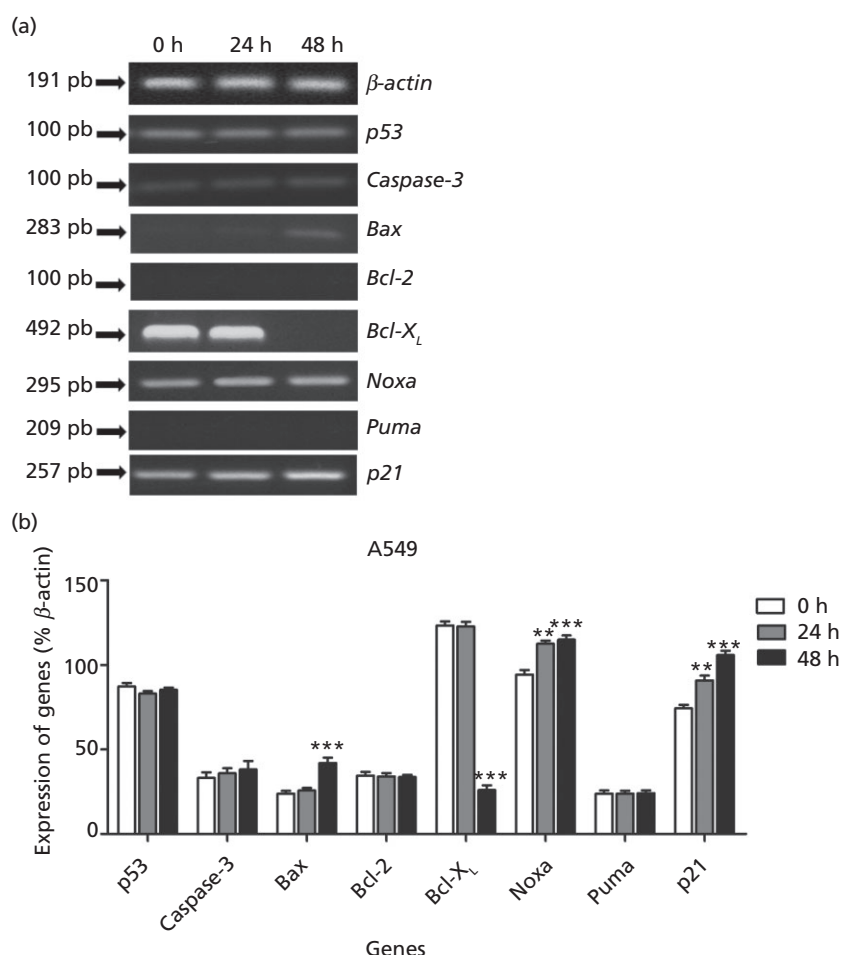


Figure 5 Effect of propolis on mRNA level by reverse transcription polymerase chain reaction assay. A564 cells were treatment with $\frac{1}{4}$ IC₅₀ of propolis for 0, 24 and 48 h. (a and b) Relative signal intensities and effect of propolis on mRNA expression of *p53*, *Caspase-3*, *Bax*, *Bcl-2*, *Bcl-x_L*, *Noxa*, *Puma* and *p21*. Data represent mean $\pm$ standard deviation ($n = 3$). ** $P < 0.01$, *** $P < 0.001$ vs 0 h.

indicated the appearance of conventional morphological signs of apoptosis. The number of apoptotic cells increased after incubation for 48 h at a propolis concentration of $\frac{1}{4}$ IC₅₀. These results indicated that the decrease in A549 cell viability by propolis was due to the induction of apoptosis. Several authors have reported that apoptosis can be the primary mechanism for the antitumour activity described of propolis.^[22,30] Considering that artemisinin is known to inhibit the growth of different tumour cell lines via the induction of apoptosis,^[25,29] our observations may be attributable to the presence of this chemical constituent.

During apoptosis, the mitochondrial membrane potential is disrupted by the formation of permeability transition pores due to the effect of different stimuli, which produce intracellular signals that cause the loss of $\Delta\Psi_m$. JC-1 staining indicated that the structure of the mitochondria had changed, suggesting that disruption of the mitochondrial

membrane is indeed an important mechanism in the induction of apoptosis by this propolis sample.

Sawicka *et al.* reported that the mechanism of apoptosis induced by different types of propolis depends on the chemical composition and the concentration of the propolis extract.^[31] Additionally, several studies have demonstrated that propolis and its isolated compounds can induce the intrinsic or extrinsic pathways of apoptosis.^[28–31] The intrinsic apoptotic pathway is mediated by mitochondria and pro-apoptotic proteins,^[32,33] and our results clearly indicate for the first time that Brazilian propolis induces apoptosis through the mitochondrial-mediated pathway in A549 cells.

Mitochondrial membrane potential, and subsequently apoptosis, is regulated by the Bcl-2 protein family. The family is divided into three classes based on their Bcl-2 homology (BH) domains: the multidomain antiapoptotic proteins (Bcl-2, Bcl-X_L, Mcl-1, Bcl-w and A1), multidomain

pro-apoptotic proteins (Bak and Bax) and BH3-only pro-apoptotic proteins (Bid, Bad, Bim, Puma and Noxa).^[33–35] The expression of genes, which encode many of these proteins, is regulated by *p53*, a tumour suppressor gene, which, together with *p21* (an inhibitor of cyclin-dependent kinases), induces cell cycle arrest and apoptosis.^[36,37]

In this study, the treatment of A549 cells with our extract resulted in the upregulation of *Bax*, *Noxa* and *p21* levels and the downregulation of *bcl-x_L*. Expression levels of *p53*, *caspase-3*, *bcl-2* and *puma* were not affected. The Bcl-2 protein family is an important endogenous regulator of cellular activity that depends on the protein encoded by *Bax* and its translocation to the mitochondrial membrane.^[38] Propolis can induce apoptosis by interfering with the Bcl-2/Bax-mediated regulation of mitochondrial membrane potential.^[39] In our case, Brazilian propolis upregulated the expression levels of *Bax* and did not alter the levels of *Bcl-2* in A564. The overexpression of *Bcl-2* is generally associated with many cancers as well as resistance to chemotherapy.^[40] However, it is reportedly expressed at low levels in alveolar epithelial cells,^[41] unlike *Bcl-X_L*, which shows a high basal expression in these cells.^[42] This observation could explain the unchanged expression of *Bcl-2* in the A549 cell line and suggests that altering *Bcl-2* expression alone is insufficient to affect a cell's susceptibility to apoptosis.

In contrast, the expression of the anti-apoptotic gene *Bcl-X_L* was also downregulated by propolis. The protein encoded by this gene is found in the outer mitochondrial membrane and plays a role in maintaining membrane integrity. Anti-apoptotic effect of Bcl-X_L protein could be suppressed by forming a heterodimer with Bax, which deactivates Bcl-X_L and compromises the integrity of the mitochondrial membrane.^[40]

Furthermore, it has been also shown that there is an association between *Noxa* and Bcl-X_L protein, suggesting that the upregulation of *Noxa* could affect the anti-apoptotic effect of Bcl-X_L.^[43] Our results, in which *Bcl-X_L* expression was decreased but *Noxa* expression was increased (thereby increasing the relative expression of *Noxa* to Bcl-xL), could be consistent with these findings. As such, we conclude that apoptosis induced by Brazilian green propolis is associated with increased *Bax* and *Noxa* expression.

Hassan *et al.* reported that expression of *Noxa* not only triggered apoptosis via the classical mitochondrial pathway, but also resulted in the activation of apoptosis signal-regulating kinase 1 and its downstream effectors c-Jun N-terminal kinase and *p38*, leading to cell death via a mechanism independent of caspase.^[44] Interestingly, we

observed that the expression of gene *Caspase-3* not was changed; however, the $\Delta\Psi_m$ was disrupted by our sample in A549 cells. Several studies revealed that mitochondria contain different apoptogenic factors, including apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which have crucial roles in caspase-independent apoptosis.^[45–47] In fact, it has been reported that Bax-induced cell death may not require caspase activity.^[48] Our results suggest that Brazilian propolis or some of its bioactive constituents could induce Caspase-independent apoptosis by two different routes.

Our results also demonstrated that *p53* deficiency does not alter apoptosis rates in A549 cells treated with Brazilian green propolis. It has previously been demonstrated that this gene plays a major role in *p53*-mediated apoptosis,^[49,50] which explains why *Puma* expression similarly remained unchanged by Brazilian green propolis.

Interestingly, elevated expression of *p21* was observed in cells treated with green propolis. Expression of this gene can be regulated by several pathways, inclusive by *p53*.^[51] The gene *p21* is a critical inhibitor of the G1 kinase of the cell cycle pathway. The mechanism by which this occurs is unclear, but several studies indicate that the modulation of pro-apoptotic or anti-apoptotic genes is responsible for *p21*-induced apoptosis.^[37,51,52] Thus, *p21* overexpression induced by Brazilian green propolis or some of its constituents could trigger two distinct pathways: cell cycle arrest or mitochondrial apoptosis in a manner independent of *p53*.

Conclusions

Brazilian propolis suppressed the proliferation of A459 cells by inducing apoptosis via the intrinsic pathway. The activation of this pathway was associated with decreased *Bcl-X_L* expression and increased *Bax* and *Noxa* expression, resulting in a loss of mitochondrial membrane potential. Furthermore, cell death was found to be independent of *Caspase-3*, and the upregulation of *p21* without *p53* in response to treatment of A549 cells with propolis suggested a novel mechanism underlying propolis-induced apoptosis in lung cancer cells. We believe that Brazilian propolis (or its bioactive constituents) could be a promising candidate for the treatment of lung cancer.

Declaration

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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5.2.- Cytotoxic effect of Cuban and Brazilian propolis in human laryngeal epidermoid carcinoma cells: a preliminary in-vitro comparison.

Yahima Frión-Herrera, Alexis Díaz-García, Jenny Ruiz-Fuentes, Hermis Rodríguez-Sánchez and José Maurício Sforcin.

Objetivo

Comparar o efeito da própolis brasileira (PB) e cubana (PC) sobre a proliferação, morte celular e mecanismo molecular envolvido na linhagem de carcinoma de laringe (HEp-2).

Materiais e Métodos

- Incubação das células com as amostras da própolis (6,25-100 µg/ml) durante 72 h.
- Determinação da viabilidade e citotoxicidade celular (MTT e LDH).
- Determinação do efeito da geração de espécies reativas de oxigênio (ERO) sobre a viabilidade celular pelas amostras.
- Identificação do evento de morte celular por coloração fluorescente.
- Determinação do potencial de membrana mitocondrial.
- Determinação da expressão de genes pro/anti-apoptóticos.

Resultado e Conclusões

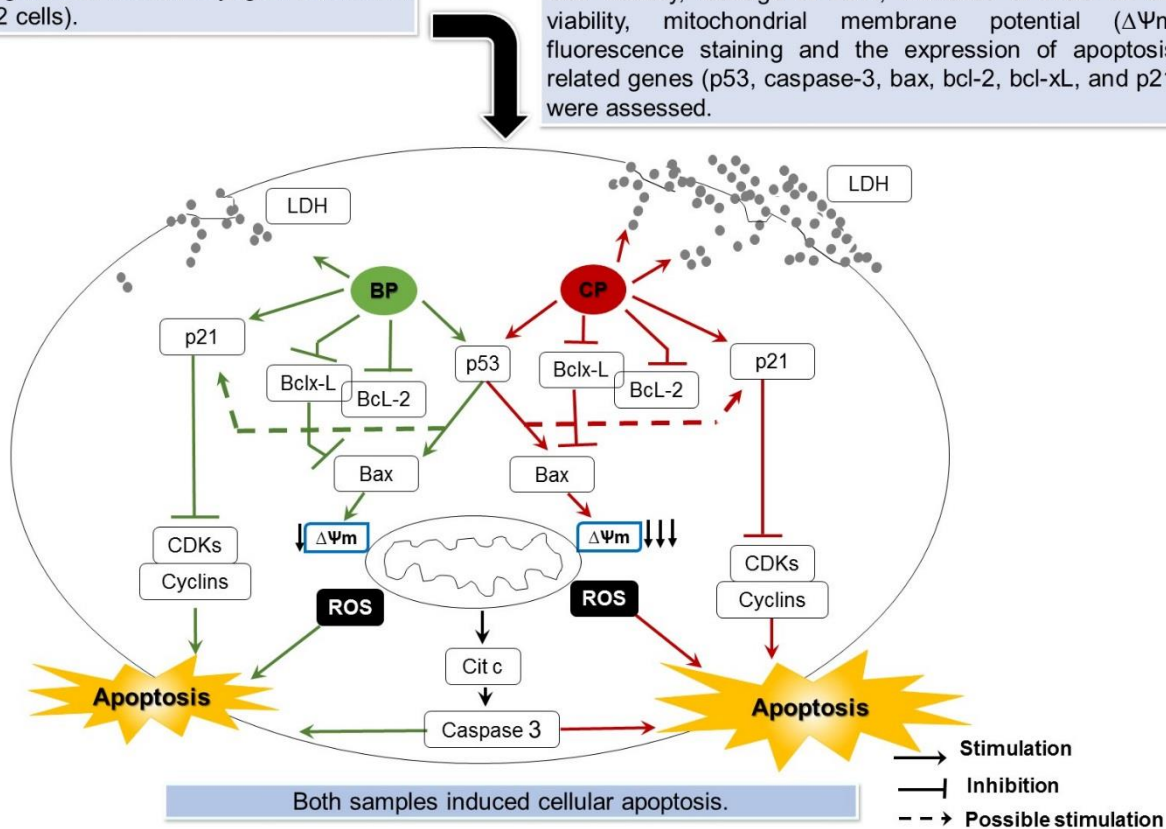
As amostras da própolis exibiram efeitos anti-proliferativos e citotóxicos de modo concentração dependente. O evento apoptótico foi observado nas células tratadas com as amostras, afetando o potencial de membrana mitocondrial, aumentando a expressão dos genes *P53*, *CASP3*, *BAX*, *P21*, e reduzindo a expressão dos genes *BCL2* e *BCL-XL*. Particularmente, a própolis cubana induziu um efeito citotóxico maior do que a própolis brasileira. Nossas descobertas abrem perspectivas para estudar os principais

componentes de cada própolis, que podem levar ao desenvolvimento de um medicamento apropriado para o tratamento do câncer.

Graphic Abstract

The cytotoxic action of Cuban red (CP) and Brazilian green propolis (BP) was investigated on human laryngeal carcinoma (Hep-2 cells).

Cell viability, leakage of LDH, influence of ROS on cell viability, mitochondrial membrane potential ($\Delta\Psi_m$) fluorescence staining and the expression of apoptosis-related genes (p53, caspase-3, bax, bcl-2, bcl-xL, and p21) were assessed.



Cytotoxic mechanism proposed of Cuban red propolis (CP) and Brazilian green propolis (BP) on HEp-2 cells. *P53*, *CASP3*, *BAX*, *BCL2*, *BCL-XL*, and *P21*: apoptosis-related genes, CDKs: Cyclin-dependent kinase, ROS: reactive oxygen species, LDH: lactate dehydrogenase.

Cytotoxic effect of Cuban and Brazilian propolis in human laryngeal epidermoid carcinoma cells: a preliminary in-vitro comparison

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ABSTRACT

Propolis has been used a traditional remedy for centuries because of its beneficial effects including anticancer properties. Due to significant differences in the chemical composition of samples originating from different geographic and climatic zones, the evaluation of antitumor effects of each type of propolis is crucial. The aim of this study was to compare the cytotoxic mechanism of Cuban red propolis (CP) and Brazilian green propolis (BP) on human laryngeal carcinoma (Hep-2). The cytotoxic effect of both samples of propolis was evaluated using different assays including cell viability, leakage of lactate dehydrogenase, fluorescence staining, mitochondrial membrane potential ($\Delta\Psi_m$), and the expression of genes *BAX*, *TP53*, *CASP3*, *BCL-XL*, *BCL2* and *P21*. Cell viability and cytotoxic assays suggested a dose-dependent effect of CP and BP extracts with possible association of intracellular reactive oxygen species production. Apoptotic potencial was confirmed by a decreased $\Delta\Psi_m$ and fluorescence staining, revealing that both samples induced apoptosis cell death via activation of *TP53*, *CASP3*, *BAX*, *P21* signaling, and downregulation of *BCL2* and *BCL-XL*. CP extract showed a higher cytotoxic effect than BP extract; however further studies, either in vitro or in vivo are needed to clarify this activity. Our preliminary findings open perspectives to study the main components of each propolis sample, what may lead to the development of strategies for the treatment of laryngeal cancer.

Keywords Propolis; apoptosis; laryngeal cancer

1. Introduction

Natural products are a promising source for the development of novel therapies, due to their potential effectiveness and low toxicity profile, and the discovery of novel natural drugs will open perspectives for the treatment of several diseases (1-4).

Propolis extract has attracted a great interest in the last decades because of its several biological and pharmacological properties (5). This natural product is collected by honeybees from the buds and exudates of different plant sources and has been used empirically for centuries as a remedy (6, 7). The large number of biological effect such as antimicrobial, antioxidant, anti-inflammatory, and antitumoral activities, is due to its chemical composition, which depends on the geographic region where it was produced and its plant source (7-10).

Different types of propolis may be found, e.g. birch propolis, poplar propolis, pacific propolis, red propolis, Brazilian green propolis, Canarian propolis and so on (11). Tropical samples show unusual compositions including lignans, prenylated organic acid derivatives, terpenoids and flavonoids. Cuba and Brazil have a huge diversity and a rich flora, and chemical studies have identified significant differences in the chemical composition of different types of propolis (12-14). Cuban red propolis (CP) from the western region, has been target of several study for its biological properties, such as hepatoprotective, antiparasitic, antimicrobial and antitumour (15-19). This propolis type is derived from the floral resin of the genus *Dalbergia ecastophyllum* (Leguminosae), whose main chemical constituents are isoflavonoids (13).

Brazilian green propolis (BP) collected in Botucatu, São Paulo State, southeast Brazil, has attracted researchers' interest because of its various biological properties such as immunomodulatory, cytotoxic and antibacterial activity, among others (20-24). The botanical sources of this propolis are *Eucalyptus citriodora* Hook. (Myrtaceae), *Baccharis dracunculifolia* DC. (Compositae) and *Araucaria angustifolia* (Bert.) O. Kuntze (Araucariaceae) (25). The major constituents of BP were artepillin C, flavonoids, triterpenes, benzoic acid, prenyl-*p*-coumaric acid, *p*-coumaric acid, dihydrocinnamic acid, trihydroxymethoxy flavonon, tetrahydroxy flavonon (26, 27).

The antitumor effect of propolis has been the subject of many studies (28). In vitro studies show different sensitiveness of tumor cells to extracts of propolis determined by its chemical

composition (11, 13, 26, 29-32). The mechanisms involved in the anticancer effects of propolis are suppressing cancer/precancerous cells proliferation (33-38). This natural product can induce cytotoxicity via apoptosis or necrosis depending on their chemical composition (29, 39). These observations are of great interest to compare two distinct propolis samples chemically characterized: Cuban red propolis (CP) and Brazilian green propolis (BP) on human laryngeal epidermoid carcinoma (HEp-2) cells, investigating the mechanisms implicated in the cytotoxic effects against these cells. The ethnopharmacological approach, combined with chemical and biological methods, may provide useful pharmacological leads about the medical effects of propolis against laryngeal cancer, which represents about 30% of all malignant tumors of the body and is one of the most common solid tumors of the head and neck region (40, 41).

2. Materials and methods

2.1. Chemicals

Complete Dulbecco's Modified Eagle Medium (DMEM), trypsin-EDTA and penicillin, streptomycin, fetal bovine serum (FBS) were purchased from Gibco/BRL (Grand Island, NY, USA). Normal agarose and Trizol reagent were obtained from Invitrogen (Carlsbad, CA, USA). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), orange acridine (OA), ethidium bromide (EB), N-acetyl-L-cysteine (NAC), dimethyl sulfoxide (DMSO), lactate dehydrogenase activity assay kit (LDH), mitochondria staining kit (JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3' tetra ethylbenzimidazolcarbocyanine iodide)) were purchased from Sigma-Aldrich (St Louis, MO, USA). One Step RT-PCR Kit was obtained from Qiagen (Hilden, Germany). Acetic acid was purchased from Quimipur (Madrid, Spain) and methanol from Merck (Darmstadt, Germany).

2.2. Cell culture

Human laryngeal cancer cells (HEp-2, ATCC-CCL23™) were grown in DMEM (90% v/v) and FBS (10% v/v) containing streptomycin (100 µg/ml) and penicillin (100 U/ml). Culture cells were maintained at 37°C under an atmosphere of 5% CO₂ for a maximum of 15 passages.

2.3. Propolis samples and extract preparation

Cuban red propolis was provided by “La Estación Experimental Apícola”, Havana, Cuba. The extract was obtained and characterized by spectroscopic techniques as described previously (13). The CP final concentrations used in all assays (6.25, 12.5, 25, 50, and 100 µg/ml) were prepared immediately from the stock solution containing 100 mg (dry weight) of CP mixed with 1 ml of ethanol 70%.

Brazilian green propolis was provided by UNESP, Campus of Botucatu (Brazil). This sample was characterized by gas chromatography and mass spectrometry techniques described previously (26). For all assays, the BP final concentrations (6.25, 12.5, 25, 50, and 100 µg/ml) were prepared immediately from the stock solution containing 110 mg (dry weight) of BP mixed with 1 ml of ethanol 70%.

2.4. Determination of cell viability by MTT assay

HEp-2 cells (2×10^5 cells/ml) were seeded into 96-well plates and treated with the concentrations of CP and BP described above. Untreated cells were used as a negative control. After 72 h, MTT was added and formazan was dissolved in DMSO (150 µl/well). The absorbance was measured at 570 nm using a spectrophotometer multiplate reader (MRX Revelation Dynex Technologies, Denkendorf, Germany). Absorbance from untreated cells was considered as 100% and viability was calculated using the formula:

$$\% \text{ Viability} = \frac{A_{570 \text{ of treated cells}}}{A_{570 \text{ of control cells}}} \times 100$$

The median inhibitory concentration (IC₅₀) values from line cells were determined.

2.5. Cytotoxicity assay

The cytotoxic effects of both propolis samples on HEp-2 cells were measured by the release of lactate dehydrogenase (LDH) into the incubation medium using the LDH test kit and reading in a spectrophotometer multiplate reader at 490 nm.

2.6. Influence of oxygen reactive species on cell viability

To examine a possible influence of reactive oxygen species (ROS) on the survival of treated cells, HEp-2 cells were co-treated with 5 µM of NAC in combination with each propolis sample

(using the IC_{50}) for 72 h. Survival percentage of HEp-2 cells was assessed by MTT assay as described above in comparison to NAC-treated cells, considered as 100%.

2.7. Mitochondrial membrane potential ($\Delta\Psi_m$) measurement

Changes in the mitochondrial structure were detected using a mitochondria staining kit (JC-1). HEp-2 cells (2×10^5 cells/ml) were cultured in a 24-well plate and treated with $\frac{1}{4} IC_{50}$ of each propolis sample for 24 and 48 h, in triplicate. The cells were stained with JC-1 according to the manufacturer's protocol and analyzed using an Olympus IX-71 inverted fluorescent microscope (Melville, NY, USA). Untreated cells were used as a negative control.

2.8. Nuclear morphological alterations in apoptotic cells

Apoptosis was analyzed by AO/EB and DAPI staining. HEp-2 cells (2×10^5 cell/ml) were grown in 24-well culture plates and treated with $\frac{1}{4} IC_{50}$ of each propolis sample. After 24 and 48 h, cells were incubated with AO/EB (100 μ g/ml AO / 100 μ g/ml EB in phosphate-buffered saline (PBS)). The nuclei of viable cells were stained by the membrane-permeable dye AO, whereas necrotic cells were stained by the highly fluorescent EB. To evaluate the changes in the chromatin morphology of cells by DAPI staining, cells were fixed with 500 μ l of fixing solution (acetic acid / methanol – 1:2 v/v) and stained with DAPI (1 μ g/ml). The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) were analyzed. Untreated cells were used as a negative control. A total of 200 cells were analyzed and counted in triplicate in three independent experiments using an Olympus IX-71 inverted fluorescent microscope.

2.9. Total RNA isolation and RT-PCR

Total RNA from HEp-2 cells was isolated using Trizol according to manufacturer's protocol. The RNA concentration was determined using a Biophotometer plus (Eppendorf, Hauppauge, NY, USA). The RT-PCRs for the genes *CASP3*, *TP53*, *BAX*, *BCL2*, *BCL-XL*, and *P21* were run on the thermocycler (AUXILAB, Navarra, Spain) according to One step RT-PCR kit and using specific primers (Table 1). The thermal cycling condition for all genes were: initial denaturation at 95°C for 1.5 min followed by 35 cycles at 94°C for 1.5 min, 59°C for 1 min and 72°C for 1 min.

The PCR products were subjected to electrophoresis at 70 V in 1.5% (w/v) agarose gel for 90 minutes. A 50-base pairs (bp) DNA ladder marker was used as a molecular marker. Gels were

visualized with EB in $1 \times$ TBE (Trisborate-EDTA) buffer and the intensity of each band was measured with Gel Doc imaging system UVIsave D-55/20M version 15.08 (UVItect, Cambridge, England).

2.10. Statistical analysis

The IC_{50} values were determined by interpolation of tendency line from linear regression curve. Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Results were analysed using Kruskal–Wallis test for multiple comparisons and Dunn's post-hoc test. Significant differences were considered significant at $P < 0.05$.

3. Results

3.1. Cell viability

To examine the antiproliferative effect of Cuban and Brazilian propolis by MTT assay, HEp-2 cells were treated with various concentrations of each sample (6.25, 12.5, 25, 50 and 100 $\mu\text{g/ml}$) during 72 h. Treatment with CP and BP resulted in proliferative inhibition of cells in a concentration-dependent manner. The differences between the control group, CP and BP treated cells were significant using the highest concentrations (50 and 100 $\mu\text{g/ml}$) (Fig. 1A and 1B). However, HEp-2 cells were significantly inhibited by CP and, to a lesser extent, by BP (Fig. 2). The inhibitory concentration (IC_{50}) for CP and BP was $56.2 \pm 9.2 \mu\text{g/ml}$ and $64.1 \pm 14.6 \mu\text{g/ml}$, respectively.

3.2. Cytotoxicity of CP and BP extracts

In vitro LDH release from HEp-2 cells provided an accurate measure of cytotoxicity effect and cell membrane integrity caused by propolis samples.

HEp-2 cells exposed to CP and BP extracts (6.25, 12.5, 25, 50 and 100 $\mu\text{g/ml}$) for 72 h exhibited a concentration-dependent increase in LDH release and significant cytotoxicity. LDH release increased significantly by CP to 57.6% and, to a lesser extent, by BP (16.1%). Data suggested that cell membrane was affected (Fig. 2).

3.3. N-acetyl-L-cysteine and ROS generation

The inhibitory effect of CP and BP on cell proliferation could be related to the production of ROS. The cytotoxic effect (IC_{50}) induced by CP and BP on HEp-2 cells after 72 h was reversed by a ROS scavenger (NAC, 5 mM) and measured by MTT assay. The co-treatment of NAC with CP and BP increased cell viability from 50.4% to 63.2% and 63.2% to 88.1%, respectively (Fig. 3).

3.4. Effect of CP and BP extracts on mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in $\Delta\Psi_m$ was measured through of cationic fluorescent indicator JC-1. J-aggregates in intact mitochondria, exhibit orange fluorescence (emission at 583 nm), and J-monomers in the cytosol, exhibit green fluorescence (emission at 525 nm). Treatment of HEp-2 cells during 24 and 48 h with CP ($\frac{1}{4} IC_{50}$) and BP ($\frac{1}{4} IC_{50}$), revealed an increase of the green fluorescence, indicating mitochondrial dysfunction and cell death (Fig. 4).

3.5. Analysis of apoptosis induction by fluorescence staining

To distinguish viable, apoptotic, and necrotic cells induced by CP and BP extracts, HEp-2 cells were stained with AO/EB and DAPI. AO/EB staining allows to distinguish viable, apoptotic and necrotic cells, whereas DAPI forms a blue fluorescent complex with DNA, showing a specific fluorescence for A-T rich regions of DNA broken chains. Treatment of HEp-2 cells with CP ($\frac{1}{4} IC_{50}$) and BP ($\frac{1}{4} IC_{50}$) extracts for 24 and 48 h revealed an appearance of labeled cells, representing apoptotic DNA fragmentation (Fig. 5A and 5B). At 48 h of treatment with each sample of propolis was evidenced an increase significant of non-viable apoptotic cells with a green fluorescence relative to the cells treated during 24 h (CP-24 h: 55.1%; CP-48 h: 64.0%; BP-24 h: 41.8%; BP-48 h: 63.1%). In control cultures, fewer or no apoptotic cells were observed. Additionally the number of apoptotic cells after 24 h treated with CP (55.1%) was higher than BP-treatment (41.8%), while at 48 h, an increased significant number of necrotic cells respect to control and BP-treatment was also visualized in CP-treatment (29.5%).

DAPI staining exhibited significant alterations (Fig. 5C). Cells incubated with each sample of propolis during 48 h showed a series of morphological changes compared with to treatment at 24 h, which include fragmentation and condensation of chromatin and nucleus, and formation of apoptotic bodies. In contrast, control cells exhibited a normal appearance.

3.6. Gene expression

Pro-apoptotic (*TP53*, *CASP3*, *BAX*, *P21*) and anti-apoptotic (*BCL2*, *BCL-XL*) genes were chosen to determine the effects of CP ($\frac{1}{4}$ IC₅₀) and BP ($\frac{1}{4}$ IC₅₀) extracts for 24 and 48 h. As expected, both CP and BP extracts drastically increased the expression of *TP53*, *CASP3*, *BAX* and *P21*. However, CP stimulated *CASP3*, *BAX* and *P21* mRNA expression more efficiently than BP after 24 h of treatment. CP decreased slightly *CASP3* mRNA expression at 48 h. On the other hand, CP exhibited a similar level of *TP53* expression at 24 and 48 h, while BP stimulated it only at 24h. In addition, *BCL2* and *BCL-XL* genes were downregulated by both propolis samples (Fig. 6).

4. Discussion

Natural products such as propolis are a precious source of novel anticancer agents but this aspect still deserves investigation to understand its usefulness to promote human health (35). In the present study, HEp-2 cells were treated with various concentrations of Cuban and Brazilian propolis, to investigate a possible cytotoxic effect by inhibiting cell proliferation and inducing apoptosis.

Data showed that CP and BP exhibited a significant dose-dependent decrease in HEp-2 cells viability compared to control after 72 h, by MTT assay. Regarding LDH release, CP exerted a cytotoxic effect on HEp-2 cells (57.6%), while BP extract had a slight cytotoxic effect on such cells (16.1%). Our research is consistent with previous studies showing that BP extract exposure can reduce cell viability, and increase slightly LDH release, revealing that the cytotoxic effect on HEp-2 cells was not related to membrane integrity (30).

The significant toxicity of CP extract may be due to its chemical composition. Orsolic *et al.* reported that various compounds of propolis could be extremely cytotoxic in experimental models depending on the composition and geographical region (42-45). Cuban red propolis contains isoflavonoids as major constituents (13). Several articles reported that flavonoids and derivatives present anticarcinogenic activity, (45-49) therefore, the cytotoxic effect of CP could be explained by the presence and proportion of isoflavonoids in our sample. Additionally, ROS-mediated cytotoxicity was partly rescued by NAC after treating HEp-2 cells with both samples of propolis. Redox imbalance may lead to a loss of cell integrity and consequent cell death (50). Based on our results, it can be suggested that oxidative stress is a possible mechanism underlying the cytotoxicity

induced by both CP and BP in HEP-2 cells. Although the antioxidant capacity of propolis has been reported depending on its composition, it may also induce oxidative damage in tumor cells (51).

ROS generation can be associated with mitochondrial dysfunction (50). Thus, the effect of both propolis sample on $\Delta\Psi_m$ of HEP-2 cells was further investigated using JC-1 staining. Fluorescence microscopic observation indicated a loss of mitochondrial membrane integrity after treatment with CP and BP extracts. These findings suggest that both propolis samples increased mitochondrial membrane permeability. Similar mechanisms have been already described to propolis samples and isolated compounds, which were able to induce mitochondrial dysfunction in tumor cells depending on the chemical composition (30, 52-54).

Mitochondria plays a key role in the apoptotic pathway and changes in its membrane permeability comprise the early events of apoptosis, via depolarization of the mitochondrial membrane (55, 56). Using AO/EB and DAPI fluorescence staining, our results indicated the appearance of classic morphological signs of apoptosis. Treatment with CP and BP resulted in an increased number of apoptotic cells after 48 h of incubation compared to control and to the treatment after 24 h. However, more apoptotic cells were counted in CP-treated cells than in BP-treated ones after 24 h. On the other hand, a higher number of necrotic cells was seen after treatment with CP for 24h. The fluorescence staining with AO/EB was in agreement with cell cytotoxicity assay, which showed that CP had a higher cytotoxic effect than BP due to loss of membrane integrity, indicating the presence of necrotic cells. The induction of two different cell death within one cell population might be a result of different expression levels or posttranslational modifications of pro-necroptotic and pro-apoptotic proteins in a particular cell (57). Thus, our findings corroborates the hypothesis that propolis could inhibit the growth of a variety of tumors cells via apoptosis, and it is highly likely that the presence of different components contribute to different pharmacological interactions, inducing mainly apoptosis in different magnitudes (30, 39, 42, 58).

Apoptosis is a physiologically active process that involving activation, expression and regulation of several genes (55, 56). It has been reported that *TP53* controls apoptosis induction and cell-cycle arrest by regulating the expression of different proteins such as p21, and Bcl-2 protein family (59, 60). The protein p53 itself translocates to mitochondria, where it activates the mitochondrial apoptotic pathway by binding to the anti-apoptotic proteins Bcl-2 and Bcl-xL (61). An increased ratio of Bax/Bcl-2 stimulate the release of cytochrome-c from mitochondria to

promote activation of a single family of proteases, the caspases, which have long been considered as the pivotal executioner of programmed cell death (55, 60, 62).

In this study, the treatment of HEP-2 cells with CP and BP extracts resulted in the upregulation of pro-apoptotic genes *TP53*, *CASP3*, *BAX* and *P21* expression and downregulation of anti-apoptotic genes *BCL2* and *BCL-XL*, indicating that both samples of propolis induced arrest of cell cycle progression; moreover, the apoptotic effect was mediated by the activation of the mitochondrial pathway. Previous reports have described that propolis and its components induce apoptosis via the mitochondrial intrinsic pathway with alterations of the Bax/Bcl-2 balance in different tumor cells (30, 61). Additionally, it has been reported that isoflavonoids and artemillin C are the main components of CP (13) and BP (63) extracts respectively, stimulating similar apoptotic mechanisms (45, 64). Thus, our findings may be attributed to the presence of such constituents in our samples. Remarkably, a more significant modulation of proapoptotic and antiapoptotic genes expression was seen after treatment of HEP-2 cells with CP than BP extract after 24 h, what indicates a higher efficacy of CP extract or some of its bioactive constituents to induce apoptosis-mediated death in HEP-2 cells. These results strongly support the idea that propolis effects regulating gene expression and/or modulating gene products may be related to the chemical composition of propolis samples, which is highly dependent on the flora where it was collected.

Conclusions

The antiproliferative effects of Cuban and Brazilian propolis were investigated, indicating that both CP and BP showed significant cytotoxic effects against HEP-2 cells by inducing apoptosis. Particularly, CP exhibited a higher cytotoxic effects than BP. Furthermore, our data suggest that the apoptotic-signaling pathway was associated with decreased of *BCL2*, *BCL-XL* expression and increased *TP53*, *CASP3*, *BAX* and *P21* expression, resulting in depolarization of the mitochondrial membrane. Further investigation in vitro or in vivo should be carried out in order to understand the mechanism of action of propolis constituents and their potential to treat laryngeal cancer.

Conflict of interest

The authors report no conflicts of interest in this work.

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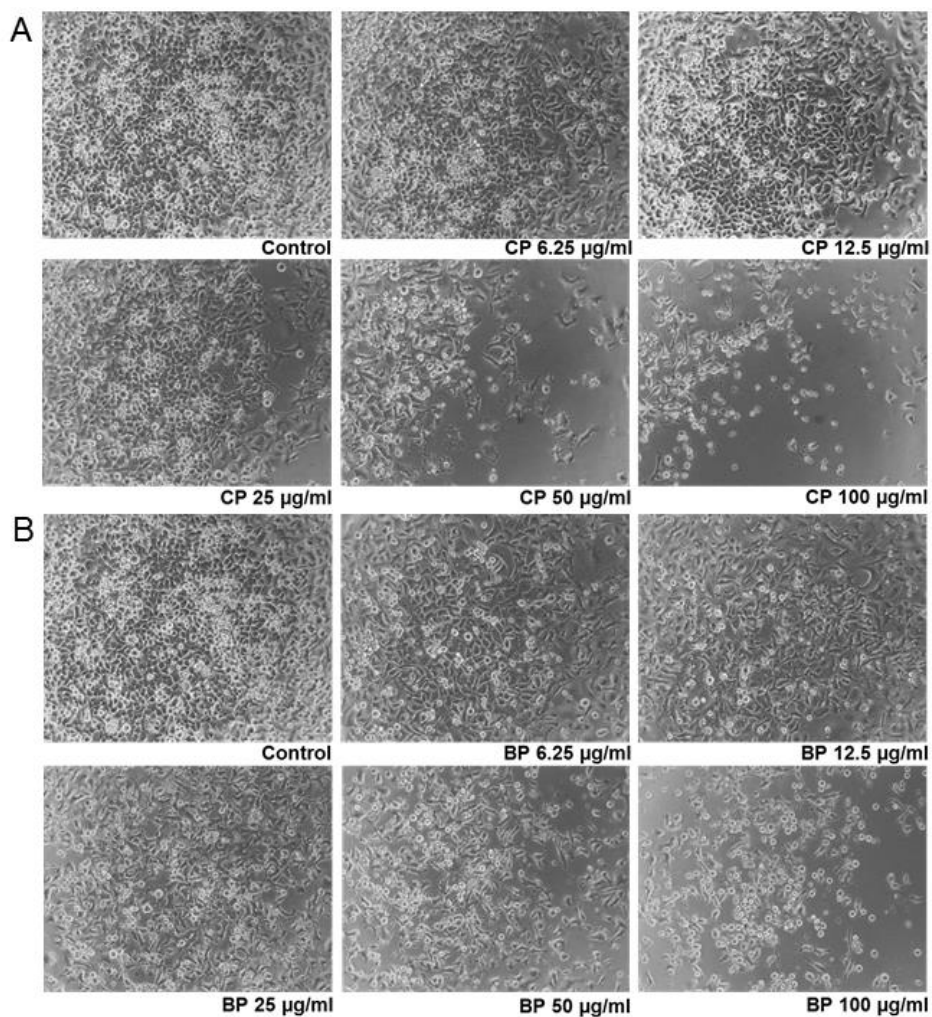


Figure 1 HEp-2 cells viability after incubation with Cuban (CP - A) and Brazilian propolis (BP - B) (6.25–100 µg/ml) for 72 h, showing a decreased viability according to the concentration (magnification 20x).

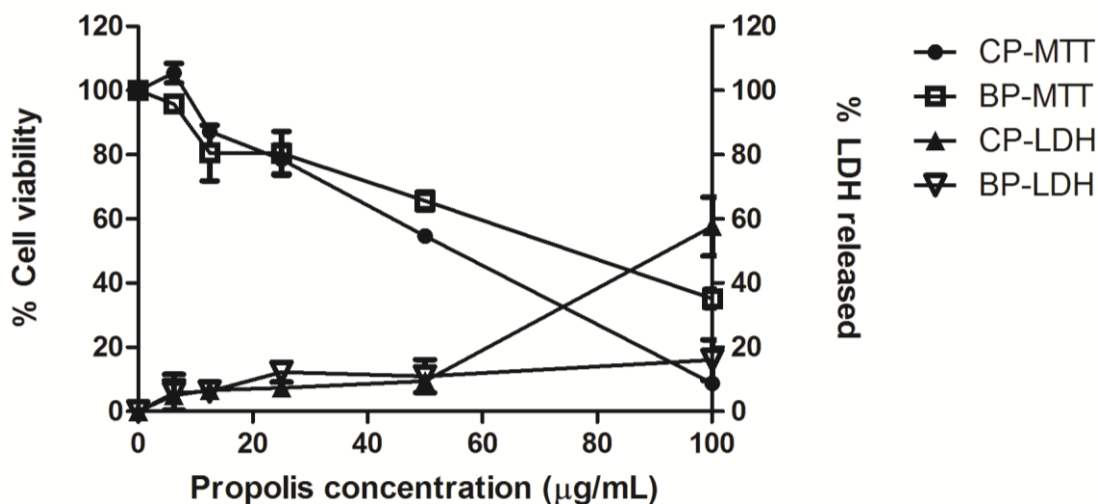


Figure 2 Viability and cytotoxicity (%) of HEp-2 cells after incubation with CP and BP extracts. Cells were treated with various concentrations of each propolis sample (6.25–100 µg/ml) for 72 h. Cell viability was measured by MTT assay and cytotoxicity by lactate dehydrogenase release. The results are expressed as the mean $\pm$ standard deviation (SD) of three independent experiments, in triplicate.

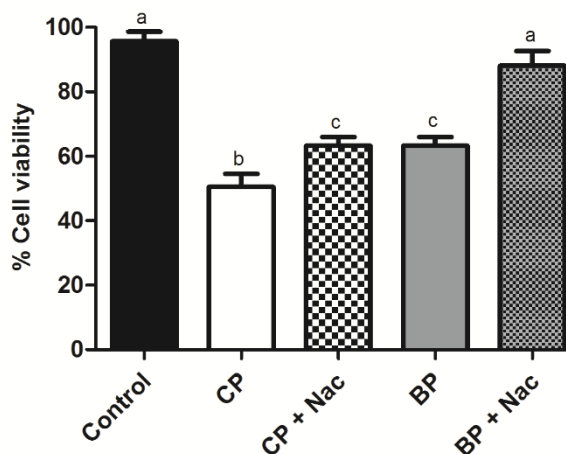


Figure 3 Effect of CP and BP extracts in association with NAC on ROS production. HEp-2 cells were co-incubated with CP or BP (IC_{50}) and NAC (5 mM) for 72 h. Cell viability was measured by MTT assay and compared to NAC-treated cells. Different letters indicate significant differences between the treatments ($P < 0.05$).

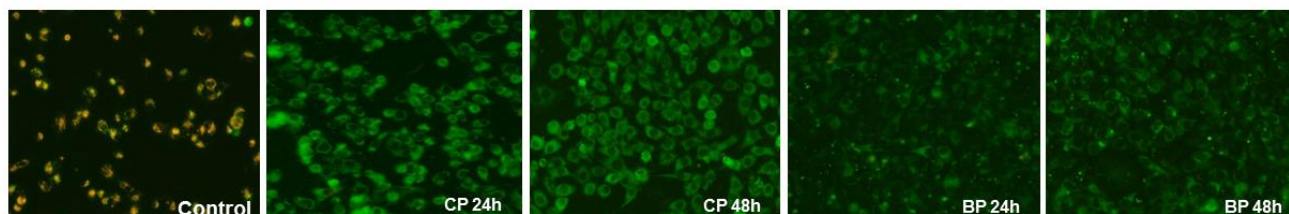


Figure 4 Effect of propolis CP and BP extracts on mitochondrial membrane potential in HEP-2 cells. Cells were incubated for 24 and 48 h with $\frac{1}{4}$ IC_{50} of each sample of propolis. Red fluorescence represents JC-1 aggregates accumulated in mitochondria due to high $\Delta\Psi_m$, whereas green fluorescence represents monomeric JC-1 in the cytoplasm indicating a lowering of $\Delta\Psi_m$. Images are magnified at 400X.

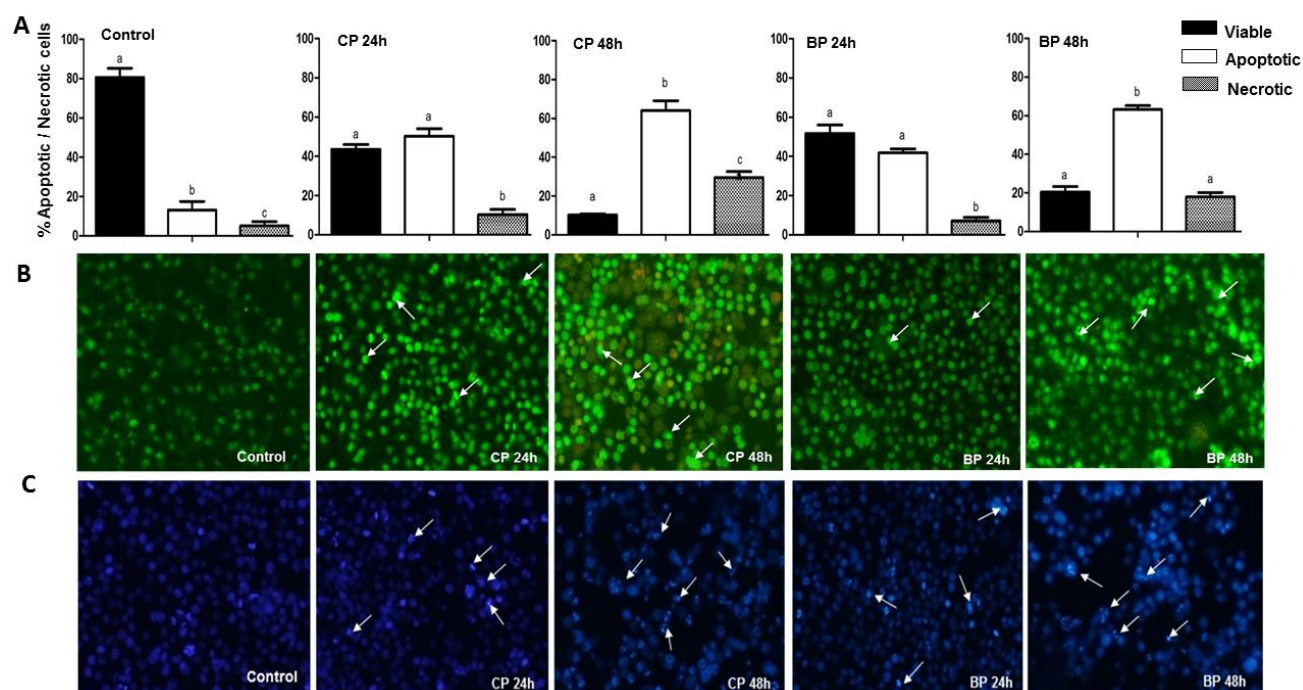


Figure 5 Morphological changes in HEP-2 cells treated with CP and BP extracts followed by Acridine Orange/Ethidium Bromide (AO/EB) and 4',6-Diamidino-2-phenylindole (DAPI) staining. The cells were incubated for 24 and 48 h with $\frac{1}{4}$ IC_{50} of each propolis. A total of 200 cells were analyzed and counted in triplicate, in three independent experiments. Images are magnified at 400 $\times$. (A) Number of viable, apoptotic and necrotic cells counted (%). Data are presented as mean $\pm$ SD. The symbols a, b, and c represent significant differences between groups ($P < 0.05$). (B) AO/EB staining, white arrows indicate apoptotic cells. (C) DAPI staining, white arrows indicate chromatin condensation and apoptotic bodies.

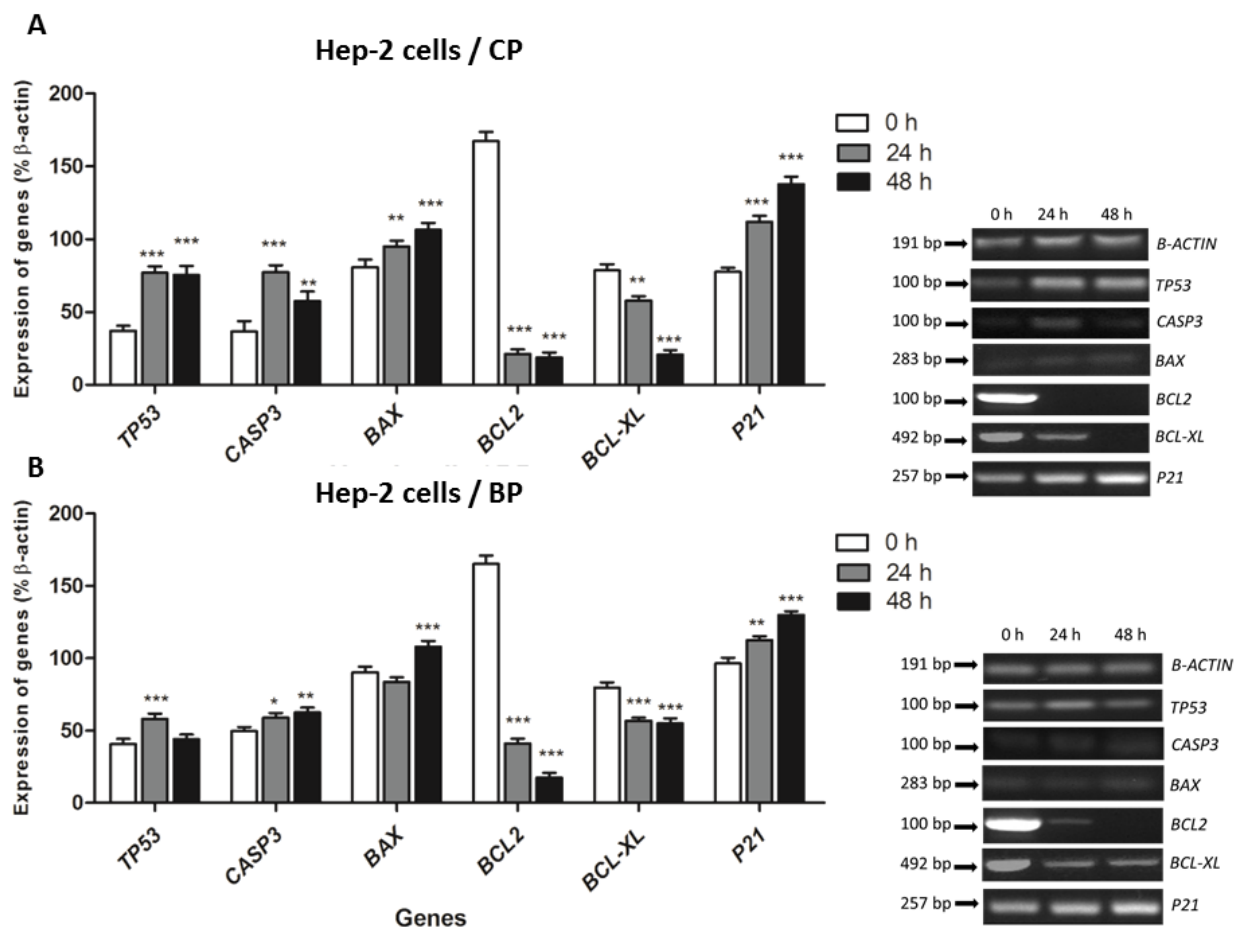


Figure 6 Relative quantification of *TP53*, *CASP3*, *BAX*, *BCL2*, *BCL-XL*, and *P21* mRNA level by reverse transcription polymerase chain reaction assay after incubation of HEp-2 cells treated with $\frac{1}{4}$ IC₅₀ of CP (A) and BP (B) extracts for 0, 24 and 48 h (left). Relative signal intensity of each gene after treatment (right). Data represent mean $\pm$ SD (n = 3). *** P < 0.05, ** P < 0.1, * P < 0.001 vs 0 h.

Table 1 Primers used in PCR reactions.

Primers	Forward (5'-3')	Reverse (5'-3')
<i>β-ACTIN</i>	CCTTCCTGGGCATGGAGTCCTG	GGAGCAATGATCTTGATCTTC
<i>TP53</i>	GGGTTAGTTTACAATCAGCCACATT	GGCCTTGAAGTTAGAGAAAATTCA
<i>CASP3</i>	AGAACTTAGGCATCTGTGGGC	ATCCAGGGGCATTGTAGCAC
<i>BCL2</i>	ATGGGCTGGACATTGGACTTC	GGGAGGTCAGCAGGGTAGAT
<i>BAX</i>	ATGGGCTGGACATTGGACTTC	CCATAAACAGCTCTGGGGCA
<i>BCL-XL</i>	ACCCCTTAGCCTCCCTGAAA	CCATAAACAGCTCTGGGGCA
<i>P21</i>	CAGGGGACAGCAGAGGAAGA	GGCAGAAGATGTAGAGCGGG

5.3.- Cuban propolis induces necrosis in breast cancer cells.

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Objetivo

Visto que o valor da IC_{50} determinado pelo ensaio de MTT no tratamento da linhagem MDA-MB-231 com a própolis brasileira foi maior a 100 $\mu\text{g/ml}$, o objetivo deste artigo foi: Avaliar somente o efeito da própolis cubana sobre a proliferação, migração, morte celular e mecanismo molecular envolvido na linhagem MDA-MB-231.

Materiais e Métodos

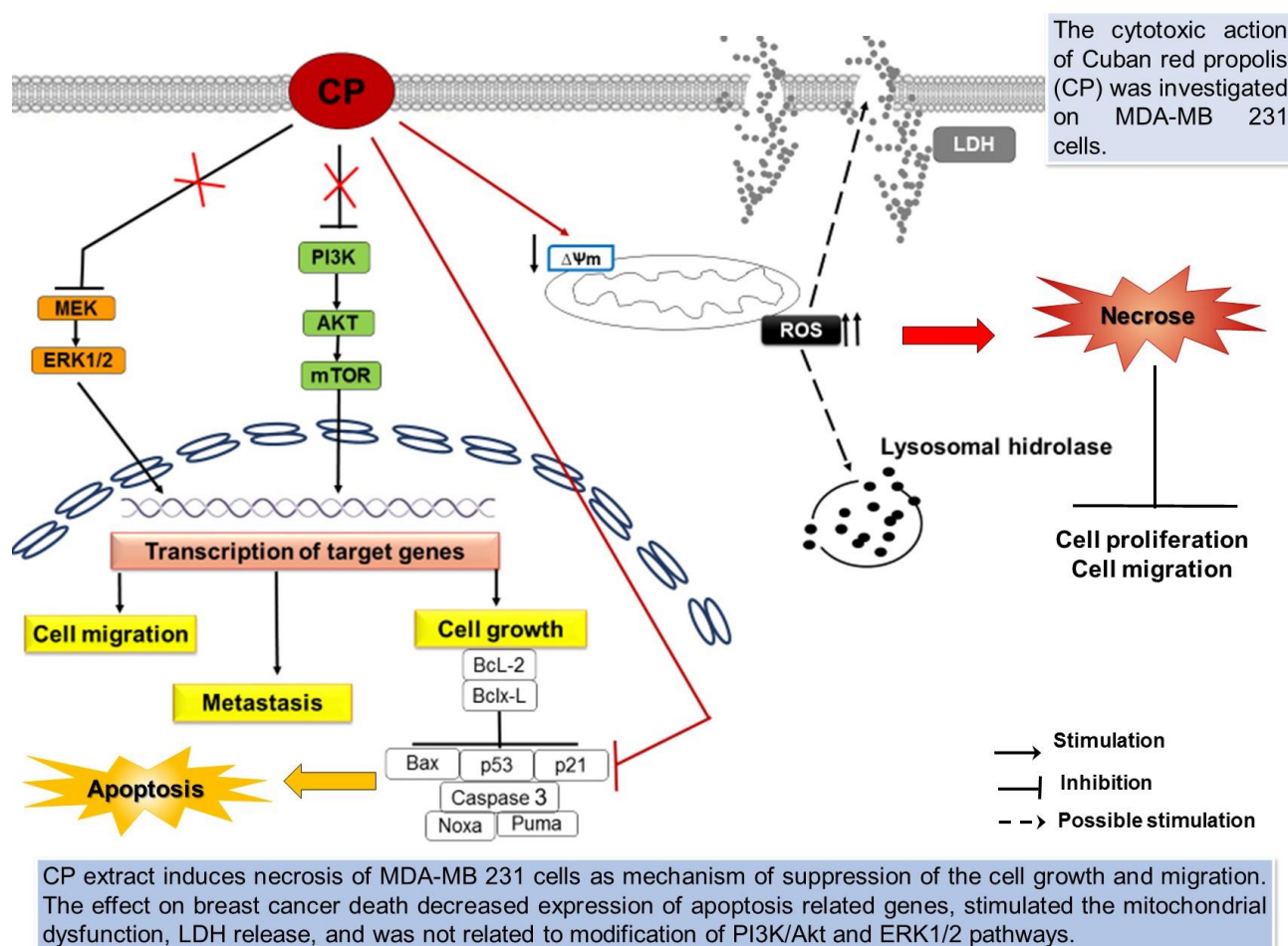
- Incubação das células com a amostra da própolis (6,25-100 $\mu\text{g/ml}$) durante 72 h.
- Determinação da viabilidade e citotoxicidade celular (MTT e LDH).
- Determinação do efeito da geração de ERO sobre a viabilidade celular pela amostra.
- Determinação do efeito sobre a migração celular
- Determinação do efeito sobre as vias de sinalização PI3K/Akt e ERK1/2.
- Identificação do evento de morte celular por coloração fluorescente.
- Determinação do potencial de membrana mitocondrial.
- Determinação da expressão de genes pro/anti-apoptóticos.

Resultado e conclusões

A própolis cubana exibiu efeito anti-proliferativo e citotóxico de modo concentração dependente sem alteração das vias de sinalização PI3K/Akt e ERK1/2. A própolis reduziu

a expressão dos genes *TP53*, *CASP3*, *BAX* e *P21* e não apresentou ação sobre os genes *BCL2*, *BCL-XL*, *NOXA* e *PUMA*. O evento de morte celular por necrose foi observado nas células tratadas de modo a concluir que houve interferência na migração celular e no potencial de membrana mitocondrial, aumentando a liberação de espécies reativas de oxigênio. Estes resultados abrem novas perspectivas para aprofundar no mecanismo de necrose induzido pela amostra de própolis cubana sobre a linhagem celular MDA MB-231.

Graphic Abstract



Cuban propolis induced necrosis in breast cancer cells

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ABSTRACT

Currently natural products such as propolis have been considered as promising antitumor agents. Cuban red propolis (CP) exhibits health benefits including antineoplastic activity. The purpose of this study was to show the cytotoxic effects of ethanolic extract of CP on human breast cancer (MDA MB-231) cell line. Antiproliferative and cytotoxic activity of CP against MDA MB-231 cells was determined by the MTT and LDH assays. Apoptosis/necrosis, inhibition of PI3K/Akt and ERK1/2 pathways, mitochondrial membrane potential dysfunction and expression of genes were investigated. Data showed that CP extract exhibited anti proliferative and cytotoxic effect on MDA MB-231 cells. The effect on breast cancer death was not related to modification of PI3K/Akt and ERK1/2 pathways. A decreased expression of apoptosis related genes *TP53*, *CASP3*, *BAX* and *P21* was seen whereas the expression of genes *BCL-2*, *BCL-XL*, *NOXA* and *PUMA* was unchanged. Furthermore, CP extract induced mitochondrial dysfunction, and LDH release, what led show cell necrosis, associated with ROS production and decreased cell migration. Our findings provided a new evidence for developing strategies against apoptosis-resistant breast cancers using Cuban red propolis.

Keywords: propolis; cytotoxicity; breast cancer; necrosis

Introduction

Breast cancer is one of the most common causes of mortality in women from all over the world. Although different therapies have shown some promise against breast cancer, there is still no effective cure for the majority of patients (1, 2). Estrogen receptor (ER)-negative breast cancers do not respond to anti-estrogen therapies and it is the most aggressive type of breast cancers (3, 4). Current therapies for ER-negative breast cancer patients are usually associated with strong toxicity and side effects (3). Therefore, the development of less toxic and more effective therapies are necessary to treat breast cancer.

Numerous phytochemicals, including propolis, have been reported to interfere with the proliferation of neoplastic cells (5-11). Folk medicine used empirically this sticky product collected by bees (*Apis mellifera*) from floral resins of plants and trees as a traditional remedy (12-14).

There are various types of propolis depending on the zone where it was collected, such as Brazilian, Canarian, European, Cuban, Indian, Chinese, New Zealand and other varieties of propolis (15-18). All of them are rich sources of polyphenol compounds. Tropical samples of propolis show unusual compositions including prenylated organic, acid derivatives, terpenoids, flavonoids and lignans (19-22). The proportion of these compounds in propolis is related to its anti-inflammatory, immunomodulatory, anti-microbial, regenerative, antioxidative, hepatoprotective and antitumor activity (11, 13, 23-28).

Several chemical studies have identified different types of Cuban propolis (brown, red and yellow) with significant differences in its chemical composition (29). Currently, Cuban red propolis (CP) has been focused due to its therapeutic properties, such as antimicrobial, antiparasitic, hepatoprotective and antitumour (24, 28, 30-32). This propolis is derived from the floral resin of the genus *Dalbergia ecastophyllum* (Leguminosae), and its main chemical constituents are isoflavonoids (29).

In the search of anticarcinogenic therapies from natural sources, we have attempted to determine in vitro the mechanisms involved in the effects of CP against MDA MB-231 cells. Apoptosis/necrosis, inhibition of PI3K/Akt and ERK1/2 pathways, mitochondrial membrane potential dysfunction and expression of genes were investigated.

Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), trypsin–EDTA, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco/BRL (Grand Island, NY, USA). Normal agarose and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA, USA). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), orange acridine (OA), ethidium bromide (EB), N-acetyl-L-cysteine (NAC), dimethyl sulfoxide (DMSO), lactate dehydrogenase activity assay kit, mitochondria staining kit (JC-1 - 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3' tetraethylbenzimidazolcarbocyanine iodide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). One Step RT-PCR Kit was obtained from Qiagen (Hilden, Germany). Acetic acid was purchased from Quimipur (Madrid, Spain) and methanol from Merck (Darmstadt, Germany). PI3K/Akt inhibitor LY-294002 and ERK1/2 inhibitor PD-98059 were purchased from Selleckchem (Houston, TX, USA)

Propolis sample and extract preparation

Cuban propolis (CP) was provided by “La Estación Experimental Apícola”, Havana, Cuba. The extract was obtained and characterized as red propolis by spectroscopic techniques as previously described (29). The CP final concentrations used in all assays (6.25, 12.5, 25, 50, and 100 µg/mL) were prepared immediately from the stock solution containing 100 mg (dry weight) of CP mixed with 1 mL of ethanol 70%.

Culture of breast cancer cells

The human breast cancer line MDA MB-231 (adenocarcinoma) derived from metastatic site pleural effusion (ATCC® HTB-26™) was grown in DMEM (90% v/v) and FBS (10% v/v) containing penicillin (100 U/ml) and streptomycin (100 µg/mL) in accordance with the manufacturer's recommendations. Culture cells were maintained at 37°C under an atmosphere of 5% CO₂ for a maximum of 18 passages.

Cell proliferation (MTT assay)

The effect of CP extract on cell proliferation was examined by MTT assay. Approximately 2×10^5 cells were seeded into 96-well plates and incubated for 24 h. Then different concentrations of CP dissolved in the media were added and cells were incubated for 72 h. Untreated cells were used as a negative control. MTT (10 μ L) stock solution (5mg/mL) was added to each well and incubated at 37°C for 3 h. MTT-formazan product in viable cells was dissolved by adding 150 μ L of DMSO. The plates were incubated 30 min at 37°C and the absorbance (A) was measured at 570 nm with Elisa reader (MRX Revelation Dynex Technologies, Denkendorf, Germany). Absorbance from untreated cells was considered as 100% and viability was calculated using the formula:

$$\% \text{ Viability} = [\text{A570 treated cells} / \text{A570 control cells}] \times 100.$$

Lactate dehydrogenase (LDH) release assay

To assess the cytotoxic effect of CP, release of the cytosolic enzyme LDH to the culture media was determined as a marker of cell membrane integrity. Briefly, cells (2×10^5 cells/mL) were seeded in 96-well plate. After overnight incubation, the medium was replaced with serum free medium containing various concentrations of CP and incubated for 72 h. LDH activity was analyzed in the supernatants using the in vitro toxicology assay kit according to manufacturer's instructions and reading in a spectrophotometer multiplate reader at 490 nm.

Effects of CP extract on PI3K-Akt and ERK1/2 signal transduction pathway

Using cellular signaling pathway-specific inhibitors and MTT assay, we explored the mechanisms underlying the inhibitory effects of CP extract on MDA MB-231 cells viability after 72 h. MDA MB-231 cells were pre-incubated in 96-well plate (2×10^5 cells/mL) for 1 h with inhibitors of ERK1/2 and PI3K/Akt pathway (PD-98059 – 80 μ M and LY-294002 – 80 μ M, respectively) prior to CP (IC_{50}) extract exposure. Cell viability was determined as a percentage of viable cells in each treatment (CP, CP/PD-98059, CP/LY-294002, PD-98059 and LY-294002) vs. untreated control.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) and reactive oxygen species (ROS) production

Changes in the mitochondrial structure were detected using a mitochondria staining kit (JC-1). MDA MB-231 cells (2×10^5 cells/mL) were cultured in a 24-well plate and treated with $\frac{1}{4}$ IC₅₀ of CP extract for 24 and 48 h in triplicate. Untreated cells were used as a negative control. The cells were stained with JC-1 according to the manufacturer's protocol and analyzed using a Olympus IX-71 (Melville, NY, USA) inverted fluorescent microscope.

To examine a possible influence of ROS on the survival of treated cells, MDA MB-231 cells were co-treated with 5 μ M of N-Acetyl-L-cysteine (NAC) in combination with CP extract (IC₅₀) for 72 h. Survival percentage of MDA MB-231 cells was assessed by MTT assay in comparison to NAC-treated cells, considered as 100%.

Fluorescence microscopic analysis of cell death

Nuclear morphological changes were determined using differential acridine orange/ethidium bromide (AO/EB) staining, DAPI staining and fluorescence microscopy Olympus IX-71. MDA MB 231 cells (2×10^5 cell/mL) were grown in 24-well culture plates and treated with $\frac{1}{4}$ IC₅₀ of CP extract. After 24 and 48 h cells were incubated with AO/EB solution in a final concentration of 100 μ g/mL. Both viable and dead cells have been stained with AO. AO emits green fluorescence by binding into RNA or single stranded DNA; on the other hand, the cells with ruptured membrane (necrotic cells) stain with AO and their DNA emit red fluorescence. To evaluate morphological aspect of chromatin condensation and fragmented nuclei by DAPI staining, cells were fixed with 500 μ L of fixing solution (acetic acid / methanol – 1:2 v/v) and stained with DAPI (1 μ g/mL). Untreated cells were used as a negative control. A total of 200 cells were analyzed, photographed and counted in triplicate in three independent experiments.

Total RNA isolation and RT-PCR

Total RNA was isolated from cultured MDA MB-231 cells using Trizol according to manufacturer's protocol. The RNA concentration was determined using a Biophotometer plus (Eppendorf, Hauppauge, NY, USA). The RT-PCRs for the genes *TP53*, *CASP3*, *BAX*, *BCL2*, *BCL-XL*, *NOXA*, *PUMA* and *P21* were run on the thermocycler (AUXILAB, Navarra, Spain) according to One step RT-PCR kit and using specific primers (Table 1). The thermal cycling condition for all

genes were: initial denaturation at 95°C for 1.5 min followed by 35 cycles at 94°C for 1.5 min, 59°C for 1 min and 72°C for 1.

Amplified PCR products were subjected to electrophoresis at 70 V in 1.5% (w/v) agarose gel for 90 minutes. A 50-base pairs (bp) DNA ladder marker was used as a molecular marker. Gels were visualized with EB in $1 \times$ TBE (Trisborate-EDTA) buffer. The gels were examined and the intensity of each band was measured by using Gel Doc imaging system UVIsave D-55/20M version 15.08 (UVItec, Cambridge, England).

Table 1 Primers used in PCR reactions.

Primers	Forward (5'-3')	Reverse (5'-3')
<i>β-ACTIN</i>	CCTTCCTGGGCATGGAGTCCTG	GGAGCAATGATCTTGATCTTC
<i>TP53</i>	GGGTTAGTTTACAATCAGCCACATT	GGCCTTGAAGTTAGAGAAAATTCA
<i>CASP3</i>	AGAACTTAGGCATCTGTGGGC	ATCCAGGGGCATTGTAGCAC
<i>BCL2</i>	ATGGGCTGGACATTGGACTTC	GGGAGGTCAGCAGGGTAGAT
<i>BAX</i>	ATGGGCTGGACATTGGACTTC	CCATAAACAGCTCTGGGGCA
<i>BCL-XL</i>	ACCCCTTAGCCTCCCTGAAA	CCATAAACAGCTCTGGGGCA
<i>NOXA</i>	TTCAATGTGTTTCCTGTTGGGC	GTGACAAGGAGCATTTTCCGA
<i>PUMA</i>	GTCCTCAGCCCTCGCTCT	CTGCTGCTCCTCTTGTCTCC
<i>P21</i>	CAGGGGACAGCAGAGGAAGA	GGCAGAAGATGTAGAGCGGG

Cell migration assay

MDA MB-231 cells (2×10^5 cells/ml) were grown on 24 well plates to form confluent monolayer. Gaps were created in confluent cell layers using micropipette tips, followed by a rinse with PBS. Cells were treated with $\frac{1}{4}$ IC₅₀ of CP extract and cells growing in complete media were considered as control. The wound closure was monitored by phase-contrast microscopy and photographed at 0, 24 and 48 h. The wound-healed distances were measured from each picture and the migrated cells were counted from five random fields using Image J software (NIH, USA). Each experiment was repeated at least three times independently and the percentage of migration was calculated using the formula:

$$\% \text{ Migration} = \frac{\text{Width of the wound at 0h} - \text{Width of the wound at (24h or 48h)}}{\text{Width of the wound at 0h}} \times 100$$

Statistical analysis

The experiments were repeated at least three times. The values are reported as mean $\pm$ standard deviation (SD). The IC₅₀ values were determined by interpolation of the graph of propolis concentration vs cell viability. Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Results were analysed using Kruskal–Wallis test for multiple comparisons and Dunn’s post-hoc test. Significant differences were considered significant at $P < 0.05$.

Results

CP extract induced morphological changes and decreased MDA MB-231 cells viability in a concentration dependent manner

The morphological changes observed in MDA MB-231 cells after treatment during 72 with CP extract (6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$) are represented in figure 1A. The cell morphology of untreated cells appeared in elongated shape and treated cells appeared with cellular shrinkage, oval or irregular in shape and condensed cytoplasm. The cells treated with highest concentrations of CP extract (50 and 100 $\mu\text{g/mL}$) displayed the detached and round shape dead cells.

A dose response curve of percentage cell viability against the concentration of CP extract after 72 h of treatment was obtained using the data obtained for MTT cell viability assay (figure1B).

The IC₅₀ value obtained for the mean of the three independent against MDA MB-231 cells was $67.27 \pm 12.8 \mu\text{g/mL}$.

CP extract induced MDA MB-231 cells cytotoxicity

Cell membrane integrity and cytotoxic effects caused by CP extract on MDA MB-231 cells were detected by LDH release. The amount of LDH release increased in a dose-dependent manner in MDA MB-231 cells, and the percentage of cytotoxic cells appeared to reach a maximum 71.07% at a CP extract concentration of 100 $\mu\text{g/mL}$ (figure 1B). These data is consistent with the cell viability assay results, which suggested a specific cytotoxic effect of CP extract against MDA MB-231 cells.

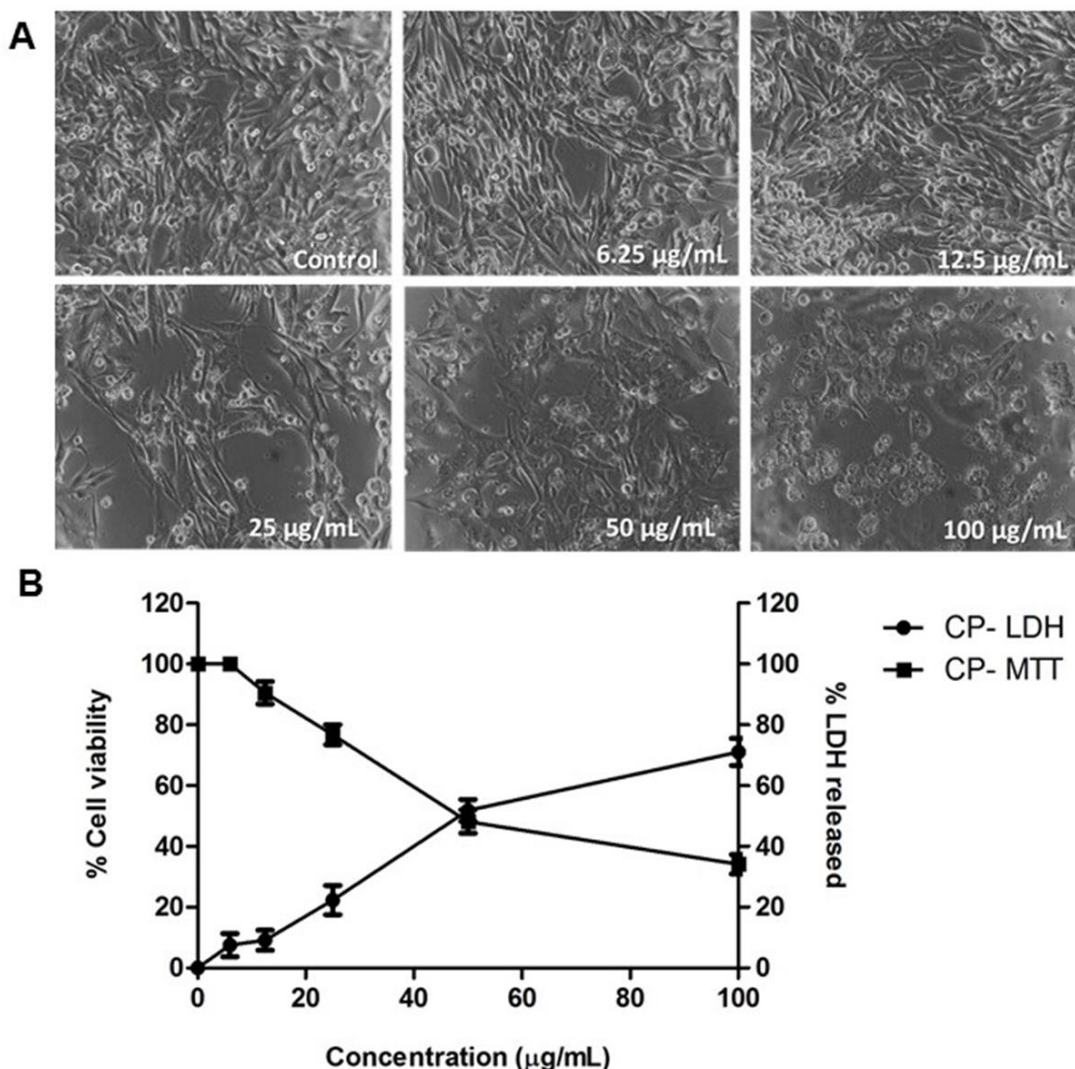


Fig.1. Viability and cytotoxicity (%) of MDA MB-231 cells after incubation with CP extract. Cells were treated with various concentrations of Cuban propolis (CP: 6.25–100 µg/mL) for 72 h. (A) Figures showing a decreased viability and morphological changes according to the concentration (magnification 20x). (B) Cell viability was measured by MTT assay and cytotoxicity by lactate dehydrogenase release. The results are expressed as mean $\pm$ SD of three independent experiments in triplicate.

Cytotoxic effects of CP extract on MDA MB-231 cells is PI3K-Akt and ERK1/2 signaling independent

The combined treatments with PD-98059 / CP extract (IC_{50}) and LY-294002 / CP extract (IC_{50}) exerted the same inhibitory effect on ERK1/2 and PI3K/Akt pathway respectively compared to the treatment with CP alone in MDA MB-231 cells. In contrast, the treatment with each inhibitor alone exhibited a different inhibitory effect compared to control and to the combined treatment with

CP extract (figure 2A and 2B). These results revealed that the ERK1/2 and PI3K/Akt pathway was not involved in the cytotoxic effect of CP extract.

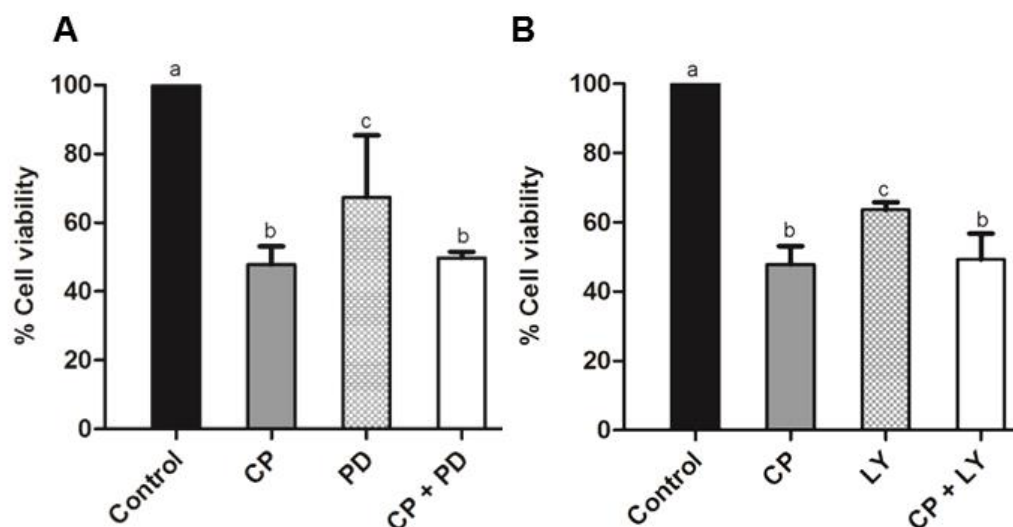


Fig.2. Effect of CP extract in association with inhibitors of ERK1/2 and PI3K/Akt signaling pathways (PD-98059 and LY-294002, respectively). MDA-MB 231 cells were treated with CP (IC_{50}) for 72 h with or without PD (80 μ M) (2A) and LY (80 μ M) (2B) co-treatment. Cell viability was measured by MTT assay and calculated according to the survival of control cells. Different letters represent significant differences between the groups ($p < 0.05$).

CP extract decreased mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in mitochondrial transmembrane potential were evaluated using the cationic fluorescent indicator JC-1. J-aggregates in intact mitochondria fluoresce with emission at 583 nm (red), and J-monomers in the cytoplasm fluoresce with emission at 525 nm (green) at 488 nm. Treatment of MDA MB-231 cells with CP ($\frac{1}{4} IC_{50}$) for 24 and 48 h revealed a markedly increased green fluorescence, indicating a significant disruption of mitochondrial potential and onset of cell death (figure 4). The loss of $\Delta\Psi_m$ might be related to the overproduction of ROS.

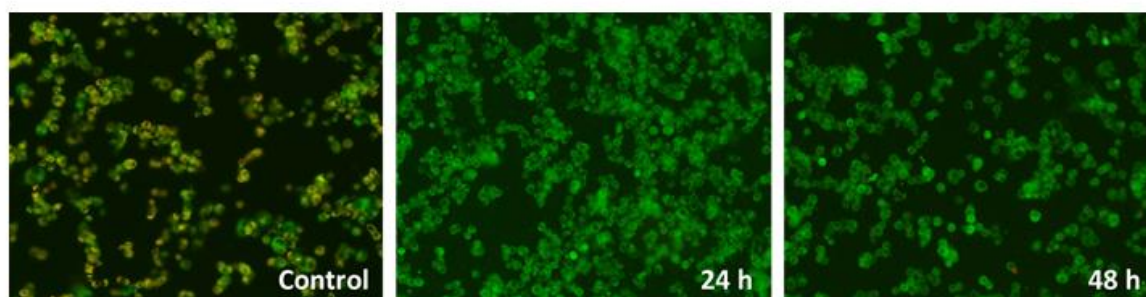


Fig. 4. Effect of propolis CP extract on mitochondrial membrane potential in MDA MB-231 cells. Cells were incubated for 24 and 48 h with $\frac{1}{4}$ IC_{50} of propolis. Red fluorescence represents JC-1 aggregates accumulated in mitochondria due to high $\Delta\Psi_m$, whereas green fluorescence represents monomeric JC-1 in the cytoplasm indicating a lowering of $\Delta\Psi_m$. Images are magnified at 400x.

A ROS scavenger (NAC) decreased the cytotoxicity of CP extract to MDA MB-231 cells

The co-treatment of NAC with CP extract increased cell viability from 48.28 % to 69.84 as shown in figure 3. These results suggested that the cytotoxic effect of CP extract on MDA MB-231 cells might be associated to the intracellular ROS production.

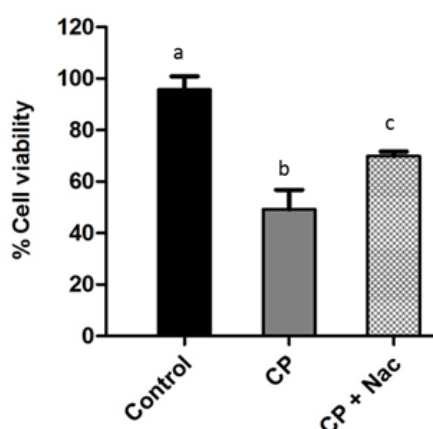


Fig. 3. Effect of CP extract in association with NAC on ROS production. MDA MB-231 cells were co-incubated with CP (IC_{50}) and NAC (5 μ M) for 72 h. Cell viability was measured by MTT assay and compared to NAC-treated cells. Different letters indicate significant differences between the treatments ($p < 0.05$).

CP extracts induced necrosis in MDA MB-231 cells

Identification of apoptosis and necrosis was analyzed by AO/EB and DAPI staining. AO/EB staining allows to distinguish viable, apoptotic and necrotic cells, whereas DAPI forms a blue fluorescent complex with DNA, showing a specific fluorescence for A-T rich regions of DNA broken chains. The percentage of viable, apoptotic and necrotic cells were calculated with AO/EB staining (figure 5A). It can be seen that the number of viable cells reduced by increasing the exposure time to CP extract ($\frac{1}{4}$ IC_{50}). Cells treated with CP extract for 24h exhibited a similar percentage of viable, apoptotic and necrotic cells, indicating that CP extract induces both apoptosis and necrosis in MDA MB-231 cells at 24 h. However, after 48 h of treatment with CP extract, the number of orange-colored nuclei increased compared to control and apoptotic cells, showing a severe membrane disruption and a higher prevalence of necrotic cells (figure 5B). Additionally,

morphological change of chromatin condensation and fragmented nuclei evaluated by DAPI staining was undetectable (figure 5C). These data revealed that CP-treated MDA MB-231 cells primarily undergone necrotic cell death in a time-dependent manner.

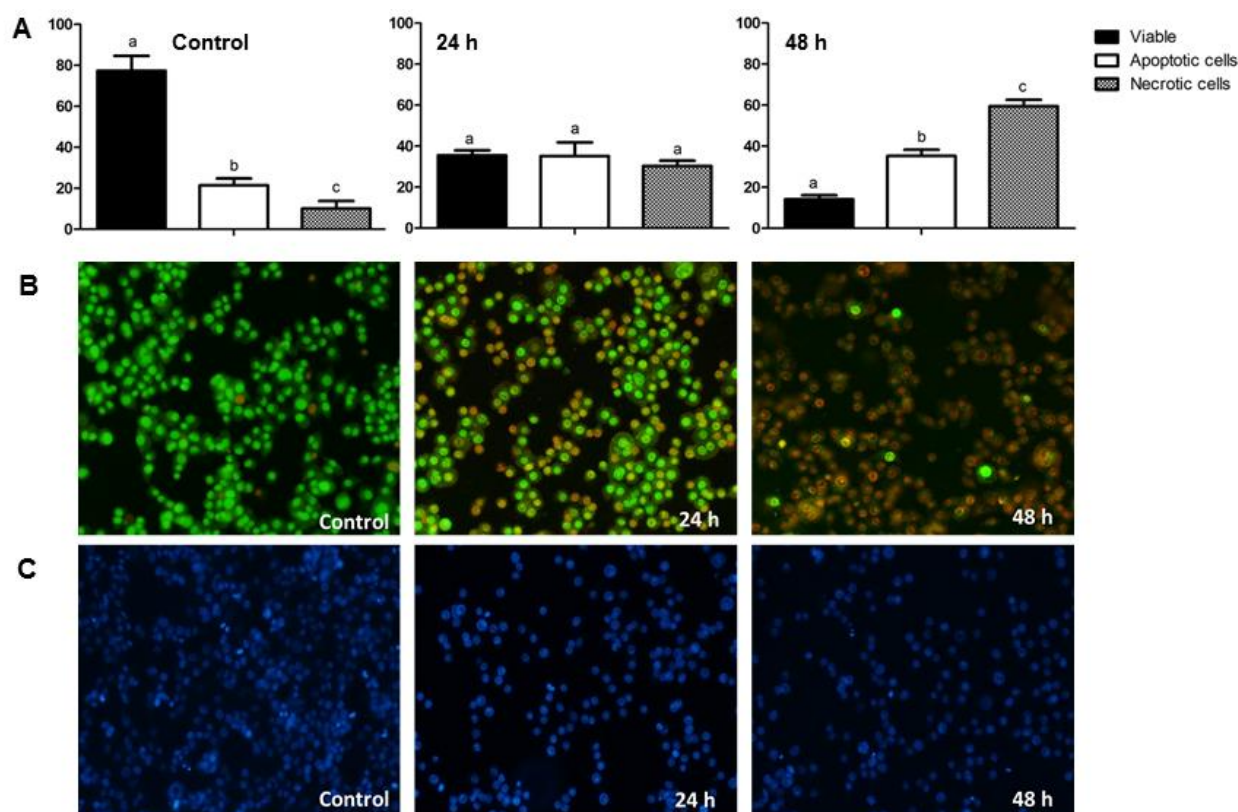


Fig. 5. (A) Percentage of viable, apoptotic and necrotic cells after incubation for 24 and 48 h with $\frac{1}{4}$ IC_{50} of CP. Data represent mean $\pm$ SD. Different letters indicate significant differences between the groups ($p < 0.05$). (B) Morphological changes in MDA MB-231 cells treated with CP extract followed by Acridine Orange/Ethidium Bromide (AO/EB) and (C) 4',6-Diamidino-2-phenylindole (DAPI) staining. A total of 200 cells were analyzed and counted in triplicate in three independent experiments. Images are magnified at 400 $\times$.

CP extract inhibited the expression of apoptosis related genes

It can be seen in figure 6 that the expression of apoptosis related genes *TP53*, *CASP3*, *BAX*, *P21* decreased in CP-treated MDA MB-231 cells at 24 and 48 h. Moreover, the expression of *BCL2*, *BCL-XL*, *NOXA* and *PUMA* in propolis-treated cells was unchanged compared to untreated cells. Data indicated that CP extract induced non-apoptotic cell death.

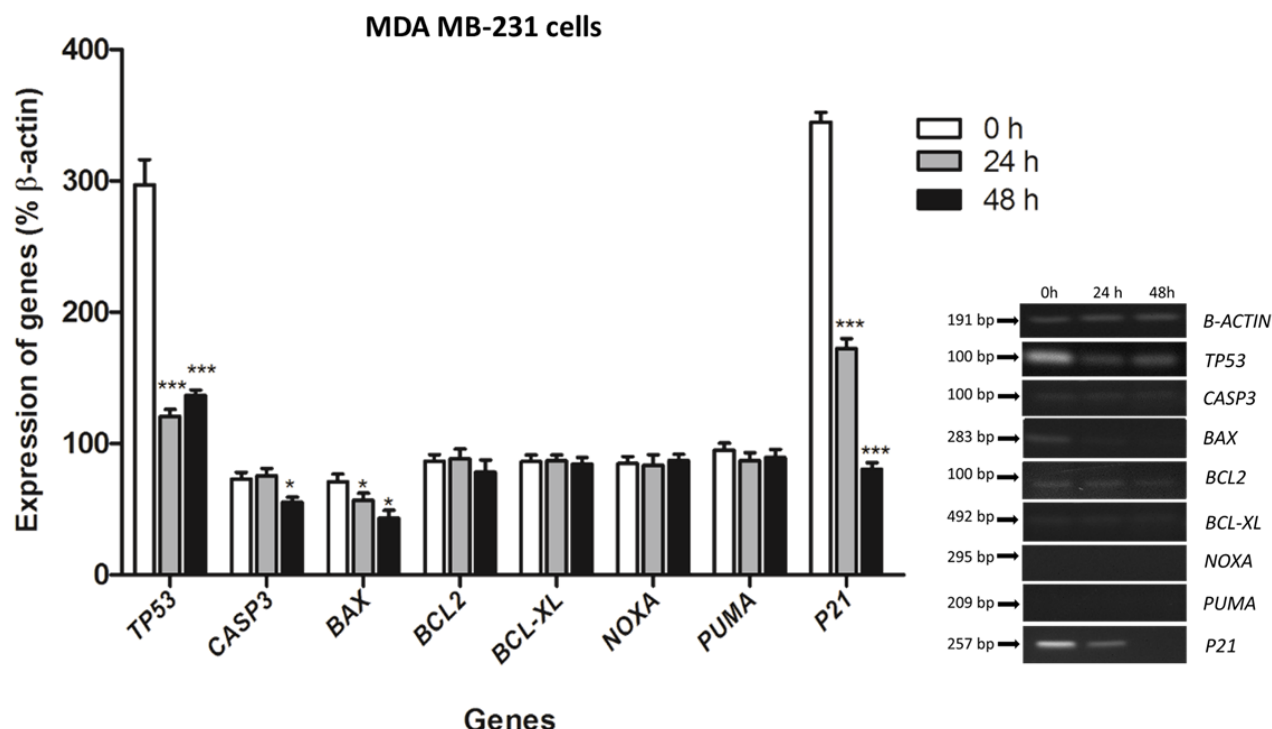


Fig 6. Relative quantification of *TP53*, *CASP3*, *BAX*, *BCL2*, *BCL-XL*, *NOXA*, *PUMA* and *P21* mRNA level by reverse transcription polymerase chain reaction assay after incubation of MDA MB-231 cells treated with $\frac{1}{4}$ IC_{50} of CP (A) extract for 0, 24 and 48 h (left). Relative signal intensity of each gene after treatment (right). Data represent mean $\pm$ SD (n = 3). *** $P < 0.05$, ** $P < 0.1$, * $P < 0.001$ vs 0 h.

CP extract inhibited MDA MB-231 cells migration

Breast cancer is associated with a higher dissemination rate (33, 34); therefore, we also performed wounding-healing experiments to determine the effects of CP on MDA MB-231 cells migration. Cells were treated with $\frac{1}{4}$ IC_{50} of CP for 24 and 48 h and the percentage of cell migration in CP treated group was of $33.24 \pm 11.02\%$ at 24 h and $51.43 \pm 9.09\%$ at 48 h vs $45.67 \pm 4.46\%$ at 24 h and $67.70 \pm 15.03\%$ at 48 h in the control group. Wound healing assay revealed that CP decreased cell migration in comparison to control (figure 7).

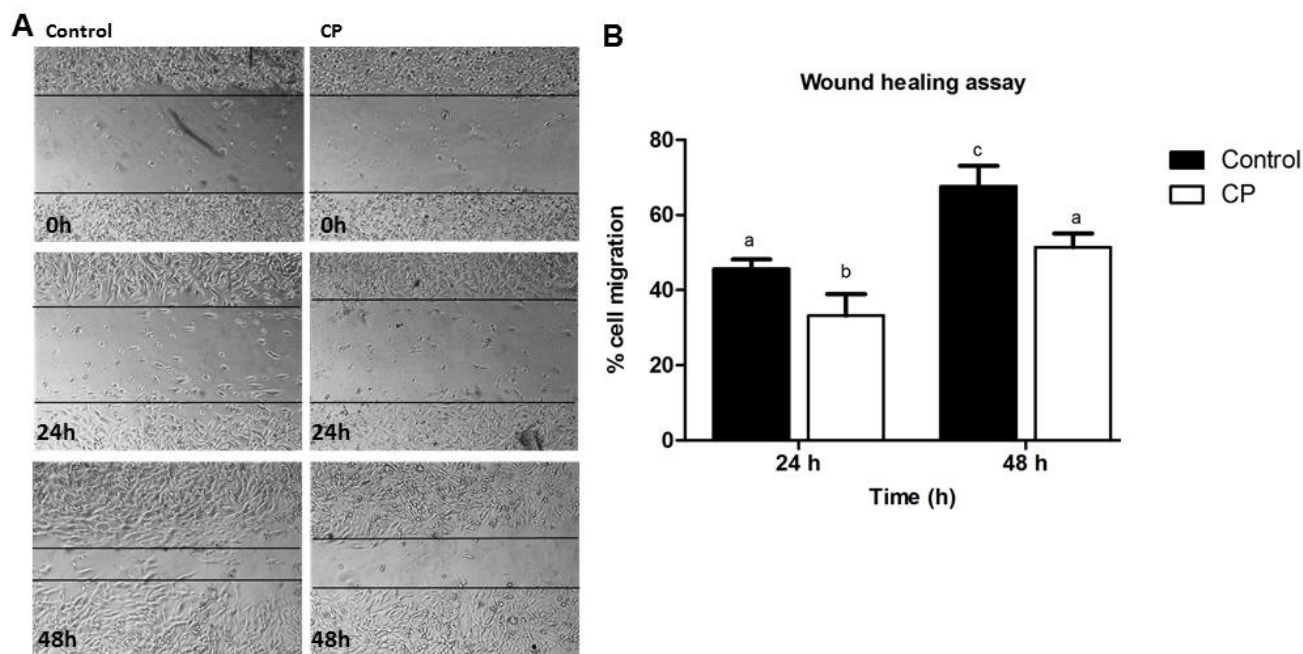


Fig 7. CP inhibited cell migration in MDA MB-231 cells. (A) Representative images of wound healing at 0, 24 and 48 h following scratch induction and CP treatment. (B) Percentage (%) of cell migration into the wound scratch with or without CP treatment.

Discussion

In this study, we demonstrated the sensitiveness of MDA MB-231 cells to the treatment with different concentrations of CP extract. MTT results showed the inhibition of cell proliferation in a concentration-dependent manner. The higher LDH release through plasma membrane by treated cells suggested that CP-induced growth inhibition occurred through its influence on membrane integrity. It has been reported that propolis cytotoxic effects on animal and human tumor cells depend on its main components (6, 7, 9-11, 13). Therefore, the main components found in our propolis, such as isoliquiritigenin, liquiritigenin, vestitol, neovestitol, isosativan, and medicarpin may be related to the cytotoxic potential of CP extract.

In breast cancer cells, constitutive activation of PI3K/Akt and ERK1/2 signal transduction pathway play an essential role in the regulation of multiple cellular processes to promote cell growth, survival, and metastasis (35-40). Based in our data comparing with inhibitors LY294002 and PD-98059, CP extract did not induced breast cancer cell death by suppression of both PI3K/Akt and ERK1/2 pathways. Several researches have shown a close relationship between PI3K/Akt, ERK1/2 pathways and apoptosis (35-40). PI3K/Akt signaling pathway can promote the

phosphorylation or inactivation of Bcl-2 family proteins, block the cytochrome C release from mitochondria and caspase function, which play an essential role in apoptosis of tumor cells (41). On the other hand, several molecules can be targeted by ERK1/2 to promote proliferation and inhibit apoptosis. They include Bcl-2 family proteins, caspase-3, cyclin D1, and death associated to protein kinase (DAPK) (40, 42, 43). These data are in agreement with the results of AO/EB and DAPI staining and indicate that CP extract induces cell death by necrosis.

In addition, CP extract decreased the expression of apoptosis related genes (*TP53*, *CASP3*, *BAX*). No alterations were seen in the expression levels of anti-apoptotic (*BCL2*, *BCL-XL*) and pro-apoptotic (*PUMA*, *NOXA*) genes. The down-regulation of *CASP3*, *BAX*, *P21* and unchanged regulation of *PUMA* and *NOXA* indicate the involvement of a transcription-p53-dependent apoptosis (44-47). It is well known that Bcl-2 family members can influence apoptosis, and alterations in the levels of anti- and pro-apoptotic genes can regulate the type of cell death program (48-51). Furthermore, the down-regulation of p21 indicated that other mechanism(s) may be triggered by CP extract to replace p21-dependent checkpoint and to induce inhibition of cell proliferation. These results prove that death pathway engaged by CP extract in MDA MB-231 cells is clearly related to necrosis. On the other hand, CP-induced ROS generation was reduced after cotreatment with the antioxidant NAC, which increased the percentage of viable cells suggesting that CP-induced necrotic cell death could be associated with ROS production.

Cell death due to necrosis is associated with mitochondrial dysfunction and we observed that our propolis sample reduced the $\Delta\Psi_m$ and increased LDH levels. When there is any cellular stresses, mitochondria exerts its pro-death function (52, 53). The consequence of mitochondrial dysfunction includes a rapid loss of $\Delta\Psi_m$, rupture of mitochondrial membrane, loss of adenosine triphosphate (ATP), excessive ROS production, intracellular $[Ca^{+2}]$ elevation, osmotic shock, loss of ion homeostasis and cell integrity, release of LDH, what finally results in necrosis (53, 54). Our findings are consistent with other reports showing that the production of ROS, LDH release, and loss of $\Delta\Psi_m$ in cancer cells are death signals related to mitochondrial-regulated necrosis pathway (55-60).

In contrast to other cytotoxic studies with propolis which showed the inhibitory effects towards a variety of tumor cells through autophagy/apoptosis (6, 61-63), our sample induced necrotic cell death. All these data, which seems initially contradictory, may be explained by

differences in the chemical composition of propolis samples obtained in different geographic zones from different botanical sources and/or by the behavior inherent to each cellular model.

Our data indicated that CP extract not only induced necrotic cell death but also the inhibition of cell migration. MDA MB-231 cancer cells are widely used as a model to study breast cancer cell metastasis (64-66). Here we found that CP extract treatment exerted a remarkably effect on the aggressive behavior of this cancer cells what is evident by decreased migration potential. Propolis composition is heterogeneous; however, this natural product and its main constituents have shown efficacy in the prevention of metastasis (67). Different components from various propolis samples conferring antimetastatic potentials have been identified as isoflavonoids, caffeic acid phenethyl ester, artemillin C, nemorosone, galangin, cardanol, among others (11, 68-71). These compounds target various genetic and biochemical pathways of cancer progression thus, chemical composition of our propolis sample could determine the good ability on inhibiting breast cancer cells metastasis.

Conclusion

This study has indicated that necrosis was the main mechanism induced by CP extract in MDA-MB 231 breast cancer cells, suppressing the growth and migration of such cells.

Therefore, CP may serve as an alternative therapy based on necrosis death mechanism and could offer an effective approach to eliminate apoptosis-resistant cancer cells. Further studies are still needed to evaluate the CP-induced necrotic pathways that provide the antitumor and antimetastatic activity of our sample propolis and its main constituents.

Competing interests

The authors declare that they have no competing interests.

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6. - CONCLUSÕES

Os resultados deste estudo permitem chegar às seguintes conclusões:

- ✓ As linhagens tumorais A549 e HEP-2 foram sensíveis ao efeito citotóxico ocasionado pela própolis brasileira. O evento envolvido na morte celular das mesmas foi a apoptose. Entretanto, a viabilidade celular da linhagem MDA MB-231 não foi afetada por esta amostra de própolis.
- ✓ As linhagens HEP-2 e MDA MB-231 foram sensíveis ao efeito citotóxico induzido pela própolis cubana. Os eventos envolvidos na morte celular das mesmas foram apoptose e necrose, respectivamente.
- ✓ O efeito citotóxico e tipo de morte celular induzido pelas amostras de própolis é dependente da composição química, a qual está relacionada à região geográfica em que a amostra foi produzida e às fontes vegetais.
- ✓ A utilização de amostras de própolis brasileira e cubana pode levar à descoberta de novas substâncias bioativas para o tratamento do câncer.