UNIVERSIDADE DE CAXIAS DO SUL

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

INSTITUTO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

Avaliação dos efeitos antitumorais da própolis vermelha em células humanas *in vitro*

Caroline Olivieri da Silva Frozza

Caxias do Sul

2016

F943a Frozza, Caroline Olivieri da Silva
Avaliação dos efeitos antitumorais da própolis vermelha em células humanas "in vitro" / Caroline Olivieri da Silva Frozza. – 2016.
125 f.: il.

Tese (Doutorado) - Universidade de Caxias do Sul, Programa de Pós-Graduação em Biotecnologia, 2016.
Orientação: João Antonio Pêgas Henriques, Mariana Roesch Ely.

1. Própolis vermelha 2. Atividade antitumoral 3. Proteômica 4. Apoptose. I. Henriques, João Antonio Pêgas, orient. II. Roesch Ely, Mariana, orient. III. Título.

Elaborado pelo Sistema de Geração Automática da UCS com os dados fornecidos pelo(a) autor(a).

Caroline Olivieri da Silva Frozza

Avaliação dos efeitos antitumorais da própolis vermelha em células humanas *in vitro*

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

Orientadores: Prof. Dr. João Antonio Pêgas Henriques

Prof[a]. Dr[a]. Mariana Roesch Ely

Caxias do Sul

2016

CAROLINE OLIVIERI DA SILVA FROZZA

AVALIAÇÃO DOS EFEITOS ANTITUMORAIS DA PRÓPOLIS VERMELHA EM CÉLULAS HUMANAS *IN VITRO*

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

Orientadores: Prof. Dr. João Antonio Pêgas Henriques

Prof.ª Dr.ª Mariana Roesch Ely

TESE APROVADA EM 05 DE DEZEMBRO DE 2016

____

Orientador: Prof. Dr. João Antonio Pêgas Henriques

____

Orientadora: Prof.ª Dr.ª Mariana Roesch Ely

____

Prof. Dr. Marcelo Maraschin

____

Prof.ª Dr.ª Dinara Jaqueline Moura

____

Prof.ª Dr.ª Suelen Osmarina Paesi

Dedico este trabalho especialmente aos meus queridos filhos Letícia Olivieri Frozza e Vítor Olivieri Frozza e ao meu amado esposo Rangel Frozza, por todo amor, incentivo e ajuda na obtenção dos meus ideais.

“*Devemos aceitar com serenidade as coisas que não podemos modificar ter coragem para modificar as que podemos e sabedoria para perceber a diferença.”*

FRANCISCO DE ASSIS

AGRADECIMENTOS

Gostaria de agradecer a Deus por iluminar a minha vida e ter me dado a oportunidade de aprender e compartilhar os meus conhecimentos.

A toda a minha família, pelo carinho e compreensão em todos os momentos que precisei, em especial, aos meus pais, por serem meus exemplos de amor e luta e aos meus irmãos por todo incentivo.

As minhas amigas Ângela e Silviane, pela amizade de longa data e companheirismo em todos os momentos.

Ao meu orientador, professor Henriques, obrigada pela colaboração e todo auxílio prestado durante o trabalho e pela ideia do projeto.

A minha orientadora, professora Mariana, obrigada pela oportunidade de desenvolver este trabalho, por acreditar no meu potencial. Além disso, agradeço pelo exemplo de amor e dedicação à pesquisa.

Ao professor Sidnei pelo auxílio com a parte química e ao professor Claus pelo empenho, apoio e confiança no projeto.

Aos professores da banca de acompanhamento, professor Sergio e professora Suelen, pelas colaborações e aos ensinamentos durante o desenvolvimento da tese.

Aos colegas do laboratório de proteômica por toda ajuda e amizade.

À secretária Lucimara Serafini Rech por todo auxílio prestado.

Por fim, a CAPES pela bolsa de estudos e ao projeto Pró-ensino na saúde, pelo auxílio financeiro. E a todos que contribuíram para a realização deste trabalho.

SUMÁRIO

LISTA DE FIGURAS ix

LISTA DE SÍMBOLOS E ABREVIATURAS x

INSTITUIÇÕES E FONTES FINANCIADORAS xi

ESTRUTURA DA TESE xii

RESUMO xiv

ABSTRACT xvi

1. INTRODUÇÃO 18

2. REVISÃO BIBLIOGRÁFICA 20

3. OBJETIVOS 39

CAPÍTULO 1 40

CAPÍTULO 2 51

CAPÍTULO 3 64

4. DISCUSSÃO 102

5. CONCLUSÕES 108

6. PERSPECTIVAS FUTURAS 109

7. REFERÊNCIAS BIBLIOGRÁFICAS 110

8. ANEXO 120

LISTA DE FIGURAS

Figura 1. A- Abelha com própolis vermelha depositada na corbícula; B- estruturas da pata da abelha. 20

Figura 2. Estrutura básica dos flavonoides e subclasses. 22

Figura 3. Estruturas químicas dos compostos Biochanina A e Genisteína. 29

Figura 4. Estrutura química do isoflavonoide Formononetina. 30

Figura 5. Estrutura química da flavona Liquiritigenina. 30

Figura 6. Morte celular por apoptose. 33

Figura 7. Representação esquemática das principais vias moleculares que conduzem à apoptose. 36

LISTA DE SÍMBOLOS E ABREVIATURAS

BH	Domínio Homólogo
DISC	Complexo de Sinalização Indutor de Morte
DNA	Ácido desoxirribonucleico (do inglês, *deoxyribonucleic acid*)
ESI	Ionização por *Electrospray*
FADD	Efetor de morte
FAZ	Receptor de morte
IC50	Concentração inibitória de 50% do crescimento celular
IG	Indicação Geográfica
INCA	Instituto Nacional do Câncer
INPI	Instituto Nacional de Propriedade Industrial
LC	Cromatografia Líquida
MALDI	Ionização e dessorção a laser assistida por matriz do inglês *Matrix-assisted laser desorption/ionization*
MOMP	do inglês *Mitochondrial Outer Membrane Permeabilization*
MS	Espectrometria de massas
MS	Ministério da Saúde
MTT	3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio
OMS	Organização Mundial da Saúde
PI	Iodeto de Propídeo
ROS	Espécies Reativas de Oxigênio (do inglês *reactive oxygen species*)
TNF	Fator de Necrose Tumoral
VEGF	Fator de crescimento vascular endotelial

INSTITUIÇÕES E FONTES FINANCIADORAS

Este trabalho foi desenvolvido principalmente nas instalações do Laboratório de Genômica, Proteômica e Reparo de DNA, do Instituto de Biotecnologia da Universidade de Caxias do Sul (UCS). Este trabalho foi subsidiado pelas agências CAPES, a qual concedeu a bolsa de doutorado através do EDITAL Nº 024 / 2010 PRÓ-ENSINO NA SAÚDE, FAPERGS, PROGRAMA PESQUISADOR GAÚCHO 02/2014 e CNPq, CHAMADA UNIVERSAL– MCTI/CNPq Nº 14/2014.

ESTRUTURA DA TESE

A presente tese está estruturada da seguinte forma: introdução geral, revisão bibliográfica, objetivos do trabalho (geral e específicos) e resultados e discussão, onde consta a descrição de três artigos separados por Capítulos. Uma discussão geral do trabalho é apresentada, seguida das conclusões obtidas, perspectivas e referências bibliográficas. O Currículo Lattes encontra-se em anexo.

A introdução apresenta aspectos relacionados à própolis e os assuntos que serão abordados na tese. A revisão bibliográfica trata sobre características importantes das própolis, em especial a própolis vermelha, proveniente da região nordeste do Brasil, sua composição química e atividades biológicas. Também aborda sua importância como fonte de agentes antitumorais e a busca de um melhor entendimento dos mecanismos celulares envolvidos nesta propriedade.

O Capítulo 1 apresenta o trabalho publicado na revista "Food and Chemical Toxicology" em 2014, o qual abordou o perfil proteico da linhagem Hep-2 tratada ou não com extrato hidroalcoólico da própolis vermelha proveniente de Sergipe, utilizando o ensaio eletroforese bidimensional, bem como, análise de indução de apoptose utilizando citometria de fluxo, análise *in situ* do anticorpo Anexina-V/Iodeto de Propídeo e coloração por Giemsa. Este trabalho também conta com a quantificação de proteínas, a qual possibilita discutir satisfatoriamente os resultados encontrados nos ensaios.

O Capítulo 2 se refere ao artigo publicado na revista "Journal of Pharmacy and Pharmacology" em 2016, o qual mostra a identificação realizada gel *free* de mais de 2000 proteínas nas duas linhagens celulares testadas, sendo elas: a linhagem Hep-2, célula tumoral de laringe, e Hek-293, célula renal não tumoral. Este artigo também apresenta a classificação destas proteínas quanto suas funções moleculares e compara o perfil proteico das linhagens que foram tratadas com o extrato hidroalcoolico de própolis

com o grupo controle. Algumas proteínas identificadas (TLN1, EPIPL, FSCN1, VINC e ROA1) apresentam-se em grande quantidade nos tecidos dos tumores de laringe comparado ao fenótipo dos tecidos sem tumor, as quais apresentaram expressão diminuída após tratamento com extratos da própolis.

O Capítulo 3 se refere ao artigo que será submetido à revista "Food and Chemical Toxicology" em 2016, o qual retrata a caracterização química do extrato hidroalcoólico da própolis vermelha proveniente de Alagoas e de duas frações provenientes desta amostra. Também apresenta o ensaio de viabilidade celular na linhagem Hep-2 pelo período de 24 h e 48 h de tratamento, bem como análise da morfologia com a coloração de Giemsa e da fluorescência com DAPI. Este artigo ainda mostra os resultados sobre a avaliação do ciclo celular, a formação de ROS, alteração do potencial de membrana das células que receberam o tratamento, bem como a marcação com Anexina-V/PI. O dano ao DNA plasmidial causado pelas amostras de própolis também é avaliado.

A discussão geral aborda os resultados dos três capítulos apresentados, a relação entre os mesmos e a importância desse estudo como contribuição científica. Por fim, é apresentada a conclusão final do trabalho desenvolvido, as perspectivas e o anexo contendo o Currículo Lattes.

RESUMO

A própolis vermelha brasileira tem atraído interesses científicos e econômicos devido às suas variadas atividades biológicas. Este produto natural possui composição química variada de acordo com a região na qual é encontrado, sendo necessária uma completa caracterização química para cada tipo de própolis, a fim de se elucidar os compostos presentes e responsáveis pela atividade biológica observada. Neste trabalho, buscou-se caracterizar quimicamente os extratos da própolis vermelha brasileira, avaliar sua atividade antitumoral, bem como alguns mecanismos de morte celular, além de investigar o padrão proteico de células tumorais de laringe (Hep-2) e não tumorais de rim (Hek-293) tratadas e não tratadas com extratos da própolis vermelha através da análise proteômica comparativa.

A caracterização química mostrou que a própolis apresenta moléculas complexas, principalmente isoflavonoides, que possuem importantes atividades biológicas, dentre elas a atividade antitumoral. A atividade citotóxica de duas frações e do extrato hidroalcoólico da própolis foi avaliada na linhagem tumoral Hep-2, a qual se revelou mais citotóxica nos tratamentos com as frações investigadas. Os ensaios de citometria mostraram que as células que receberam tratamento apresentaram menor potencial de membrana mitocondrial, maior quantidade de células em apoptose tardia (TUNEL e Anexina-V/PI), menor geração de espécies reativas do oxigênio e o aumento de fragmentação do DNA. Além disto, as colorações revelaram a presença de corpos apoptóticos e condensação de cromatina.

A análise proteômica permitiu a comparação dos mapas proteicos da linhagem Hep-2, na ausência ou presença de extratos da própolis vermelha, com a linhagem não tumoral Hek-293. Estas proteínas foram classificadas de acordo com os processos biológicos as quais estão envolvidas. Além disto, doze proteínas identificadas na

linhagem Hep-2 apresentaram expressão reduzida comparada ao grupo controle, dentre as quais muitas estão presentes em grande quantidade em outros tumores e são consideradas biomarcadores tumorais.

Os resultados, de forma geral, mostram que a própolis interfere em um conjunto de eventos intracelulares e, assim, passa a ser uma candidata promissora para inibir o crescimento celular e contribuir para os diferentes passos relacionados com o processo de carcinogênese. Embora os mecanismos moleculares pelos quais a própolis vermelha interaja com o metabolismo das células permaneçam ainda desconhecidos, estudos adicionais servirão para melhor elucidar a atividade antitumoral da própolis vermelha brasileira e de suas frações.

Palavras-chave: própolis vermelha, antitumoral, proteômica

ABSTRACT

The Brazilian red propolis has attracted scientific and economic interests due to its varied biological activities. This natural product has diversify chemical composition according to the region in which it is found, then it is necessary a complete chemical characterization for each type of propolis, in order to elucidate the present compounds and responsible for the biological activity observed. In this work, we attempted to chemically characterize the extracts of Brazilian propolis, evaluate their antitumor activity as well as some cell death mechanisms, and to investigate the protein profile of larynx tumor cells treated and not treated with propolis extracts through comparative proteomic analysis.

The chemical characterization showed that propolis has complex molecules, particularly isoflavones, which present significant biological activities, such as antitumor activity. The cytotoxic activity of two fractions and of the hydroalcoholic extract of propolis was assessed in Hep-2 tumor cell line, which proved to be more cytotoxic treatments in the investigated fractions. Flow cytometric assays showed that treated cells had lower mitochondrial membrane potential, increased amount of cells in late apoptosis (TUNEL and Annexin-V / PI), less generation of reactive oxygen species and increase in DNA fragmentation. Furthermore, the staining revealed the presence of apoptotic bodies and chromatin condensation.

The proteomic analysis has allowed comparison of the maps of the protein Hep-2 cell line, in the absence or presence of propolis extracts, with non-tumor cell line Hek-293. These proteins were classified according to the biological processes which they are related. Besides, twelve identified proteins in Hep-2 showed reduced expression from

the control group. Between them, many are presented in large quantities in other tumors and are considered tumor biomarkers.

The results in general show that propolis interferes with a series of intracellular events and hence becomes a promising candidate to inhibit cell growth and contribute to the different steps relating to the process of carcinogenesis. Although the molecular mechanisms by which propolis interact with the metabolism of the cells remain unknown, further study will serve to better elucidate the antitumor activity of Brazilian propolis and its fractions.

Keywords: red propolis, antitumor, proteomic

1. INTRODUÇÃO

Os produtos naturais apresentam estruturas químicas complexas e são muito utilizados em estudos para a descoberta de novas drogas, as quais são aplicadas como agentes inovadores na terapêutica de doenças de alta prevalência e morbidade, como o câncer. As propriedades medicinais da própolis têm sido muito estudadas ao longo da história e seus extratos vêm sendo utilizados para diversas finalidades. A própolis é um produto resinoso elaborado pelas abelhas, as quais a utilizam para se protegerem contra a invasão de insetos e para assepsia da colmeia.

No Brasil há diversos tipos de própolis, que possuem composições químicas que variam de acordo com as espécies de abelhas e a flora das diferentes regiões em que são encontradas. A própolis vermelha, encontrada no nordeste brasileiro, vem sendo estudada por apresentar características físico-químicas e biológicas distintas das demais. Dentre todas as suas atividades já descritas, destaca-se sua ação citotóxica contra células tumorais. Sabe-se que o câncer é uma doença crônica, e segundo o Instituto Nacional de Câncer (INCA), a segunda causa de morte registrada no mundo, precedida apenas de doenças de origem vascular. Desta forma, busca-se um melhor entendimento quanto à sua etiopatogenia e o desenvolvimento de novas estratégias preventivas e terapêuticas no seu combate.

A indução de apoptose tem sido um dos mecanismos propostos para os efeitos terapêuticos antitumorais da própolis. Assim, estudos citoquímicos quanto à atividade de extratos da própolis vermelha em células tumorais que avaliem marcadores relacionados à regulação de apoptose são de grande importância, uma vez que evidências demonstram que a resistência a apoptose é uma das características mais marcantes da maioria dos tumores malignos. Já os estudos proteômicos podem

contribuir para o entendimento dos mecanismos pelos quais a própolis atua nas células, uma vez que indicam o perfil de proteínas presentes no grupo de células tratadas, o qual pode ser comparado ao grupo não tratado e ainda podendo ser feita a correlação de suas quantidades. A proteômica utiliza uma combinação de métodos analíticos, tais como eletroforese bidimensional, cromatografia líquida e espectrometria de massas, o que permite esta identificação de alterações específicas e perfis proteicos sob determinadas condições, como, por exemplo, associados com as neoplasias. De modo geral, os estudos proteômicos contribuem para determinação de diagnósticos precoces, novas possibilidades terapêuticas e melhor entendimento dos mecanismos biológicos.

Tendo em vista os benefícios que a própolis vermelha tem mostrado a saúde humana, aliado a carência de estudos relacionados ao potencial biológico do seu extrato e frações em células tumorais e não tumorais, torna-se importante obter um melhor entendimento dos complexos mecanismos celulares e principalmente as suas propriedades antitumorais. A caracterização química do extrato e de suas frações, somado a estudos de expressão gênica e proteômicos, deve contribuir para o melhor entendimento das interações moleculares no que diz respeito ao uso da própolis vermelha, a qual tem se apresentado como um produto promissor com variadas possibilidades terapêuticas.

2. REVISÃO BIBLIOGRÁFICA

2.1 PRÓPOLIS

A palavra própolis é derivada do grego *pro-,* em defesa, e *polis*-, comunidade, considerado assim, um produto utilizado pelas abelhas na defesa da colmeia (Toreti *et al.*, 2013). A própolis é um produto não tóxico resinoso coletado de várias plantas, brotos ou exsudatos, misturado com enzimas salivares, pólen e cera, elaborado pelas abelhas principalmente do gênero *Apis mellifera* (Sforcin, 2016; Silva *et al.*, 2008). Para a elaboração da própolis, as abelhas fragmentam os ápices vegetativos das plantas com as mandíbulas, coletando, neste processo, as resinas liberadas e com o primeiro par de patas, transferem a massa de resina para as patas medianas e então para a corbícula (parte posterior da tíbia), nas patas posteriores (Fig. 1) (Teixeira *et al.*, 2005). A resina obtida das plantas é mastigada pelas abelhas, que adicionam enzimas salivares e o material, parcialmente digerido, é misturado com a cera de abelha e usado na colmeia (Burdock, 1998). Os resultados desta modificação enzimática são hidrólises dos compostos fenólicos como, por exemplo, os flavonoides heterosídeos (Najafi *et al.*, 2007).

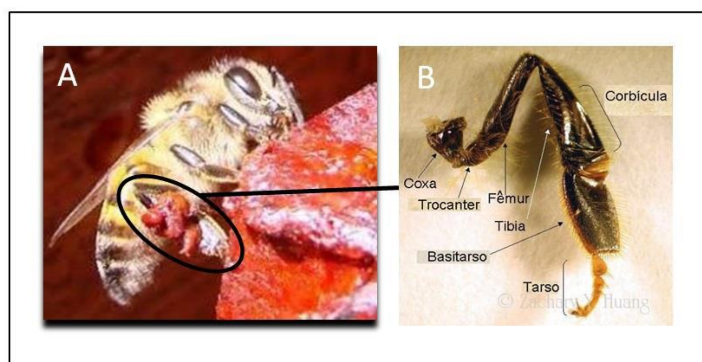


Figura 1. A- Abelha com própolis vermelha depositada na corbícula; B- estruturas da pata da abelha. Fonte: Little Blossoms

As abelhas usam a própolis para protegê-las contra insetos e microrganismos, no reparo de frestas ou danos à colmeia, no preparo de locais assépticos para a postura de ovos da abelha rainha e na mumificação de insetos invasores (Bankova, 2009; Toreti *et al.*, 2013). Nas últimas décadas, a própolis tornou-se objeto de vários estudos realizados em todo o mundo, e sua composição química e propriedades biológicas têm sido extensivamente estudadas (Silva-Carvalho *et al.*, 2015).

A própolis é constituída de aproximadamente 55% de resinas (contendo flavonoides e compostos fenólicos), 30% de ceras, 10% de óleos essenciais e aromáticos e 5% de pólen. É uma rica fonte de elementos essenciais como magnésio, cálcio, ferro e zinco, sendo que, mais de 300 componentes já foram identificados em diferentes amostras de própolis (Khalil, 2006). Os flavonoides são considerados os principais compostos responsáveis pelos efeitos benéficos da própolis, sendo a eles atribuídas as propriedades antibacteriana, antiviral, antioxidante (Barbosa *et al.*, 2009) e antitumoral (Watanabe *et al.*, 2011). Os flavonoides (Fig. 2) apresentam estrutura hidrocarbonada do tipo C6-C3-C6 nas quais as duas unidades C6 (anel A e anel B) possuem natureza fenólica (Tsao, 2010). Tais estruturas podem ser divididas em subclasses de acordo com o grau de oxidação do anel heterocíclico como: flavan-3-ol, flavona, flavonol, flavanona, antocianina e isoflavonas (Singh *et al.*, 2008). Estas últimas podem ser principalmente encontradas nas plantas da família de leguminosas e possuem na sua estrutura o anel B ligado na posição C3 do anel C (Fig. 2) (Tsao, 2010). Devido a grande quantidade de ceras a própolis deve ser purificada através da extração com solventes, os quais removem o material inerte e preservam as frações polifenólicas, neste caso o álcool é particularmente adequado para esta finalidade (Pietta *et al.*, 2002).

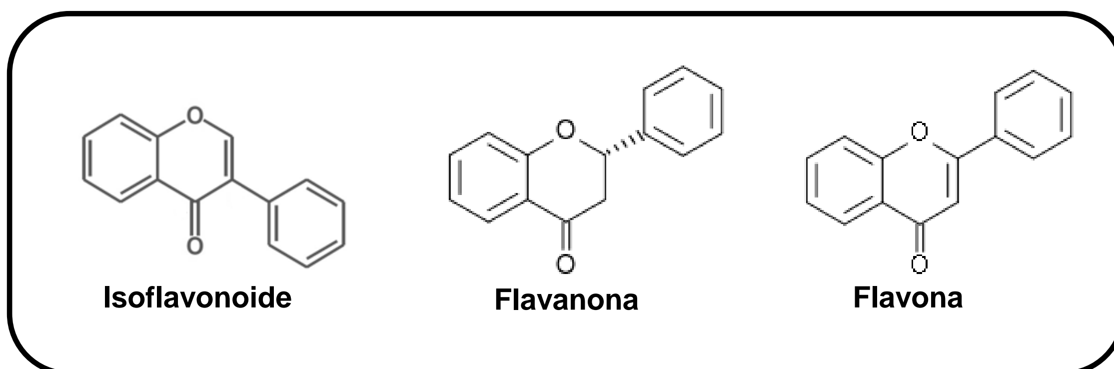


Figura 2. Estrutura básica dos flavonoides e subclasses.

A própolis é considerada uma das misturas biológicas mais heterogêneas encontradas em fontes naturais. A diversidade dos componentes químicos presentes nas própolis está relacionada aos diferentes ecossistemas de onde as abelhas fazem a coleta do material para a sua elaboração (Daleprane & Abdalla, 2013). Desta forma, o uso de amostras com caracterização química nos ensaios biológicos é a forma de investigar suas propriedades e fazer estudos comparativos, oferecendo sinais para a padronização e controle de qualidade de uma determinada amostra de própolis (Sforcin, 2016) o que é imprescindível para se garantir a eficácia e segurança deste produto natural para um mercado cada vez maior e exigente em todo o mundo (Lustosa *et al.*, 2008). Contudo há uma grande dificuldade para se estabelecer uma uniformidade deste material o qual pode proceder de diversas origens geográficas (Machado *et al.*, 2016).

A própolis é um dos muitos produtos naturais que vem sendo utilizado desde a antiguidade (Kuropatnicki *et al.*, 2013). Os egípcios conheciam as propriedades antiputrefativas da própolis e a empregavam para embalsamar cadáveres (Toreti *et al.*, 2013). Além disso, a própolis tem sido amplamente utilizada por várias civilizações para o tratamento de resfriados, feridas e úlceras devido a suas propriedades antissépticas e anestésicas e ela também tem sido utilizada na medicina complementar

devido a suas atividades antimicrobianas, anti-inflamatórias, antitumorais, imunomoduladoras, antioxidantes, entre outras (Sforcin, 2016).

2.2 PRÓPOLIS VERMELHA

As colorações das própolis podem variar de amarelo claro até marrom avermelhado dependendo de sua procedência, elas possuem sabor resinoso e odor aromático (Salatino *et al.*, 2011). A própolis vermelha, denominada assim devido a sua intensa coloração vermelha, é encontrada principalmente na região Nordeste brasileira, nos estados de Sergipe, Alagoas, Bahia, Pernambuco e Paraíba (Daugsch *et al.*, 2008). Segundo Silva *et al.*, (2008), a sua origem botânica é a planta *Dalbergia ecastophyllum* (L.) Taub. da família *Leguminosae*, chamada popularmente como rabo-de-bugio. Geograficamente a *D. ecastophyllum* distribui-se no continente americano, da Flórida (EUA) ao Brasil, desde o litoral Norte até Palhoça-SC, ocorrendo também no continente africano (Souza, 2010). É uma espécie escandente, associada a estuários, mangues e dunas e bem adaptada às condições de alta salinidade (Souza, 2010).

O Brasil, por meio do Estado de Alagoas, passou a ser reconhecido como produtor dessa espécie de própolis com a denominação de origem (IG 201101) obtida em julho de 2012, pelo Instituto Nacional de Propriedade Industrial (INPI, 2016).

Uma quantidade de grupos de compostos tem sido identificada na própolis vermelha brasileira, incluindo isoflavonoides, pterocarpanos, chalconas, flavanona (Liquiritigenina), benzofenonas preniladas, terpenos e taninos (Lopez *et al.*, 2014). Muitos destes compostos nunca foram citados nos outros 12 tipos de própolis brasileiras, o que torna a composição química da própolis vermelha exclusiva. Os marcadores químicos da própolis vermelha brasileira são os isoflavonoides, incluindo a

Formononetina, Biochanina A, Pinocembrina e Medicarpina (Freires *et al.*, 2016). Os isoflavonoides, os quais pertencem à classe dos flavonoides, têm sido tema de intensa pesquisa médica nas últimas décadas com funções benéficas contra várias doenças humanas, dentre elas o câncer (Ahmad *et al.*, 2013). As isoflavonas obtidas a partir de soja são as mais amplamente caracterizadas e estudadas, sendo em particular, a genisteína (Fig. 3) a mais amplamente investigada (Ahmad *et al.*, 2013).

A composição química complexa da propolis vermelha brasileira remete a várias atividades biológicas. A própolis vermelha é conhecida pelos seus benefícios à saúde humana e entre as atividades biológicas estudadas encontram-se a antibacteriana (Lopez *et al.*, 2015), antifúngica (Pippi *et al.*, 2015; Siqueira *et al.*, 2009), anti-inflamatória (Cavendish *et al.*, 2015), antiulcerativa (Pinheiro, 2009), antioxidante (Cabral, 2009; Righi *et al.*, 2011), antiparasitária (Ayres *et al.*, 2007), cicatrizante de feridas (Jacob *et al.*, 2015) e antitumoral (Begnini *et al.*, 2014; Frozza *et al.*, 2013; Li *et al.*, 2008). No entanto, uma vez que existem diferenças na composição químicas de diferentes amostras, estas atividades podem não estar presentes em todas as própolis vermelha.

2.3 ATIVIDADE ANTITUMORAL DA PRÓPOLIS VERMELHA

O câncer é um importante problema de saúde pública mundial (Siegel *et al.*, 2016) sendo que o número de novos casos estimados para 2020, excluindo o câncer de pele não melanoma, chegará a mais de 17 milhões e o número de mortes ultrapassará 10 milhões (OMS, 2016). Segundo o Instituto Nacional do Câncer (INCA/MS, 2016) o termo câncer é empregado para designar mais de uma centena de diferentes doenças que têm em comum o crescimento desordenado de células, que invadem tecidos e órgãos podendo espalhar-se para outras regiões do corpo. O último processo é referido como metástase, as quais são a principal causa de morte por câncer (OMS, 2016).

No Brasil, as estimativas realizadas para o biênio 2016-2017 apontam a ocorrência de mais de 420 mil novos casos de câncer, excluindo os casos de pele não melanoma, reforçando a magnitude do problema do câncer no país (INCA/MS, 2016). Os tipos mais incidentes para o sexo masculino são próstata, pulmão e intestino; e para o sexo feminino, mama, intestino e colo do útero (INCA/MS, 2016). Estas estimativas reforçam a necessidade de compostos anticancerígenos, os quais podem ser obtidos da triagem a partir de fontes naturais.

O câncer resulta de mutações em genes que normalmente controlam crescimento, a divisão, o reparo de DNA e morte celular (Dunn, 2012). Estas mutações podem ser acidentais, devido à predisposição genética, oriundas de fatores ambientais (radiações, produtos químicos), ou devido à presença de vírus, os quais podem danificar o DNA. Podem-se levar anos ou décadas, para que exista uma combinação de mutações, as quais não sofreram reparo, que possa levar ao surgimento de um câncer, por isto que geralmente ele acomete pessoas com maior idade (Dunn, 2012). Este processo, chamado carcinogênese é composto por três estágios. No estágio de iniciação, as células sofrem o efeito dos agentes cancerígenos ou carcinógenos que provocam modificações em alguns de seus genes. No estágio de promoção, a célula iniciada sofre o efeito dos agentes cancerígenos classificados como oncopromotores e é transformada em célula maligna, de forma lenta e gradual. No terceiro, estágio de progressão, as células alteradas se multiplicam de forma descontrolada e irreversível (INCA/MS, 2016).

A busca por substâncias bioativas é um dos principais objetivos da química medicinal. No entanto, a descoberta de substâncias seletivas, ou seja, que atuem exclusivamente em células cancerígenas permanece um desafio na pesquisa do tratamento do câncer. Os atuais medicamentos antitumorais disponíveis no mercado não são alvo específicos e possuem efeitos adversos e complicações, o que destaca uma

necessidade urgente para uma abordagem terapêutica mais efetiva e menos tóxica (Patel & Goyal, 2012).

Existe hoje uma grande tendência para o aproveitamento de recursos naturais na terapêutica de tumores (Newman & Cragg, 2016). Os produtos naturais desempenham um papel importante na terapia do câncer, uma vez que numerosos agentes anticancerígenos usados na clínica são naturais ou derivados de produtos naturais, os quais podem ser provenientes de diversas fontes, como plantas, animais, micro-organismos, inclusive de origem marinha (Khalid *et al.*, 2016; Nobili *et al.*, 2009). Neste contexto, a própolis e seus derivados que possuem suas propriedades antitumorais já comprovadas são de grande interesse para a comunidade científica.

Mesmo na área do câncer, é comum a utilização de produtos naturais a fim de descobrir e desenvolver um medicamento. Desde a década de 1940 até o fim de 2014, das 175 pequenas moléculas aprovadas, 131 (75%), eram produtos naturais ou compostos diretamente derivados deles (Newman & Cragg, 2016). Watanabe *et al.,* (2011) relataram que amostras de própolis de vários países (Brasil, China, Grécia, México, entre outros) foram testadas em diferentes células tumorais e apresentaram capacidade antitumoral dose e linhagem dependentes, sendo que os principais mecanismos descritos foram: indução de apoptose, parada do ciclo celular e interferência nas vias metabólicas. Os testes com culturas celulares (método *in vitro*) vêm, neste contexto, sendo utilizados com sucesso por serem reprodutíveis, rápidos, sensíveis e financeiramente viáveis. Extratos de própolis vermelha brasileira, ou frações destes, já foram testados em várias células humanas, conforme apresentado no Quadro 1.

Quadro 1. Relação das células tumorais sensíveis ao tratamento com amostras de própolis vermelha brasileira ou compostos derivados.

Linhagem Celular	IC50 µg/mL (tempo de trat.)	Referência*
Mieloma múltiplo (RPMI-8226) Leucemia promielocítica (HL60) Leucemia mielóide crônica (K562)	n.d.	Silva, (2007)[a]
Adenocarcinoma cervical (HeLa)	7,45 (48 h)	Alencar *et al.,* (2007)[b]
Câncer pancreático (PANC-1)	n.d.	Awale *et al.,* (2008)[c]
Carcinoma de colon (26-L5) Melanoma murino (B16-BL6) Adenocarcinoma de pulmão (A549) Fibrosarcoma (HT-1080)	n.d.	Li *et al.,* (2008)[b]
Carcinoma hepatocelular (HepG-2)	n.d.	Filardi, (2010)[d]
Câncer de próstata (RC-58T/h/AS)	2,75 (48 h)	Moraes *et al.,* (2010)[e]
Leucemia crônica mieloide (K562) Leucemia promielocítica (HL60 e NB4) Linfoma de Burkitt (Raji e Ramos) Células precursora de leucemia (B15, REH, Nalm16, Nalm6, RS4)	Aprox. 15 (48 h) Aprox. 15 (48 h) Aprox. 15 (48 h) Aprox. 15 (48 h)	Franchi *et al.,* (2012)[b]
Câncer de mama (MCF-7)	n.d.	Kamiya *et al.,* (2012b)[b]
Carcinoma de laringe (Hep-2) Adenocarcinoma Cervical (HeLa)	63,4 (24 h) 81,4 (24 h)	Frozza *et al.,* (2013)[b]
Carcinoma de bexiga (5637)	95 (24 h)	Begnini *et al.,* (2014)[b]
Leucemia crônica mieloide (K562) Leucemia promielocítica aguda (HL60) Multiplomieloma (RPMI 8226) Melanoma murino (B16F10)	42,1 (48 h) 29,7 (48 h) 36,5 (48 h) 32,8 (48 h)	Novak *et al.,* (2014)[b]
Glioblastoma (SF-295) Câncer de ovário (OVCAR-8) Câncer de colon (HCT-116)	34,27 (72 h) 28,76 (72 h) 25,26 (72 h)	de Mendonça *et al.,* (2015)[b]

n.d. Não disponível. *Metodologia: [a] azul de tripan, [b] MTT, [c] Kit WST-8, [d] Vermelho neutro, [e] MTS.

Os principais mecanismos antiproliferativos da própolis vermelha brasileira em geral e dos seus compostos isolados incluem a indução de apoptose através da ativação da caspase-3, a inibição da proliferação celular e da angiogênese *in vitro* e a inibição do desenvolvimento de tumores em roedores (Freires *et al.*, 2016; Pinheiro *et al.*, 2014). Porém, o mecanismo exato de atuação contra as células tumorais permanece indefinido, uma vez que cada amostra possui particularidades únicas.

Os resultados descritos até o momento sugerem que a própolis vermelha brasileira e alguns de seus constituintes agem através de todos esses mecanismos e, portanto, poderiam ser bons candidatos para desenvolvimento futuro de uma droga anticâncer (Freires *et al.*, 2016). Alguns compostos presentes na própolis possuem ação antitumoral já comprovadas, dentre estes estão a Biochanina A, a Formononetina e a Liquiritigenina.

Biochanina A (isoflavona 4'-metoxi-5,7-di-hidroxi) é uma isoflavona bioativa encontrada em trevo vermelho, grão de bico, e várias outras fontes vegetais, e assim como outras moléculas pequenas, ela tem se tornado popular nos últimos anos como opção de tratamento de diferentes tipos de cânceres (Jain *et al.*, 2015). Como exemplos, a Biochanina A reduziu a sobrevivência de células de carcinoma da mama (Sehdev *et al.*, 2009), em carcinoma de células escamosas orais (Johnson *et al.*, 2010) e de câncer pancreático (Bhardwaj *et al.*, 2014). Estes estudos demonstraram que ela reduz a progressão do câncer principalmente através da inibição da proliferação, invasão e sinalização celular. A Biochanina A é uma análoga da genisteína (Fig. 3) e é considerada menos tóxica, além do mais, ela representa um melhor agente preventivo do câncer de mama do que a genisteína uma vez que ela proporcionou alterações

quimiopreventivas benéficas em concentrações que podem ser obtidas através da ingestão diária (Moon *et al.*, 2007).

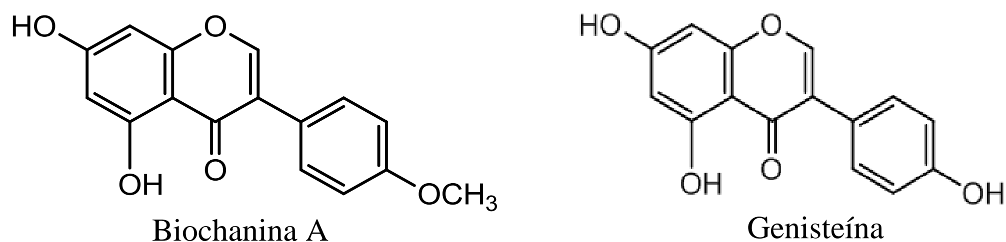
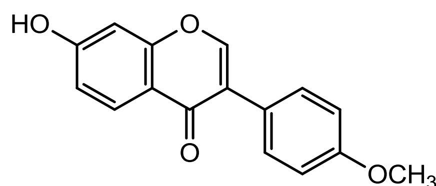


Figura 3. Estruturas químicas dos compostos Biochanina A e Genisteína.

A Formononetina (7-hidroxi, 4'-metoxi-isoflavona) (Fig. 4) é uma isoflavona encontrada nas raízes de *Astragalus membranaceus*, *Trifolium pratense* (Trevo Vermelho), *Glycyrrhiza glabra* (Alcaçuz) e *Pueraria lobata* (chamada pelos japoneses de Kudzu) (Zhou *et al.*, 2014). Essa substância e seus derivados apresentam ação anti-inflamatória, antioxidante (Cavendish *et al.*, 2015), anti-hipertensiva (Sun *et al.*, 2013) dentre outras. Ela é responsável, pelo menos parcialmente, pela atividade antimicrobiana da própolis vermelha (Neves *et al.*, 2016) e inibe a proliferação de diversas células cancerosas *in vivo* e *in vitro* (Yang *et al.*, 2014; Zhou *et al.*, 2014).

Com relação ao mecanismo de ação antitumoral a Formononetina inibe a proliferação de células de câncer de pulmão através da parada de ciclo celular e indução de apoptose (Yang *et al.*, 2014). Inibe a migração e invasão de células de câncer de mama através da redução da expressão de metaloproteinases de matriz 2 e 9, as quais se apresentam com elevada expressão no câncer de mama através da via de sinalização do fosfatidilinositol-3-quinase/serina-treonina quinase (PI3K/Akt) (Zhou *et al.*, 2014).



Formononetina

Figura 4. Estrutura química do isoflavonoide Formononetina.

O flavonoide Liquiritigenina, 4′,7-diidroxiflavanona (Fig. 5) é uma flavanona presente em *Radix glycyrrhizae*, que possui estrutura polifenólica. Há registros que este composto possui múltiplos efeitos biológicos, tais como anti-inflamatório e atividade citotóxica contra diversas linhagens cancerosas humanas *in vitro* (Zhang *et al.*, 2009) e *in vivo* (Zhou *et al.*, 2010). Os mecanismos propostos para a inibição do crescimento de células tumorais *in vitro* foram pela via apoptótica, a qual foi provavelmente mediada pela geração de espécies reativas de oxigênio em células de carcinoma hepatocelular (Zhang *et al.*, 2009) e apoptose pela via mitocondrial, a qual foi associada ao aumento da expressão de p-53, liberação do citocromo C e aumento da atividade das caspases 3 e 9 em células de câncer cervical (HeLa) (Liu *et al.*, 2011). Além disso, ela reduziu a angiogênese de maneira dose dependente, sendo este efeito associado à inibição do fator de crescimento vascular endontelial (VEGF) (Liu *et al.*, 2012).

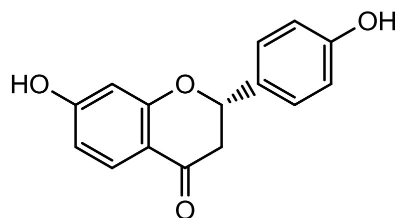


Figura 5. Estrutura química da flavona Liquiritigenina.

2.4 VIAS DE MORTE CELULAR E RESPOSTA CELULAR

O balanço entre a proliferação celular e a morte celular regula e controla o número de células no organismo. As características de cada tipo de morte celular estão intimamente ligadas à cascata de eventos bioquímicos e fisiológicos que levam a mudança na síntese de macromoléculas, na homeostase celular, na regulação do volume celular e por último na perda da viabilidade celular (Tinari *et al.*, 2008).

A morte celular pode ser classificada de acordo com suas características morfológicas (apoptose, necrose ou relacionada à mitose); por critérios enzimáticos (com ou sem o envolvimento de nucleases ou de classes diferentes de proteases, tais como caspases, calpaínas, catepsinas e transglutaminases); por características funcionais (programada ou acidental, fisiológica ou patológica); ou por aspectos imunológicos (imunogênico ou não imunogênico) (Kroemer *et al.*, 2009). Além desta classificação, existem diferentes tipos de mortes celulares classificadas pela via de sinalização, as quais são: morte celular autofágica, cornificação, netosis, partahanatos e Anoikis (Vural *et al.*, 2013). A utilização de termos adicionais, definidos por uma série de propriedades bioquímicas, demonstra que a presença de características morfológicas específicas não é suficiente para estabelecer todo o processo de morte celular. Ao menos, *in vitro*, a ativação das caspases, deixa de ser uma exigência para os múltiplos casos de apoptose. A geração de espécies reativas de oxigênio e espécies reativas de nitrogênio, que eram associadas a apoptose, é reconhecida, também, em alguns casos de necrose. Da mesma maneira, a exposição da fosfatidilserina, um componente fosfolipídico, deixa de ser uma prerrogativa de apoptose e passa a constituir uma característica precoce de parthanatos e netosis (Galluzzi *et al.,* 2012).

Dentre os diversos mecanismos existentes, a indução de apoptose tem sido um dos mecanismos propostos para os efeitos terapêuticos antitumorais da própolis (Begnini *et al.*, 2014). O termo apoptose, também conhecida como morte celular programada, é definido por várias características morfológicas e bioquímicas únicas, sendo suas diferentes formas essenciais para a homeostase do tecido (Misra *et al.*, 2016). É caracterizada por encolhimento celular, formação de vesículas de membrana, condensação do núcleo e a fragmentação (Misra *et al.*, 2016). A desregulação da apoptose e sua disfunção pode contribuir para uma variedade de condições patológicas, tais como o câncer, infecções virais e doenças imunológicas (Misra *et al.*, 2016)

As células cancerosas podem utilizar alguns dos vários mecanismos moleculares para evitar a apoptose e adquirir resistência aos agentes apoptóticos, por exemplo, expressando proteínas anti-apoptóticas, como a Bcl-2, ou diminuindo a expressão ou provocando a mutação de proteínas que são pró-apoptóticas, tais como a BAX (Hassan *et al.*, 2014).

A apoptose é causada pelas proteases, conhecidas como caspases. Estas proteínas decompõem componentes celulares necessários para a função celular normal, incluindo proteínas estruturais, proteínas do citoesqueleto e nucleares, como enzimas para o reparo de DNA. As caspases também podem ativar outras vias de degradação enzimática, tais como DNAases, que começam a clivar o DNA do núcleo (Hassan *et al.*, 2014).

Durante o processo de apoptose, as células exibem morfologia distinta. Tipicamente, a célula começa a encolher após a clivagem de filamentos de actina no citoesqueleto. A repartição apoptótica da cromatina no núcleo, muitas vezes leva à condensação nuclear e/ou uma aparência de "ferradura". As células continuam a encolher, empacotam-se em uma forma que permita a sua remoção por macrófagos (Fig. 6). Estas células fagocíticas são responsáveis pela eliminação de células apoptóticas a partir de tecidos de um modo limpo e

organizado que evita muitos dos problemas associados com a morte celular por necrose. A fim de promover a sua fagocitose por macrófagos, as células apoptóticas frequentemente sofrem alterações de membrana plasmática que desencadeiam a resposta de macrófagos. Uma dessas modificações é a translocação de fosfatidilserina a partir do interior da célula para a superfície exterior. As fases finais de apoptose são muitas vezes caracterizadas pelo prolongamento da membrana celular ou aparecimento de pequenas vesículas, que dão origem aos chamados corpos apoptóticos (Hassan *et al.*, 2014).

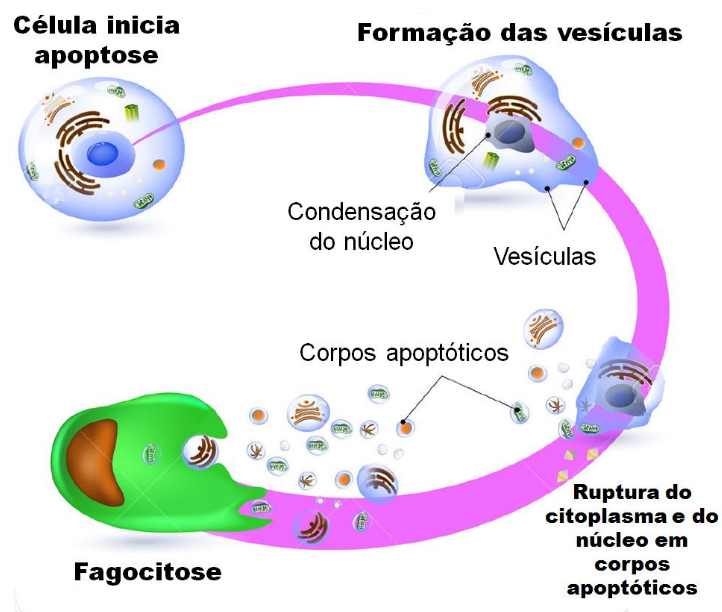


Figura 6. Morte celular por apoptose. Fonte: Banco de imagens *Royalty Free*

A apoptose extrínseca indica uma forma de morte induzida por sinais extracelulares que resultam na ligação de ligantes a receptores de transmembrana específicos, conhecidos como receptores de morte que pertencem à família TNF (Fator de Necrose Tumoral) (Fig. 7). Todos os receptores de morte funcionam de forma semelhante, ou seja, várias moléculas receptoras de ligantes são reunidas e submetidas a alterações conformacionais que permitem a montagem de um grande complexo multiproteína conhecido como Complexo de

Sinalização Indutor de Morte (DISC) que conduz à ativação da cascata de caspases. Neste complexo, o ligante FAS (receptor de morte), através de um domínio com 80 aminoácidos altamente conservados, conhecido como domínio de morte, se liga a uma molécula adaptadora chamada FADD, proteína associada a um domínio efetor de morte. Essas moléculas têm a capacidade de recrutarem a caspase-8 que irá ativar a caspase-3, desencadeando a morte por apoptose (Favaloro *et al.*, 2012).

A via intrínseca é ativada em resposta a condições estressantes, incluindo danos no DNA, o estresse oxidativo e muitos outros, as quais levam a permeabilização da membrana mitocondrial externa (MOMP do inglês *mitochondrial outer membrane permeabilization*). Esta por sua vez resulta na dissipação do potencial de membrana mitocondrial e, por conseguinte, na cessação da produção de ATP, assim como a liberação de uma série de proteínas que contribuem para ativação das caspases.

As proteínas da família Bcl-2, são reguladoras essenciais deste tipo de apoptose e são todas caracterizadas pela presença de pelo menos um domínio homólogo (BH). Do ponto de vista funcional, elas podem ser classificadas em membros anti-apoptóticos que contêm três ou quatro BH (tais como Bcl-2, Bcl-XL, Bcl-w, Mcl-1) e em membros pró-apoptóticos com dois ou três BH (tais como Bax, Bak, Bcl-xs, Bok) ou com apenas um BH (como Bad, Bik, Bid, Bim, Noxa, Puma). Proteínas pró-apoptóticas mediam a apoptose por perturbar a integridade da membrana que formam poros, enquanto os membros anti-apoptóticos impedem a apoptose através da interferência com a agregação dos membros pró-apoptótico.

Em qualquer caso, uma vez que MOMP ocorre, numerosas proteínas são libertadas a partir da mitocôndria. Uma vez que o citocromo C é liberado, ele se liga a APAF-1 induzindo a formação de um grande complexo, conhecido como apoptossomo, que recruta a caspase 9 a qual é ativada, clivada e, desta forma, ativa a caspase 3 que vai desencadear a apoptose (Fig. 7).

Há vários estudos com própolis, oriundos de diversas localidades do mundo, e seus compostos que comprovam o aumento da indução de apoptose (Oršolić, 2010), tais como: nas células endoteliais (Ohta *et al.*, 2008), células cancerígenas da próstata (Szliszka *et al.*, 2009), células ganglionares da retina (Inokuchi *et al.*, 2006) e em células leucêmicas (Bufalo *et al.*, 2009). Segundo Sawicka et al., (2012), a própolis e os seus compostos podem induzir a via intrínseca de apoptose através da liberação de citocromo C, da mitocôndria para o citoplasma, levando a cascata de caspase e ativando as proteínas pró-apoptóticas: Bax, Bad, p53, e p21.

O estado morfofuncional celular por apoptose pode ser detectado por várias técnicas, entre elas: externalização da fosfatidilserina detectada por Anexina V, alterações morfológicas evidenciadas por microscopia eletrônica, fragmentação de DNA pela técnica de TUNEL, cometa, coloração com DAPI, gel de eletroforese e citometria de fluxo, porém cada uma desta apresenta vantagens e desvantagens. Desta forma, é necessário que a sua detecção seja realizada em vários estágios para que o mecanismo de indução apoptose seja determinado de forma precisa e o mesmo possa ser utilizado como alvo terapêutico (Archana *et al.*, 2013).

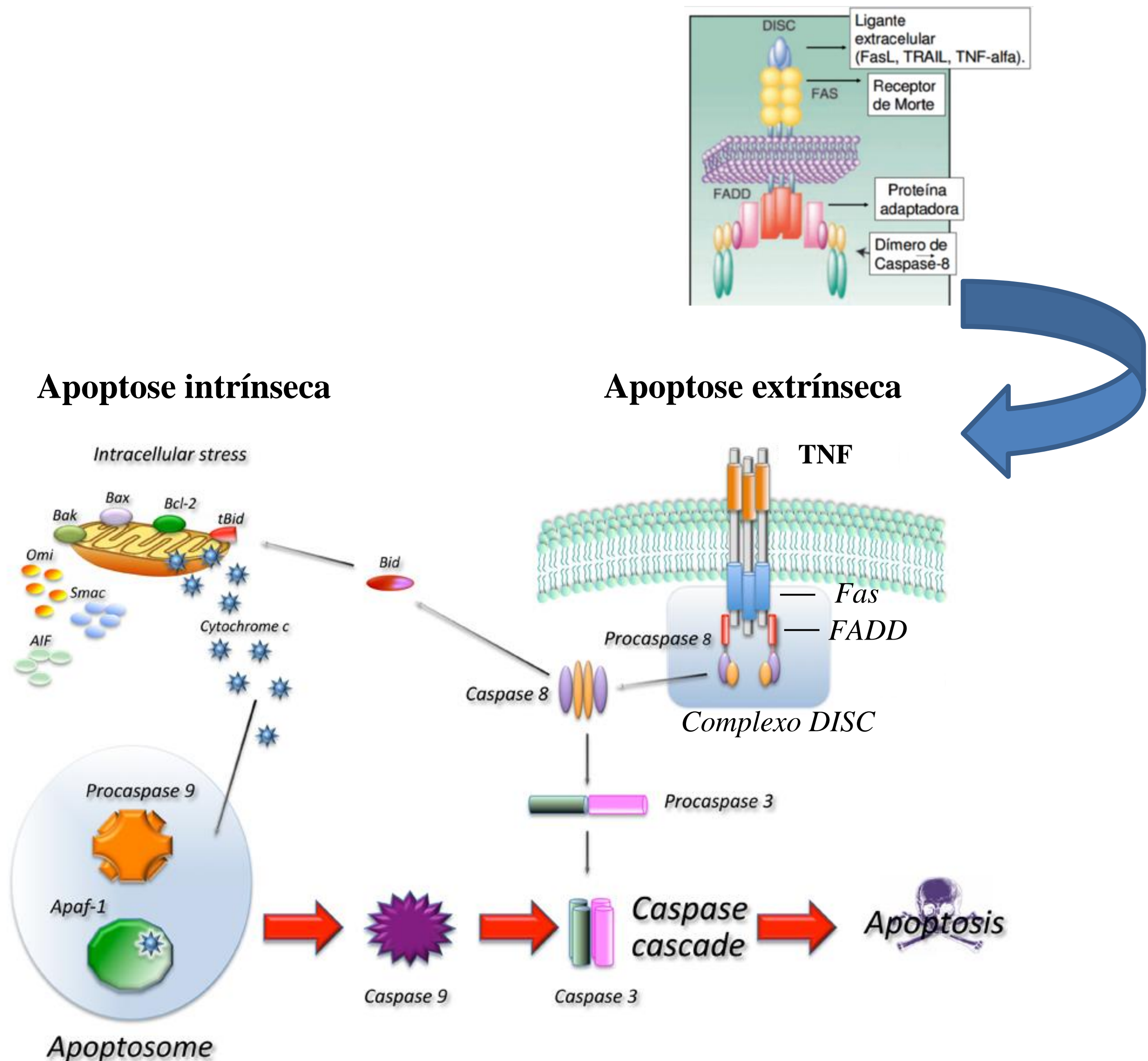


Figura 7. Representação esquemática das principais vias moleculares que conduzem à apoptose. Na via extrínseca por ligação a receptores específicos o DISC complexo é formado e caspase 8 é ativada. Na via intrínseca de liberação de citocromo C das mitocôndrias resulta na formação do apoptossomo e a ativação da caspase 9. Ambas as caspase 8 e 9, ativam a caspase 3, resultando em morte celular. Os dois percursos estão ligados através da clivagem de BID, proteína que contém domínios homólogos aos encontrados nas proteínas Bcl. Fonte: Favarolo *et al.,* (2012).

Conforme foi relatado, nas vias de sinalização celular ocorre a alteração de muitas proteínas que estão envolvidas tanto na replicação celular quanto na morte celular. A abordagem proteômica tem permitido estudos em larga escala da expressão proteica em diferentes tecidos e fluidos corporais, em condições e/ou momentos distintos. Ao longo das últimas décadas, avanços consideráveis foram realizados, tanto no diagnóstico precoce quanto na precisão do prognóstico de muitos tipos de câncer, devido ao desenvolvimento de novas estratégias proteômicas, no entanto, continua sendo difícil a padronização das abordagens proteômicas (Huang *et al.*, 2016). Atualmente, a proteômica abrange diversos campos de pesquisa, que se estendem desde o perfil proteico para análise das vias de sinalização até o desenvolvimento de métodos para testar biomarcadores proteicos. É importante notar que dentro de cada área de pesquisa, são realizados questionamentos científicos distintos e, desta forma, devem ser aplicadas abordagens proteômicas diferentes, sendo que estas abordagens podem variar amplamente quanto a sua versatilidade, maturidade técnica, dificuldade e custo (Mallick & Kuster, 2010).

Duas tecnologias foram fundamentais para avanço recente e rápido da proteômica: a emergência de novas estratégias para o sequenciamento de peptídeos usando a espectrometria de massas (MS), incluindo o desenvolvimento das técnicas de *soft* ionização, tais como a ionização por *electronspray* (ESI) e ionização/dessorção a laser assistida por matriz (MALDI); e segundo a automação da cromatografia liquida. Ambas as tecnologias permitem a medida e a identificação de peptídeos em uma taxa de milhares de sequencias por análise com melhor sensibilidade em amostras biológicas complexas (Mallick & Kuster, 2010).

Entre os estudos proteômicos apresentados, a linhagem tumoral Hep-2 tem sido utilizada como modelo experimental para avaliar o padrão proteico obtido após tratamento

com diversas drogas antitumorais, ou para fins diagnósticos e prognósticos (Bianchi *et al.*, 2011; Frozza *et al.*, 2016; Kim *et al.*, 2010), inclusive em 2014, o tecido de carcinoma de laringe foi submetido a analise proteômica comparada ao tecido adjacente normal com busca a alvos terapêuticos e de diagnóstico (Li *et al.*, 2014), sendo que neste trabalho foram encontrados quatro novos biomarcadores e uma proteína (PFN1) relacionada à metástase do câncer de laringe. Embora haja ainda desafios, não existem dúvidas de que os resultados de abordagens proteômicas são potencialmente úteis em diversas áreas da pesquisa clínica, entre elas, diagnóstico, monitoramento de resposta à terapia, predição de desfecho clínico, classificação de subtipos de doenças, determinação de riscos, caracterização de vias metabólicas, quantificação de biomarcadores e geração de alvos terapêuticos (Walsh *et al.*, 2010).

3. OBJETIVOS

3.1 OBJETIVO GERAL

Caracterizar quimicamente e avaliar o efeito antitumoral do extrato hidroalcoólico e das frações da própolis vermelha em cultivos de células tumorais, visando ampliar os conhecimentos dos mecanismos de ação da própolis.

3.2 OBJETIVOS ESPECÍFICOS

- Fracionar o extrato hidroalcoólico da própolis vermelha;
- Caracterizar quimicamente os compostos presentes nas frações e no extrato hidroalcoólico da própolis vermelha;
- Comparar o extrato hidroalcoólico da própolis vermelha de Sergipe e de Alagoas quimicamente e quanto à capacidade de induzir apoptose em células tumorais;
- Avaliar a viabilidade celular frente ao efeito biológico do extrato hidroalcoólico e das frações da própolis vermelha em culturas de células humanas tumorais;
- Avaliar a morfologia celular e indução de apoptose das culturas tratadas com extrato hidroalcoólico e frações da própolis vermelha;
- Analisar a geração de espécies reativas de oxigênio, o potencial de membrana mitocondrial e o ciclo celular nas células tumorais tratadas com extrato hidroalcoólico e frações da própolis vermelha;
- Analisar o dano ao DNA plasmidial após exposição ao extrato hidroalcoólico e frações da própolis vermelha;
- Identificar e comparar as proteínas expressas em células tumorais e não tumorais, após exposição ao extrato hidroalcoólico da própolis vermelha brasileira;

CAPÍTULO 1

Proteomic analysis identifies differentially expressed proteins after red propolis treatment in Hep-2 cells

Artigo publicado na revista *Food and Chemical Toxicology*

Food and Chemical Toxicology 63 (2014) 195–204

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Proteomic analysis identifies differentially expressed proteins after red propolis treatment in Hep-2 cells

Caroline Olivieri da Silva Frozza [a], Tanara da Silva Ribeiro [a], Gabriela Gambato [a], Caroline Menti [a], Sidnei Moura [b], Paulo Marcos Pinto [c], Charley Christian Staats [d], Francine Ferreira Padilha [e], Karine Rech Begnini [f], Priscila Marques Moura de Leon [f], Sibele Borsuk [f], Lucielli Savegnago [f], Odir Dellagostin [f], Tiago Collares [f], Fabiana Kömmling Seixas [f], João Antonio Pêgas Henriques [a,*], Mariana Roesch-Ely [a,*]

[a] *Laboratory of Genomics, Proteomics and DNA Repair, Biotechnology Institute, University of Caxias do Sul, RS, Brazil*
[b] *Laboratory of Biotechnology of Natural and Synthetics Products, Biotechnology Institute, University of Caxias do Sul, RS, Brazil*
[c] *Laboratory of Applied Proteomics Campus São Gabriel, Federal University of Pampa, São Gabriel, RS, Brazil*
[d] *Biotechnology Center, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, Porto Alegre, RS, Brazil*
[e] *Program on Health and Environment, Tiradentes University, Aracaju, SE, Brazil*
[f] *Biotechnology Unit, Center for Technology Development, Federal University of Pelotas, RS, Brazil*

A R T I C L E I N F O

Article history:
Received 9 August 2013
Accepted 5 November 2013
Available online 13 November 2013

Keywords:
Red propolis
Hep-2
Proteomics
Apoptosis
TUNEL
Annexin-V

A B S T R A C T

Here we investigated alterations in the protein profile of Hep-2 treated with red propolis using two-dimensional electrophoresis associated to mass spectrometry and apoptotic rates of cells treated with and without red propolis extracts through TUNEL and Annexin-V assays. A total of 325 spots were manually excised from the two-dimensional gel electrophoresis and 177 proteins were identified using LC–MS–MS. Among all proteins identified that presented differential expression, most were down-regulated in presence of red propolis extract at a concentration of 120 μg/mL (IC50): GRP78, PRDX2, LDHB, VIM and TUBA1A. Only two up-regulated proteins were identified in this study in the non-cytotoxic (6 μg/mL) red propolis treated group: RPLP0 and RAD23B. TUNEL staining assay showed a markedly increase in the mid- to late-stage apoptosis of Hep-2 cells induced by red propolis at concentrations of 60 and 120 μg/mL when compared with non-treated cells. The increase of late apoptosis was confirmed by *in situ* Annexin-V analysis in which red propolis extract induced late apoptosis in a dose-dependent manner. The differences in tumor cell protein profiles warrant further investigations including isolation of major bioactive compounds of red propolis in different cell lines using proteomics and molecular tests to validate the protein expression here observed.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Propolis is a natural resinous product of honeybees that has been used for centuries in traditional medicine all over the world (Burdock, 1998; Castaldo and Capasso, 2002; Daugsch et al., 2008). The propolis' composition varies according to its botanical origin; generally contains resin, wax, pollen, essential and aromatic oils (Lustosa et al., 2008; Sforcin, 2007). Since 2007, the red propolis type from the northeast of Brazil has been an important source of investigation by local and international research groups. Its botanical origin is from *Dalbergia ecastophyllum* (*L*) *Taud.* (*Leguminosae*), a plant that characterizes the red color found in the propolis (Daugsch et al., 2008; Franchi et al., 2012; Piccinelli et al., 2011; Silva, 2007). Red propolis has shown several important biological activities such as: antibacterial (Cabral et al., 2009; Righi et al., 2011), antifungal (Siqueira et al., 2009), anti-inflammatory (Barreto, 2008; de Almeida et al., 2013), antiulcerative (Pinheiro, 2009), antioxidant (Cabral et al., 2009; Righi et al., 2011), and antitumor (Alencar et al., 2007; Li et al., 2008). The propolis antitumor activity has been widely studied (Valente et al., 2011) because of growing interest on strategies for prevention and treatment of cancer (Akyol et al., 2013). The resistance mechanisms to cancer drugs promote the development of new medicines with potential source of novel bioactive molecules (Umthong et al., 2011).

We have recently reported that red propolis extracts are composed of complex molecules and exhibits important biological properties with capacity to scavenge free radicals and to inhibit tumor cell growth in a selective manner when compared to

* Corresponding authors. Address: Universidade de Caxias do Sul, Rua Francisco Getúlio Vargas 1130, 95070-560 Caxias do Sul, RS, Brazil. Tel.: +55 54 3218 2100; fax: +55 54 3218 2664 (M. Roesch-Ely).
E-mail address: mrely@ucs.br (M. Roesch-Ely).

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.11.003

non-tumor cell lines (Frozza et al., 2013). In the present study, we used 2-DE associated to mass spectrometry to reveal the protein profiles of Hep-2 cells and apoptotic rates of cells treated with and without red propolis extracts through TUNEL and Annexin-V assays.

2. Materials and methods

2.1. Red propolis sample and preparation of red propolis extract

Red propolis was collected in the year 2010, one sample per month, in the Capivara Apiary in Brejo Grande, state of Sergipe in the northeast of Brazil (10°28′25″S and 36°26′12″W). The propolis was protected from light and frozen at −20 °C until extract preparation was proceeded. The twelve samples of red propolis was ground to a fine powder forming a pool and 1 g (dry weight) was mixed with 10 mL of EtOH–H_2O 70–30% (v/v) and shaken at room temperature for 24 h. After extraction, the mixture was filtered and the solvent was evaporated and produced a red fine powder. The dry extract was kept frozen at −20 °C and prepared at different concentrations with EtOH–H_2O 50% (v/v), and finally filtered through a 0.22 μm polyethersulfone membrane (TPP, Techno Plastic Products, Switzerland). This sample was chemically analyzed as well its antioxidant and antitumor activities, with results cited by Frozza et al. (2013).

2.2. Cell culture and red propolis treatment

Hep-2 (human laryngeal epidermoid carcinoma cells) was obtained by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in Dulbecco's modified eagle's medium–high glucose (DMEM) supplemented with 10% (w/v) heat-inactivated fetal bovine serum (FBS) and 1% (w/v) penicillin–streptomycin. The culture was maintained in a humidified atmosphere at 37 °C, 5% CO_2 and 95% air. The study was performed when cells reached 70–80% confluence.

Cells (2.6×10^6) were seeded into 75 cm^2 culture flasks with supplemented culture medium. After 24 h, cells were treated and exposed for 1 h at different propolis concentrations. The negative control group (Group A) was treated with the same amount of hydroalcoholic solution used in the group with propolis extract. The other two groups received red propolis extract at two different concentrations, 6 μg/mL (Group B) and 120 μg/mL (Group C) in free FBS medium and incubated at 37 °C in 5% CO_2.

Extract concentrations were determinated according to previous 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide (MTT) survival experiments reported by our group (Frozza et al., 2013), showing non-cytotoxic dose (6 μg/mL) and dose that inhibits cell growth by 50% – IC50 (120 μg/mL). Experimental procedures were performed at least in triplicate.

2.3. Protein extraction

Cells were harvested and lysed in 0.5 mL of lysis buffer (7 M ureia, 2 M thiourea, 40 mM Tris base pH 8.5, 20 mM DTT, 4% (w/v) CHAPS and 1% (w/v) protease inhibitor). After 30 min at room temperature, shaking at 90 rpm, the lysate was scraped, transferred into microtubes and centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was collected and frozen at −20 °C. The procedure was repeated twice and a pool of extract was analyzed.

The samples were dialyzed for 24 h, at 4 °C, with gentle agitation on a magnetic stirrer, changing four times Milli-Q water every 6 h. Bradford assay (BioRad Laboratories, Hercules, CA) was used to determine protein quantification according to manufacturer's instructions. The samples were frozen at −80 °C, lyophilized (Labconco FreeZone, Labconco Corporation, USA) and the pellets were suspended in 25 mM (w/v) Tris HCl pH 7.4 and processed with the Clean Up Kit (GE Healthcare, USA).

2.4. 2-D gel electrophoresis

Protein extracts containing 900 μg of total protein were diluted in IPG rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 0.01% (w/v) bromophenol blue, 0.2% (v/v) ampholytes). The protein samples were refrigerated at 4 °C for 40 min and centrifuged at 20,800×g for 40 min at 20 °C. The supernatants were loaded onto 17 cm IPG strips with pH 4–7 gradient. Proteins were focused at 20 °C according to the following program: 15 min, 250 V; 3 h, gradient to 10,000 V; 6 h, 10,000 V until 60 kVh in a Protean IEF cell system (BioRad Laboratories, Hercules, CA). Samples were equilibrated for 15 min in a buffer containing 6 M urea, 30% (v/v) glycerol, and 1% (w/v) DTT and for 15 min in the same buffer but with DTT replaced by 4% (w/v) iodoacetamide. The second dimension was SDS-PAGE in 12% polyacrylamide in a PROTEAN II xi 2D Cell apparatus (BioRad Laboratories, Hercules, CA). Electrophoresis was performed at 40 mA/gel for 30 min and at 50 mA/gel. The spots were stained with Colloidal Coomassie (2% (v/v) phosphoric acid, 20% (v/v) methanol, 10% (w/v) ammonium sulfate, 0,05% (w/v) Coomassie Brilliant Blue G-250.

2.5. Image analysis of differential protein expression

Two-dimensional images were captured by scanning the stained gels with Image Scanner III (GE Healthcare, USA), digitalized with LabScan™ v. 6.0 software and analyzed with the ImageMaster™2D Platinum 7.0 software (GE Healthcare, USA). Three gels of each treatment obtained from a pool of three independent cultures were analyzed to guarantee representative results. Each treatment showed less than 10% variability in the number of protein spots detected among replicates. Spot normalization, as an internal calibration to make the data independent from experimental variations among gels, was made using relative volumes to quantify and compare the gel spots.

2.6. In-gel digestion and protein identification by mass spectrometry

The protein spots of interest were manually excised from Colloidal Commassie stained gels by biopsy punches, placed in a microtube, and washed twice with 50% (v/v) acetonitrile and 25 mM ammonium bicarbonate for 15 min each. Subsequent washing with acetonitrile was performed before the samples dried at room temperature and digested overnight using modified trypsin (Sigma) diluted in 25 mM ammonium bicarbonate at 37 °C. Next, peptides were extracted in two washing steps with 50% (v/v) acetonitrile and 5% (v/v) trifluoroacetic acid for 1 h and dried in a vacuum centrifuge. To identify proteins, we used a liquid chromatography (LC) separation (reversed-phase HPLC) (nanoACQUITY®, Waters, Milford, USA) coupled with tandem mass spectrometry (MS) as detector with an electrospray ionization (ESI) quadrupole and time-of-flight (Q-ToF) (Q-ToF Micro, Waters Micromass, Milford, USA) coupled to a capillary liquid chromatography system (nanoAcquity UPLC, Waters, Milford, USA). A nanoflow ESI source was used with a lockspray source for lockmass measurement during all the chromatographic runs.

Peptides were identified by matching the measured monoisotopic masses to theoretical monoisotopic masses generated using the MASCOT search via *in silico* digest through the NCBI-NR (Non-Redundant Protein Sequences database). For protein identification, the resulting MS data was interpreted by MASCOT software (Matrix Science, London, UK) against NCBInr database. During the database searching, one missed cleavage *per* peptide was set as the maximum allowance, a mass tolerance of 0.2 Da, and MS/MS tolerance of 0.2 Da was also used according to predefined protocol. Other possible variations, such as carbamidomethylation for cysteine and oxidation for methionine, were also taken into account.

2.7. Flow cytometry TUNEL apoptosis assay

Hep-2 cells were treated with 6, 60 and 120 μg/mL of red propolis for 1 h, followed by cultivation in extract-free medium for 24 h and the Guava® TUNEL assay (Guava Technologies) was conducted following the manufacturer's instructions. Briefly, Hep-2 treated cells were subjected to cells fixation procedure with 50 μL of 4% (w/v) paraformaldehyde in PBS for 60 min at 4 °C and then with 200 μL of ice-cold 70% (v/v) ethanol at −20 °C for at least 18 h. For staining procedure, 1.5×10^4 to 1.0×10^5 of fixed cells was washed twice and was added to 25 μL of DNA Labeling Mix for 60 min at 37 °C. At the end of the incubation time, cells were centrifuged and resuspended in 50 μL of the Anti-BrdU Staining Mix. Cells were incubated in the dark at room temperature for 30 min and samples were acquired on the flow cytometry (Guava® Flow Cytometry easyCyte™ System; Millipore Corporation™). In this assay, terminal deoxynucleotidyl transferase (TdT) catalyzes the incorporation of BrdU residues into the fragmenting nuclear DNA of apoptotic cells at the 3′-hydroxyl ends by nicked-end labeling. TRITC-conjugated anti-BrdU antibody binds to the incorporated BrdU residues, labeling the mid- to late-stage apoptotic cells.

2.8. Morphological examination of cancer cells

Cells were seeded into 24-well plates containing coverslips. After 24 h incubation, the cells were treated with red propolis hydroalcoholic extract at 6, 60 and 120 μg/mL for 1 h, followed by cultivation in extract-free medium for 24 h. The negative control group was treated with EtOH–H_2O 50% (v/v) instead of extract for the same period. Then they were stained with Giemsa Stain (Accustain® – Sigma–Aldrich®) according standard giemsa procedure established by the supplier. Morphological changes of the cells were observed and photographed under a light microscope at 200× (Olympus®).

2.9. Detection of late apoptosis by in situ analysis

Apoptotic cell death was detected and visualized by fluorescence microscopy using Annexin-V staining. Apoptosis is associated with characteristic morphological and biochemical changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and cell surface changes (Reed, 2000). Annexin is presented in several cellular functions and mainly characterized by their ability to reversibly interact with membranes in a calcium-dependent manner (Lizarbe et al., 2013). Annexin-V is used for apoptosis detection in cell cultures, and also for *in vivo* molecular imaging (Lizarbe et al., 2013). The assay is based on the observation of

phosphatidylserine translocation to the cell surface during apoptosis in most cell types and by many apoptotic stimuli. On the cell surface it can be detected by binding fluorochrome-tagged Annexin-V (Reed, 2000).

The Hep-2 cells were seeded into the coverslips into 24-well plates at 1.0×10^5 cells/mL in 500 μL of supplemented culture media. After 24 h, cells were treated with 60 and 120 μg/mL of red propolis extract and negative controls were treated with the same amounts of hydroalcoholic solution, incubated at 37 °C in 5% CO_2 for 1 h, followed by cultivation in extract-free medium for 24 h. Next, the medium was removed and the cells were incubated with 2.5 μg/mL of Annexin V (abcam® – ab14196) primary antibody diluted in biding buffer (Hepes 10 mM, NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM pH 7.4) for 30 min. They were washed once with binding buffer, then incubate with 2.5 μg/mL Dylight® 488 (abcam® – ab115637) secondary antibody. The cells in the late apoptosis or necrosis were staining either with propidium iodate (Sigma–Aldrich® – P4170). Briefly, they were fixed with formalin 4% for 15 min. The apoptosis was analyzed by fluorescent microscope (Olympus®). The percentage of cells undergoing early (Annexin-V positive) or late (Annexin-V positive/PI positive) apoptosis was estimated by counting at least 500 cells in a total of three test and expressed as a percentage of the total number of cells.

2.10. Statistical analysis

The results are expressed as the means ± SD obtained from three independent experiments. Statistical significance was evaluated using the Levene test followed by analysis of variance (ANOVA) and Tukey's post-hoc test for Annexin-V assay, for TUNEL was used Chi-Square test and for proteomic data, statistical analysis was performed using *t* tests. A *p*-value <0.05 was considered significant using the Statistical Package for Social Sciences (SPSS, version 19.0) for Windows.

3. Results

Protein spots were manually excised from 3 groups of pH 4–7 and 2-DE map gels that received different concentration of red propolis extract compared to an untreated control group. Altogether, 325 spots were excised and submitted to identification. All spots from group A (control) were excised ($n = 251$) and only differentially expressed spots from the treated groups (B and C) were submitted to identification according to differences in protein profiles observed in the ImageMaster™ 2D Platinum software (Fig. 1). Therefore, 65 spots were excised from group B and only 9 spots from group C. The identified proteins are listed in Supplementary Table S1 and Table 1.

Altogether, MS allowed the identification of 177 proteins out of 325 spots analyzed. The molecular mass (MW) and the isoelectric point (pI) of each protein spot were experimentally determined and compared with theoretical molecular mass and pI taken from Mascot database and are listed in Supplementary Table S1. Many of the gel-estimated MW/pI corresponded well to the theoretical values. Different levels of post-translational modification, however, could reveal distinct values from experimentally determined MW and/or pI from the predicted ones. In addition, in most cases, different isoforms were identified for a given protein.

All proteins identified were distributed according to the Eukaryotic Orthologous Groups (KOG-NCBI) functional classification (Supplementary Table S1). The proteins from control are presented in Supplementary Fig. S1 by percentage of their KOG classification. Most of the proteins are related to posttranslational modification, protein turnover and chaperones (O), cytoskeleton (Z), translation, ribosomal structure and biogenesis (J), transcription (K), RNA processing and modification (A) and intracellular trafficking, secretion and vesicular transport (U). Only 8% of the identified proteins could not be classified (X). Selections of identified proteins that presented expression in the control group and were down- and up-regulated in the red propolis treated group are represented with fold changes in Table 1.

Five proteins were identified as down-regulated in the group treated with 120 μg/mL of hydroalcoholic extract of red propolis (Fig. 1C) compared to the control group (Fig. 1A): 78 kDa glucose-regulated protein (GRP78 – control spot 54/propolis IC50 spot 268), thiol-specific antioxidant protein (PRDX2 – control spot 122/propolis IC50 spot 314), L-lactate dehydrogenase B chain (LDHB – control spot 138/propolis IC50 spot 293), vimentin (VIM-control spot 79/propolis IC50 spot 307) and alfa-tubulin (TUBA1A – control spot 74/propolis IC50 spot 298). Two proteins were up-regulated in the red-propolis treated group (Fig. 1B) using non-cytotoxic concentrations of extract (6 μg/mL) compared to the control group (Fig. 1A): UV excision repair protein RAD23 homolog B isoforms 1 (RAD23B – control spot 67/propolis non-cytotoxic spot 321) and 60 acidic ribosomal protein P0 (RPLP0 – control spot 143/propolis non-cytotoxic spot 323). All spot numbers are presented in Supplementary Table S1 and differential expressed proteins are seen in Figs. 1 and 2, at a maximized perspective.

Some of the proteins identified above are related to induction or suppression of apoptosis. Thus, tests were carried out to verify the relationship between the extract of red propolis and apoptosis induction. Red propolis activity has shown to promote apoptosis in other cancer cells models like MCF-7 (Kamiya et al., 2012) and Nalm16 (Franchi et al., 2012).

TUNEL staining assay was performed to further elucidate the mid- to late-stage apoptosis of Hep-2 cells induced by red propolis. TRITC-conjugated anti-BrdU antibody binds to the incorporated BrdU residues in those cells that exposed 3′-hydroxyl ends in the nuclear DNA, a characteristic feature of apoptotic cells. Fig. 3 demonstrates that red propolis at concentrations of 60 and 120 μg/mL presented a higher percentage ($P < 0.05$) of TUNEL–staining cells when compared with the control group, with mean values of 33.5%, 63.5% and 4.5%, respectively. Moreover, red propolis at 6 μg/mL presented a similar percentage of apoptosis compared with the control group (4.7%) and no difference between these two groups were observed ($P < 0.05$).

The examination of cancer cells through Giemsa staining revealed altered morphological structure according to the concentration of red propolis extract used. The cells in the negative control group that received hydroalcoholic solution of 50% (v/v) presented a rounded-like morphology (Fig. 4A), surrounded by abundant cytoplasm, well defined and intact, represented as healthy cells. Similar morphological patterns were observed in the cell culture that received 6 μg/mL red propolis extract treatment (Fig. 4B). However, the cells that received treatment higher concentrations of red propolis like 60 μg/mL (Fig. 4C) and 120 μg/mL (Fig. 4D) presented detachment and shrinkage, indicating several biological changes upon extract activity that suggests cytotoxic activity against the tumor cell lineHep-2.

Red propolis extract induced apoptosis in Hep-2 cancer cells in a dose-dependent manner. According to Fig. 5, Annexin-V labeling without PI uptake (green fluorescence) indicates phosphatidylserine detection on the external surface of an intact plasma membrane. PI staining (red fluorescence) in conjunction with Annexin-V labeling (Merge – yellow fluorescence) indicates a compromised membrane that may result from either late apoptosis or necrosis. According to Fig. 6 the signaling to late apoptosis/necrosis increased in a significant way in the cells treated with 120 μg/mL (98.46 ± 0.77%) comparing with that ones that received 60 μg/mL (8.68 ± 2.84%) of red propolis extract and control group (1.26 ± 0.41%).

4. Discussion

We have recently shown that red propolis is composed of complex molecules and exhibits important biological properties with capacity to scavenge free radicals and to inhibit tumor cell growth in a selective manner (Frozza et al., 2013). It is expected that propolis affects the metabolism pathways and cellular activity after treatment and cells may switch towards several different functions before being rescued and programmed to die. To investigate how

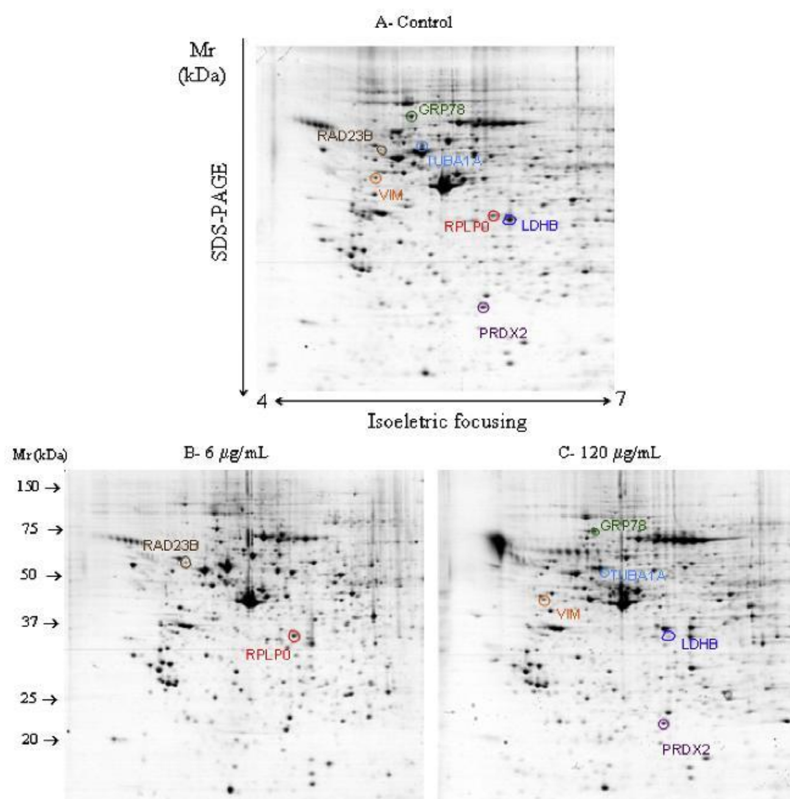


Fig. 1. 2-DE profiles of the control (A) and red propolis treated group (B, 6 µg/mL; C, 120 µg/mL). Total protein extracts were separated on pH 4–7, 17 cm IPG strips, following by 12% SDS-PAGE and staining with CBB R-250. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Identification of differentially expressed proteins in Hep-2 cells from control and treated groups.

Gene name	Expression (%Vol)[a]		Fold change[b]		KOG[c]	Protein description
	Control[d]	Treatment[e]		Control/treatment		
		120 µg/mL	6 µg/mL			
DOWN-REGULATED						
GRP78	0.416 ± 0.022	0.208 ± 0.028		2.00	O	78 kDa Glucose-regulated protein precursor
PRDX2	0.272 ± 0.011	0.200 ± 0.010		1.36	O	Thiol-specific antioxidant protein
LDHB	0.548 ± 0.022	0.080 ± 0.006		6.85	C	L-Lactate dehydrogenase B chain
VIM	0.167 ± 0.007	0.119 ± 0.010		1.40	Z	Vimentin
TUBA1A	0.193 ± 0.007	0.030 ± 0.001		6.43	Z	Alfa-tubulin
UP-REGULATED						
RAD23B	0.069 ± 0.001		0.087 ± 0.001	0.79	L	UV excision repair protein RAD23 homolog B isoforms 1
RPLPO	0.240 ± 0.010		0.319 ± 0.002	0.75	J	60 Acidic ribosomal protein P0

In *t*-test, all changes in%Vol between control and treated groups were significant ($P < 0.05$).
[a] %Vol represents a normalized value of a relative volume of a spot taken from ImageMaster™ 2D Platinum 7.0.
[b] Fold change (control vs. treatment) calculated dividing the% (vol) from control gels by the% (vol) from propolis treated gels.
[c] KOG database functional classes: C – energy production and conversion; J – translation, ribosomal structure and biogenesis; L – replication, recombination and repair; O – post-translational modification, protein turnover, chaperons; Z – cytoskeleton.
[d] Control group, treatment with hydroalcoholic solution instead of propolis.
[e] Propolis treated groups: 120 µg/mL (IC50) and 6 µg/mL (not cytotoxic).

red propolis extract modulates several cellular metabolic responses in Hep-2, this study generated protein profiles from cells treated with or without red propolis extracts.

This is the first study using red propolis from northeast of Brazil associated to 2-DE SDS-PAGE and mass spectrometry that reports alterations in the protein profile using Hep-2 cells as experimental model. The results showed differences in protein patterns between the analyzed treated and untreated groups. There are several proteomic studies on Hep-2 models submitted to a variety of extracts and culture conditions, which allow the identification of differentially expressed proteins (Bianchi et al., 2011; Mukhopadhyay et al., 2006; Okoli et al., 2007; Rodrigues-Lisoni et al., 2010). So far, however, there are no reports using proteomics with Brazilian red propolis in any kind of tumor cell line.

Altogether, a total of 325 spots from both control and treated groups were excised, in-gel digested with trypsin, and 177 proteins were identified. Different isoforms were identified for a given protein, considering this, 113 different proteins were found. All spots

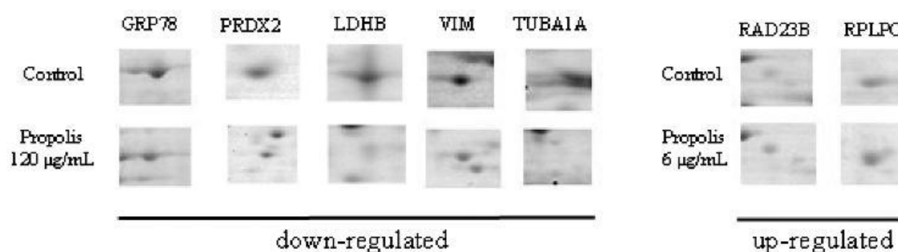


Fig. 2. Magnified view expression profile of the 7 significant altered proteins: 5 down-regulated in the C panel from Figs. 1 and 2 up-regulated in the B panel form Fig. 1.

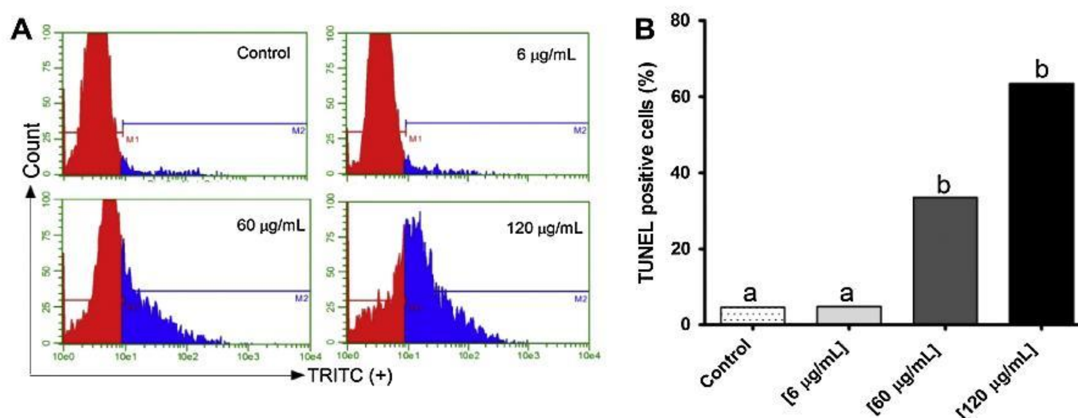


Fig. 3. TUNEL assay analysis of Hep-2 cells treated with 6, 60 and 120 μg/mL of red propolis and control group, after 1 h of exposure, followed by cultivation in extract-free medium for 24 h. (A) Flow cytometry graphs and (B) percentage of mid- to late-apoptotic cells. Different letters (a and b) indicate significant differences between the means and differences were considered significant at $P < 0.05$ (Chi-Square test).

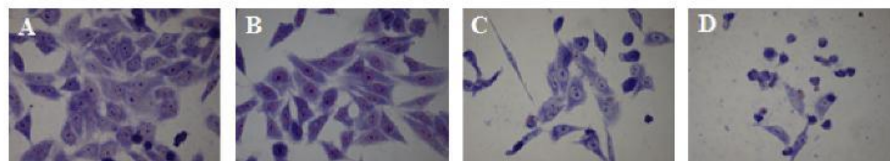


Fig. 4. Morphological structure of cancer cells Hep-2 through Giemsa Stain. (A) Control, (B) treatment with 6 μg/mL red propolis extract, (C) Treatment with 60 μg/mL red propolis extract and (D) treatment with 120 μg/mL red propolis extract. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

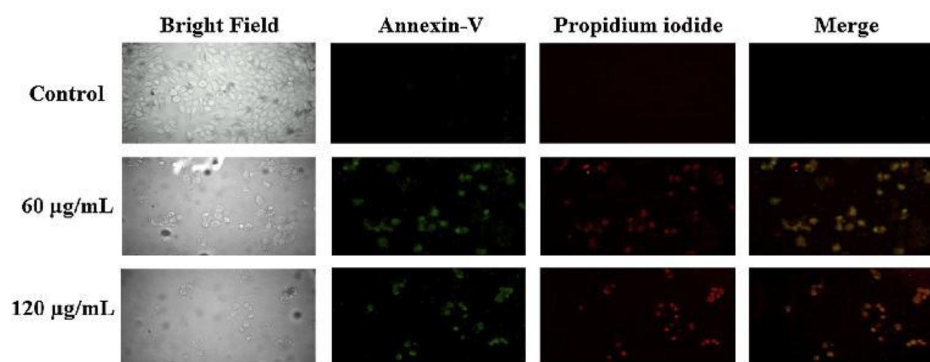


Fig. 5. Detection of cell apoptosis by *in situ* Annexin-V immunostain. Tumor cells were grown on glass slides and treated for 1 h (control, 60 μg/mL, 120 μg/mL) and then were submitted to indirect immunofluorescence. Representative photographs of the triplicate were taking under fluorescent microscopy at 200×. Healthy cells presented no staining, early apoptotic cells (stained with Annexin-V) emitted green fluorescence and late apoptotic cells (stained with Annexin-V and PI) are shown in yellow (Merge). The cells stained only with PI emitted red fluorescence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from the control group were digested and only a few selections of spots from the red propolis extract treated groups were removed according to differential protein maps generated from ImageMaster™ 2D Platinum software. We have found several cases in which more than one spot was assigned to the same protein. These variants or isoforms may be attributed to biologically

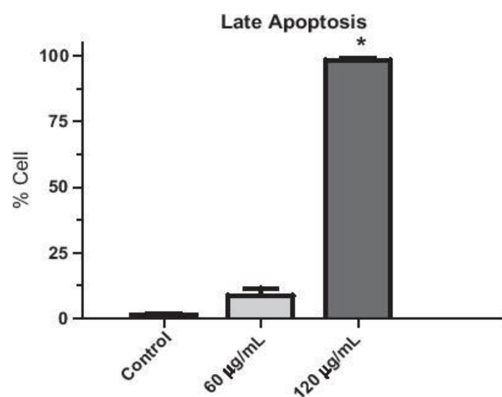


Fig. 6. Percentage of cells immunostained in late apoptosis (Annexin V positive + PI positive) by *in situ* analysis. Results were obtained from three independent experiments expressed in median ± SD. *represent statistical significance according Levene test followed by analysis of variance and Tukey's post hoc test ($p \leqslant 0.05$).

important post-translational modifications, ranging from chemical modifications to proteolytic cleavage. Among all proteins identified that presented differential expression, the majority were down-regulation in presence of red propolis extract at a concentration of 120 µg/mL (IC50): GRP78, PRDX2, LDHB, VIM, TUBA1A (Table 1).

Cell rescue proteins like heat-shock (Hsps) or Hsp-related are a family of proteins and examples of molecules involved in the degradation or reactivation of damaged molecules and expected to be up-regulated under stress conditions. Hsps are normally found in response to stressful stimuli, including chemotherapeutic agents, and response in carcinogenesis may be related to their activities as chaperones, altering protein expression on tumor cells, especially proteins related to tumor progression and components of the cell cycle (Myung et al., 2004). There are reports, however, indicating down-regulation of some of the members of the heat-shock protein family under stress conditions. HSp27 expression was reduced in human breast adenocarcinoma adriamycin-resistant and paclitaxel-resistant cell lines in comparison to the parental cell ones (Chuthapisith et al., 2007) and in human cells of hepatocarcinoma in response to capsaicin treatment (Baek et al., 2008). We observed that a member of the HSP70 family, the GRP78 protein, was down-regulated in the propolis treated group. It has been previously reported that HSP27 and HSP70 function as antiapoptotic proteins, while HSP60 and HSP10 as proapoptotic (Garrido et al., 2001), which could explain the down-regulation of a member of the HSP70 (GRP78) associated to cytotoxic response of propolis in Hep-2 cells at IC50 levels.

GRP78 is a protein that modulates endoplasmatic reticulum function showing prosurvival and anti-apoptotic properties in cancer metabolism (Chiu et al., 2008; Wang et al., 2009). It is believed that GRP78 can interact and inhibit the activation of apoptosis pathway components related to a series of molecules. It is reported to be induced in a variety of cancer cells and drug-resistance lines (Li and Lee, 2006). Chiu et al. (2008) demonstrated that knockdown by siRNA of GRP78 reduced the malignant phenotype, inhibiting cell growth and preventing cell migration and colony formation on several types of head and neck cancers. Knockout of GRP78 also seems to enhance apoptosis in colon cancer cells, generate resistance to epirubicin (Chang et al., 2012) and paclitaxel (Mhaidat et al., 2011). Chemoresistance of various cells correlates with GRP78 expression and apoptosis inhibition (Jiang et al., 2009; Wang et al., 2009; Zhang et al., 2006; Zhou et al., 2011). Despite most studies indicate prosurvival and anti-apoptotic properties of GRP78, some reports reveal the increase expression of this protein upon treatment with drugs as vorinostat (Bianchi et al., 2011; Kahali et al., 2010).

Tumors present a metabolic environment normally acidic, hypoxic, and deprived from nutrients like glucose and amino acids, due to rapid proliferation, elevated glucose intake, and sometimes poor vascularization (Wang et al., 2009). Microenvironment of tumor cells may be similar to a physiological stress condition and cells manage to acquire a survival function (Koumenis, 2006), PRDX2 is a member of the peroxiredoxin family of antioxidant enzymes (PRDX 1–6), which perform important functions in the maintenance of the intracellular redox homeostasis (Shen et al., 2012). Peroxiredoxins have been linked with regulation of proliferation, differentiation, and cellular apoptosis (Park et al., 2006). Peroxiredoxin 2 has been attributed to have both proliferative and antiapoptotic functions, inducing carcinogenic changes (Noh et al., 2001).

A mechanism of protecting cancer cells from oxidative stress and mediating resistance to therapy has been reported upon alterations in PRDX2 expression. PRDX2 in glioma cells was decreased by RNA silencing and increased hyperoxidation, suggesting a more oxidizing redox environment, but apparently not altering levels of other antioxidant enzymes, like catalase superoxide dismutase, among others (Smith-Pearson et al., 2008). In addition, PRDX2 protein was shown to be up-regulated in radiation-resistant breast cancer cells compared with radiation-sensitive breast cancer cells and plays an important protective role from oxidative radical damage (Wang et al., 2005). Another recent study compared the protein expression of radiation-resistant lines of Hep-2 (RR-Hep-2) to parental Hep-2 cells, where RR-Hep-2 acquired a more resistant profile and anti-apoptotic phenotypes than the parental line (Kim et al., 2010). Protein levels for PRDX2 were up-regulated in the RR-Hep-2 cells, known to play a protection function against radiation induced oxidative stress (Kim et al., 2010). Stresing et al. (2012) have related that overexpression of PRDX2 in lung metastatic cells effectively removes the intracellular Reactive Oxygen Species (ROS), suggesting that this condition is favorable in the lung microenvironment to metastatic cell colonization. Most importantly, lung cells silenced for PRDX2 were significantly more sensitive to oxidative stress than the parental cells. High PRDX2 expression has also been associated with renal tumors and tumors with fewer metastases (Soini et al., 2006).

It is known that is important for developing an efficacious therapy to monitor the ROS in cancer cells, since most of the chemotherapy substances induce ROS generation with the propose to eliminate cancer cells by apoptosis through common molecular pathways (Maiti, 2012). But prolonged treatment with combined drugs may also bring deleterious consequences, like reduction in ROS level, generating resistant cells with lower ROS content than drug sensitive cancer cells (Maiti, 2012).

Sonveaux et al. (2008) proposed a "metabolic symbiosis" between regions of hypoxic and aerobic cancer cells in tumors, which contain well-oxygenated (aerobic) and poorly oxygenated (hypoxic) regions. The glucose is converted to lactate in human cancer cells when cultured under hypoxic conditions, while well-oxygenated cancer cells take up lactate via monocarboxylate transporter 1 (MCT1) and use it for oxidative phosphorylation by an oxygen-dependent expression of lactate dehydrogenase (LDHB) (Semenza, 2008). LDH has a central function in cellular metabolism, showing a tetrameric spatial conformation composed of 2 subunits (LDHA and LDHB). Depending upon the LDH isoform and the concentration of pyruvate and lactate, the enzyme can interconvert these two compounds. LDHB is a key enzyme that converts lactate to pyruvate and its expression was significantly increased in primary melanomas compared with nevi (Ho et al., 2012). LDH is also expressed in breast tumors (Hussien and Brooks, 2011) and gastric tumors (Liu et al., 2009).

Recently, the role of LDHB was examined with loss-of function studies using transfected maxillary sinus squamous cell carcinoma and generated significant reduced in cell proliferation upon down-regulation of LDHB (Kinoshita et al., 2012). These results are in accordance to the down-regulation expression of LDHB observed in this study. Cytotoxic activity of red propolis in the IC50 cell samples presented reduced expression for LDHB, suggesting an inhibition on cell proliferation activity or a proapoptotic environment. Apparently, overexpression of both isoforms A and B suggests that pyruvate metabolism has been deviated from the tricarboxylic acid cycle to lactic acid (Liu et al., 2009). The production of aerobic lactate where the final step is executed by lactate dehydrogenase is one of the characteristic phenotypes in invasive tumor development, thus suppression of LDHB expression plays important role in tumor invasiveness (Kim et al., 2011).

From the cytoskeleton compartment and organization, we have identified a class II intermediate filament protein vimentin (VIM), which was down-regulation in the red propolis treated group. Although vimentin is normally expressed in stromal cells, regulation of its expression levels have been recently associated to many epithelial transformed tumor cells. VIM is frequently overexpressed in tumors undergoing epithelial to mesenchymal transition (EMT), a condition correlated with invasiveness and poor prognosis (Bozzuto et al., 2010). EMT is an important procedure necessary during embryonic development, also associated to an invasive cancer phenotype (Gavert and Ben-Ze'ev, 2008), characterized by a modification in the morphology with loss of polarity and contacts between cells by the epithelial cells with increased vimentin expression and concomitant decrease of E-cadherin (Chen et al., 2010).

Vimentin expression in epithelial tumors is considered a marker of de-differentiation and aggressiveness, which has been recently indicated as a novel therapeutic target (Lahat et al., 2010) and a promising serological marker in detecting metastatic potential in human hepatocellular carcinoma cells (Pan et al., 2012). The down-regulation of vimentin seems to be correlated with inhibition of tumor cell proliferation and invasion (Nodale et al., 2012). Corroborating with our results, VIM was down-regulated in Hep-2 cells under vorinostat and/or gefitinib treatment (Bianchi et al., 2011), in the erlotinib-sensitive head and neck cancer cells (Haddad et al., 2009) and in chemotherapy resistance breast cancer tissues (Hodgkinson et al., 2012).

Also as part of a group of the cytoskeleton compartment, the microfilament-associated tubulin alpha-1A chain (TUBA1A) is a major constituent of microtubules, also down-regulated in this study. Cytoskeletal proteins play an important role in a series of cell functions like as mitotic progression, proper positioning of the nucleus, cell motility, actin filament growth, mitochondrial tubulation, among others. In the structure of the cytoskeletal microtubules are the α and β-tubulins (Sahab et al., 2012). Identification of differential modifications in tubulin is especially important in the research area of cancer therapeutics. These proteins are very significant in cell division, which make them interesting targets for anticancer drug development (Kavallaris, 2010). Drugs elaborated with natural products that target the tubulin and the microtubule system, also known as anti-mitotics, are considered a crucial component in combination chemotherapy for the treatment of many cancers (Kavallaris, 2010).

The two up-regulated proteins identified in this study were from the non-cytotoxic (6 μg/mL) red propolis treated group. One of the proteins is a constituent of ribosome, the 60S acidic ribosomal protein P0 (RPLP0), the other protein identified is related to the DNA-repair machinery, the UV excision repair protein RAD23 homolog B isoforms 1 (RAD23B).

RAD23B is involved in nucleotide excision repair (NER) in humans by acting as a component of the XPC complex and a sensor to bind at the sites of DNA damage (Janicijevic et al., 2003; Sugasawa et al., 2009). The XPC complex recognizes a wide spectrum of damaged DNA characterized by distortions of the DNA helix (Sugasawa et al., 2009). Together with other recognition factors like XPA and RPA, RAD23B is part of the pre-incision (or initial recognition) process of DNA repair and preferentially binds to platinum based chemotherapeutic agents, like cisplatin and UV-damaged double-stranded DNA (Neher et al., 2010). A recent model in which XPC–RAD23B is the actual damage sensor has been proposed, since after recognition RAD23B displays the XPC damage-binding sites by decoupling it, permitting XPC (without RAD23B) to stably bind the DNA damage (Bergink et al., 2012).

DNA repair mechanisms are important for maintaining DNA integrity and preventing carcinogenesis. The presence of RAD23B protein was observed in damage recognition and DNA repair of smoking and alcohol induced DNA bulky adducts in laryngeal cancer risk (Abbasi et al., 2009). RAD23B was identified among a group of up-regulated proteins that were differentially expressed as a result of transformation following deletion of the p53 gene in lymphomas (Honore et al., 2008). We have identified up-regulation of RAD23B in cells treated with low concentrations of red propolis (6 μg/mL), where no cytotoxic activity was presented. However, as cytotoxic activity started and presumably an apoptotic environment established, RAD23B expression in cells treated with higher concentrations of red propolis (120 μg/mL) was altered. Upon higher concentrations of propolis, cells were signaled to a proapoptotic profile and RAD23 dependent DNA-repair was no more requested.

Brockstedt et al. (1998) investigated human apoptosis-associated proteins to lymphoma cell line and found up-regulation of UV excision repair protein RAD23B and 60S acidic ribosomal protein P0 (RPLP0) on the 2-DE pattern of non-apoptotic cell group. The apoptosis pathways are activated when other cellular response pathways will not be efficient enough (Friedberg, 2001).

RPLP0 is a protein component of the 60S subunit of the ribosome, localized in both cytoplasm and nucleus, which is involved in mRNA translation. Recent reports pointed to an up-regulation of RPLP0 under stress conditions induced by the pro-apoptotic agent tumor necrosis factor related-apoptosis-inducing-ligand (TRAIL) in breast cancer cells (Wilmet et al., 2011) and in platinum-based chemotherapy for cisplatin-resistant lung cancer line (Kageyama et al., 2011). Alternative regulation for RPLP0 was reported in proteomic approach to study global changes in protein expression in Ras-transformed NIH3T3 mouse fibroblast cell lines (presenting overexpression of Ras), showing down-regulation of RPLP0 in Ras transformed cells. Ras oncoproteins play important role in the malignancy through apoptosis, invasion and deregulation of cellular growth of tumors (Ji et al., 2007). Phosphor 60S acidic ribosomal protein P0 was reduced by the action of oxythiamine (OT) into pancreatic cells, OT has been demonstrated in many studies to reduce cancer cell proliferation through inhibition of the cell cycle (Zhang et al., 2010). A recent proteomic report using yeast cells model showed that grapefruit seed extract induced apoptosis by down-regulation of the mitochondrial 60S ribosomal compartment (L-14A) and up-regulation of RPLP0 (Cao et al., 2012).

Traditional anticancer treatments, such as chemotherapy and radiotherapy, destroy tumor cells primarily by the induction of apoptosis (Vangestel et al., 2009). The great number of publications on the antitumor action of propolis and its compounds reveals their potential in the development of new anticancer agents (Sforcin and Bankova, 2011) since that propolis and its compounds have influence in the apoptotic process on cancer cells (Sawicka et al., 2012). Several *in vitro* studies have shown a proapoptotic effect of propolis in different types of cancer cell lines such as MCF-7 (Seda Vatansever et al., 2010), HeLa (Orsolic and Basic, 2003) and HL-60 (Eom et al., 2010). The apoptosis mechanism induced by propolis seems to be through activating caspase-dependent pathway (Aso et al., 2004), however it can

be dependent on the type of substances and the concentration of the propolis extract (Sawicka et al., 2012; Watanabe et al., 2011). Our TUNEL and Annexin-V data showed that red propolis treatment markedly increased the mid- to late-apoptotic levels of Hep-2 cells after treatment, which are in agreement with protein profiles from cells treated with red propolis, demonstrated by altered morphology and thus potentially may be an effective anticancer agent for this kind of cancer cells.

In summary, we were able to identify candidate proteins that participate of the regulatory metabolism of Hep-2 cells upon red propolis treatment. Among all proteins identified with different expression, most were down-regulated in the propolis treated cells. Hep-2 cells treated with red propolis revealed differentially expressed proteins related to energy production and conversion, carbohydrate transport and metabolism, post-translational modification, protein turnover and chaperons, cytoskeleton, ribosomal structure and repair pathway. These differences in tumor cell protein profiles warrant further investigations focusing on protein validation trough *in situ* studies and protein identification of other tumor lines treated with red propolis extracts.

5. Conclusions

Our group has previously reported that red propolis from Brazil is composed of complex molecules and exhibits important biological properties with capacity to scavenge free radicals and to inhibit tumor cell growth in a selective manner. Our results show that red propolis interacts with a whole set of intracellular events and turns to be a promising candidate to inhibit cell growth and contribute to different steps related to the carcinogenesis process. Effects of some of the proteins here and elsewhere identified that contribute to regulate the metabolism may provide further applications as molecular targets of therapeutic intervention for several cancer types, including the ones found for Hep-2. Cell-based proteomic assays are particularly valuable when searching for therapeutic agents once they can reveal activity against a particular molecular target and present desirable properties such as the protein–protein interactions to permeate cells and to retain biological activity. Although the molecular mechanisms by which red propolis interacts with cell metabolism remains still unclear, further studies including isolation of major bioactive compounds of red propolis in different tumor and non-tumor cell lines using proteomics and molecular tests to validate the differential protein expression here observed will contribute to elucidate the antioxidant and anticancer activities reported.

6. Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by CAPES and FAPERGS.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.11.003.

References

Abbasi, R., Ramroth, H., Becher, H., Dietz, A., Schmezer, P., Popanda, O., 2009. Laryngeal cancer risk associated with smoking and alcohol consumption is modified by genetic polymorphisms in ERCC5, ERCC6 and RAD23B but not by polymorphisms in five other nucleotide excision repair genes. Int. J. Cancer 125, 1431–1439.

Akyol, S., Ozturk, G., Ginis, Z., Armutcu, F., Yigitoglu, M.R., Akyol, O., 2013. In vivo and in vitro antineoplastic actions of caffeic acid phenethyl ester (CAPE): therapeutic perspectives. Nutr. Cancer 65, 515–526.

Alencar, S.M., Oldoni, T.L., Castro, M.L., Cabral, I.S., Costa-Neto, C.M., Cury, J.A., Rosalen, P.L., Ikegaki, M., 2007. Chemical composition and biological activity of a new type of Brazilian propolis: red propolis. J. Ethnopharmacol. 113, 278–283.

Aso, K., Kanno, S., Tadano, T., Satoh, S., Ishikawa, M., 2004. Inhibitory effect of propolis on the growth of human leukemia U937. Biol. Pharm. Bull. 27, 727–730.

Baek, Y.M., Hwang, H.J., Kim, S.W., Hwang, H.S., Lee, S.H., Kim, J.A., Yun, J.W., 2008. A comparative proteomic analysis for capsaicin-induced apoptosis between human hepatocarcinoma (HepG2) and human neuroblastoma (SK–N–SH) cells. Proteomics 8, 4748–4767.

Barreto, A.L.S., 2008. Estudo histomorfológico do efeito de membranas de colágeno contendo própolis vermelha sobre o processo de reparo cicatricial por segunda intenção em ratos. Universidade Tiradentes, Aracaju.

Bergink, S., Toussaint, W., Luijsterburg, M.S., Dinant, C., Alekseev, S., Hoeijmakers, J.H., Dantuma, N.P., Houtsmuller, A.B., Vermeulen, W., 2012. Recognition of DNA damage by XPC coincides with disruption of the XPC-RAD23 complex. J. Cell Biol. 196, 681–688.

Bianchi, L., Bruzzese, F., Leone, A., Gagliardi, A., Puglia, M., Di Gennaro, E., Rocco, M., Gimigliano, A., Pucci, B., Armini, A., Bini, L., Budillon, A., 2011. Proteomic analysis identifies differentially expressed proteins after HDAC vorinostat and EGFR inhibitor gefitinib treatments in Hep-2 cancer cells. Proteomics 11, 3725–3742.

Bozzuto, G., Ruggieri, P., Molinari, A., 2010. Molecular aspects of tumor cell migration and invasion. Ann. Ist Super Sanita 46, 66–80.

Brockstedt, E., Rickers, A., Kostka, S., Laubersheimer, A., Dorken, B., Wittmann-Liebold, B., Bommert, K., Otto, A., 1998. Identification of apoptosis-associated proteins in a human Burkitt lymphoma cell line. Cleavage of heterogeneous nuclear ribonucleoprotein A1 by caspase 3. J. Biol. Chem. 273, 28057–28064.

Burdock, G.A., 1998. Review of the biological properties and toxicity of bee propolis (propolis). Food Chem. Toxicol. 36, 347–363.

Cabral, I.S.R., Oldoni, T.L.C., Prado, A., Bezerra, R.M.N., Alencar, S.M.d., 2009. Composição fenólica, atividade antibacteriana e antioxidante da própolis vermelha brasileira. Quim Nova 32, 1523–1527.

Cao, S., Xu, W., Zhang, N., Wang, Y., Luo, Y., He, X., Huang, K., 2012. A mitochondria-dependent pathway mediates the apoptosis of GSE-induced yeast. PLoS One 7, e32943.

Castaldo, S., Capasso, F., 2002. Propolis, an old remedy used in modern medicine. Fitoterapia 73, S1–S6.

Chang, Y.J., Huang, Y.P., Li, Z.L., Chen, C.H., 2012. GRP78 knockdown enhances apoptosis via the down-regulation of oxidative stress and Akt pathway after epirubicin treatment in colon cancer DLD-1 cells. PLoS One 7, e35123.

Chen, L.F., Cohen, E.E., Grandis, J.R., 2010. New strategies in head and neck cancer: understanding resistance to epidermal growth factor receptor inhibitors. Clin. Cancer Res. 16, 2489–2495.

Chiu, C.C., Lin, C.Y., Lee, L.Y., Chen, Y.J., Kuo, T.F., Chang, J.T., Liao, C.T., Wang, H.M., Yen, T.C., Shen, C.R., Liao, S.K., Cheng, A.J., 2008. Glucose-regulated protein 78 regulates multiple malignant phenotypes in head and neck cancer and may serve as a molecular target of therapeutic intervention. Mol. Cancer Ther. 7, 2788–2797.

Chuthapisith, S., Layfield, R., Kerr, I.D., Hughes, C., Eremin, O., 2007. Proteomic profiling of MCF-7 breast cancer cells with chemoresistance to different types of anti-cancer drugs. Int. J. Oncol. 30, 1545–1551.

Daugsch, A., Moraes, C.S., Fort, P., Park, Y.K., 2008. Brazilian red propolis–chemical composition and botanical origin. Evid Based Complement Alternat Med 5, 435–441.

de Almeida, E.B., Cordeiro Cardoso, J., Karla de Lima, A., de Oliveira, N.L., de Pontes-Filho, N.T., Oliveira Lima, S., Leal Souza, I.C., de Albuquerque-Junior, R.L., 2013. The incorporation of Brazilian propolis into collagen-based dressing films improves dermal burn healing. J. Ethnopharmacol. 147, 419–425.

Eom, H.S., Lee, E.J., Yoon, B.S., Yoo, B.S., 2010. Propolis inhibits the proliferation of human leukaemia HL-60 cells by inducing apoptosis through the mitochondrial pathway. Nat. Prod. Res. 24, 375–386.

Franchi Jr, G.C., Moraes, C.S., Toreti, V.C., Daugsch, A., Nowill, A.E., Park, Y.K., 2012. Comparison of effects of the ethanolic extracts of brazilian propolis on human leukemic cells as assessed with the MTT assay. Evid Based Complement Alternat Med. http://dx.doi.org/10.1155/2012/918956.

Friedberg, E.C., 2001. How nucleotide excision repair protects against cancer. Nat. Rev. Cancer 1, 22–33.

Frozza, C.O., Garcia, C.S., Gambato, G., de Souza, M.D., Salvador, M., Moura, S., Padilha, F.F., Seixas, F.K., Collares, T., Borsuk, S., Dellagostin, O.A., Henriques, J.A., Roesch-Ely, M., 2013. Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. Food Chem. Toxicol. 52, 137–142.

Garrido, C., Gurbuxani, S., Ravagnan, L., Kroemer, G., 2001. Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem. Biophys. Res. Commun. 286, 433–442.

Gavert, N., Ben-Ze'ev, A., 2008. Epithelial–mesenchymal transition and the invasive potential of tumors. Trends Mol. Med. 14, 199–209.

Haddad, Y., Choi, W., McConkey, D.J., 2009. Delta-crystallin enhancer binding factor 1 controls the epithelial to mesenchymal transition phenotype and resistance to the epidermal growth factor receptor inhibitor erlotinib in human head and neck squamous cell carcinoma lines. Clin. Cancer Res. 15, 532–542.

Ho, J., Barbi de Moura, M., Lin, Y., Vincent, G., Thorne, S., Duncan, L.M., Lin, H.M., Kirkwood, J.M., Becker, D., van Houten, B., Moschos, S., 2012. Importance of glycolysis and oxidative phosphorylation in advanced melanoma. Mol. Cancer 11, 76.
Hodgkinson, V.C., Agarwal, V., D, E.L., Fox, J.N., McManus, P.L., Mahapatra, T.K., Kneeshaw, P.J., Drew, P.J., Lind, M.J., Cawkwell, L., 2012. Pilot and feasibility study: comparative proteomic analysis by 2-DE MALDI TOF/TOF MS reveals 14-3-3 proteins as putative biomarkers of response to neoadjuvant chemotherapy in ER-positive breast cancer. J. Proteomics 75, 2745–2752.
Honore, B., Buus, S., Claesson, M.H., 2008. Identification of differentially expressed proteins in spontaneous thymic lymphomas from knockout mice with deletion of p53. Proteome Sci. 6, 18.
Hussien, R., Brooks, G.A., 2011. Mitochondrial and plasma membrane lactate transporter and lactate dehydrogenase isoform expression in breast cancer cell lines. Physiol. Genomics 43, 255–264.
Janicijevic, A., Sugasawa, K., Shimizu, Y., Hanaoka, F., Wijgers, N., Djurica, M., Hoeijmakers, J.H., Wyman, C., 2003. DNA bending by the human damage recognition complex XPC-HR23B. DNA Repair (Amst) 2, 325–336.
Ji, H., Moritz, R.L., Kim, Y.S., Zhu, H.J., Simpson, R.J., 2007. Analysis of Ras-induced oncogenic transformation of NIH-3T3 cells using differential-display 2-DE proteomics. Electrophoresis 28, 1997–2008.
Jiang, C.C., Mao, Z.G., Avery-Kiejda, K.A., Wade, M., Hersey, P., Zhang, X.D., 2009. Glucose-regulated protein 78 antagonizes cisplatin and adriamycin in human melanoma cells. Carcinogenesis 30, 197–204.
Kageyama, T., Nagashio, R., Ryuge, S., Matsumoto, T., Iyoda, A., Satoh, Y., Masuda, N., Jiang, S.X., Saegusa, M., Sato, Y., 2011. HADHA is a potential predictor of response to platinum-based chemotherapy for lung cancer. Asian Pac. J. Cancer Prev. 12, 3457–3463.
Kahali, S., Sarcar, B., Fang, B., Williams, E.S., Koomen, J.M., Tofilon, P.J., Chinnaiyan, P., 2010. Activation of the unfolded protein response contributes toward the antitumor activity of vorinostat. Neoplasia 12, 80–86.
Kamiya, T., Nishihara, H., Hara, H., Adachi, T., 2012. Ethanol extract of Brazilian red propolis induces apoptosis in human breast cancer MCF-7 cells through endoplasmic reticulum stress. J. Agric. Food Chem. 60, 11065–11070.
Kavallaris, M., 2010. Microtubules and resistance to tubulin-binding agents. Nat. Rev. Cancer 10, 194–204.
Kim, J.S., Chang, J.W., Yun, H.S., Yang, K.M., Hong, E.H., Kim, D.H., Um, H.D., Lee, K.H., Lee, S.J., Hwang, S.G., 2010. Chloride intracellular channel 1 identified using proteomic analysis plays an important role in the radiosensitivity of HEp-2 cells via reactive oxygen species production. Proteomics 10, 2589–2604.
Kim, J.H., Kim, E.L., Lee, Y.K., Park, C.B., Kim, B.W., Wang, H.J., Yoon, C.H., Lee, S.J., Yoon, G., 2011. Decreased lactate dehydrogenase B expression enhances claudin 1-mediated hepatoma cell invasiveness via mitochondrial defects. Exp. Cell Res. 317, 1108–1118.
Kinoshita, T., Nohata, N., Yoshino, H., Hanazawa, T., Kikkawa, N., Fujimura, L., Chiyomaru, T., Kawakami, K., Enokida, H., Nakagawa, M., Okamoto, Y., Seki, N., 2012. Tumor suppressive microRNA-375 regulates lactate dehydrogenase B in maxillary sinus squamous cell carcinoma. Int. J. Oncol. 40, 185–193.
Koumenis, C., 2006. ER stress, hypoxia tolerance and tumor progression. Curr. Mol. Med. 6, 55–69.
Lahat, G., Zhu, Q.S., Huang, K.L., Wang, S., Bolshakov, S., Liu, J., Torres, K., Langley, R.R., Lazar, A.J., Hung, M.C., Lev, D., 2010. Vimentin is a novel anti-cancer therapeutic target; insights from in vitro and in vivo mice xenograft studies. PLoS One 5, e10105.
Li, J., Lee, A.S., 2006. Stress induction of GRP78/BiP and its role in cancer. Curr. Mol. Med. 6, 45–54.
Li, F., Awale, S., Tezuka, Y., Kadota, S., 2008. Cytotoxic constituents from Brazilian red propolis and their structure-activity relationship. Bioorg. Med. Chem. 16, 5434–5440.
Liu, R., Li, Z., Bai, S., Zhang, H., Tang, M., Lei, Y., Chen, L., Liang, S., Zhao, Y.L., Wei, Y., Huang, C., 2009. Mechanism of cancer cell adaptation to metabolic stress: proteomics identification of a novel thyroid hormone-mediated gastric carcinogenic signaling pathway. Mol. Cell Proteomics 8, 70–85.
Lizarbe, M.A., Barrasa, J.I., Olmo, N., Gavilanes, F., Turnay, J., 2013. Annexin-phospholipid interactions. Functional implications. Int. J. Mol. Sci. 14, 2652–2683.
Lustosa, S.R., Galindo, A.B., Nunes, L.C.C., Randau, K.P., Neto, P.J.R., 2008. Própolis: atualizações sobre a química e a farmacologia. Rev. Bras. Farmacogn. 18, 447–454.
Maiti, A.K., 2012. Reactive oxygen species reduction is a key underlying mechanism of drug resistance in cancer chemotherapy. Chemotherapy 1. http://dx.doi.org/10.4172/2167-7700.1000104.
Mhaidat, N., Jaradat, S., Aldaher, A., Ghabkari, A., 2011. Inhibition of GRP78 sensitizes colorectal cancer cells to paclitaxel-induced apoptosis by activation of caspase-4. Int. J. Biol. Biomed. Eng. 5, 41–48.
Mukhopadhyay, S., Good, D., Miller, R.D., Graham, J.E., Mathews, S.A., Timms, P., Summersgill, J.T., 2006. Identification of *Chlamydia pneumoniae* proteins in the transition from reticulate to elementary body formation. Mol. Cell Proteomics 5, 2311–2318.
Myung, J.K., Afjehi-Sadat, L., Felizardo-Cabatic, M., Slavc, I., Lubec, G., 2004. Expressional patterns of chaperones in ten human tumor cell lines. Proteome Sci. 2, 8.
Neher, T.M., Rechkunova, N.I., Lavrik, O.I., Turchi, J.J., 2010. Photo-cross-linking of XPC-Rad23B to cisplatin-damaged DNA reveals contacts with both strands of the DNA duplex and spans the DNA adduct. Biochemistry 49, 669–678.
Nodale, C., Sheffer, M., Jacob-Hirsch, J., Folgiero, V., Falcioni, R., Aiello, A., Garufi, A., Rechavi, G., Givol, D., D'Orazi, G., 2012. HIPK2 downregulates vimentin and inhibits breast cancer cell invasion. Cancer Biol. Ther. 13, 198–205.
Noh, D.Y., Ahn, S.J., Lee, R.A., Kim, S.W., Park, I.A., Chae, H.Z., 2001. Overexpression of peroxiredoxin in human breast cancer. Anticancer Res. 21, 2085–2090.
Okoli, A.S., Fox, E.M., Raftery, M.J., Mendz, G.L., 2007. Effects of Helicobacter hepaticus on the proteome of HEp-2 cells. Antonie Van Leeuwenhoek 92, 289–300.
Orsolic, N., Basic, I., 2003. Immunomodulation by water-soluble derivative of propolis: a factor of antitumor reactivity. J. Ethnopharmacol. 84, 265–273.
Pan, T.L., Wang, P.W., Huang, C.C., Yeh, C.T., Hu, T.H., Yu, J.S., 2012. Network analysis and proteomic identification of vimentin as a key regulator associated with invasion and metastasis in human hepatocellular carcinoma cells. J. Proteomics 75, 4676–4692.
Park, J.H., Kim, Y.S., Lee, H.L., Shim, J.Y., Lee, K.S., Oh, Y.J., Shin, S.S., Choi, Y.H., Park, K.J., Park, R.W., Hwang, S.C., 2006. Expression of peroxiredoxin and thioredoxin in human lung cancer and paired normal lung. Respirology 11, 269–275.
Piccinelli, A.L., Lotti, C., Campone, L., Cuesta-Rubio, O., Campo Fernandez, M., Rastrelli, L., 2011. Cuban and Brazilian red propolis: botanical origin and comparative analysis by high-performance liquid chromatography–photodiode array detection/electrospray ionization tandem mass spectrometry. J. Agric. Food Chem. 59, 6484–6491.
Pinheiro, M.S., 2009. Avaliação da atividade antimicrobiana e citoprotetora gástrica dos extratos de mangaba, caju e própolis vermelha. Universidade Tiradentes, Aracaju.
Reed, J.C., 2000. Methods in Enzymology. Academic Press, San Diego, CA.
Righi, A.A., Alves, T.R., Negri, G., Marques, L.M., Breyer, H., Salatino, A., 2011. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. J. Sci. Food Agric. 91, 2363–2370.
Rodrigues-Lisoni, F.C., Peitl Jr., P., Vidotto, A., Polachini, G.M., Maniglia, J.V., Carmona-Raphe, J., Cunha, B.R., Henrique, T., Souza, C.F., Teixeira, R.A., Fukuyama, E.E., Michaluart Jr., P., de Carvalho, M.B., Oliani, S.M., Head Neck Genome Project, G., Tajara, E.H., Cury, P.M., de Carvalho, M.B., Dias-Neto, E., Figueiredo, D.L., Fukuyama, E.E., Gois-Filho, J.F., Leopoldino, A.M., Mamede, R.C., Michaluart-Junior, P., Moyses, R.A., Nobrega, F.G., Nobrega, M.P., Nunes, F.D., Ojopi, E.F., Serafini, L.N., Severino, P., Silva, A.M., Silva Jr., W.A., Silveira, N.J., Souza, S.C., Tajara, E.H., Wunsch-Filho, V., Amar, A., Bandeira, C.M., Braconi, M.A., Brandao, L.G., Brandao, R.M., Canto, A.L., Cerione, M., Cicco, R., Chagas, M.J., Chedid, H., Costa, A., Cunha, B.R., Curioni, O.A., Fortes, C.S., Franzi, S.A., Frizzera, A.P., Gazito, D., Guimaraes, P.E., Kaneto, C.M., Lopez, R.V., Macarenco, R., Magalhaes, M.R., Meneses, C., Mercante, A.M., Pinheiro, D.G., Polachini, G.M., Rapoport, A., Rodini, C.O., Rodrigues-Lisoni, F.C., Rodrigues, R.V., Rossi, L., Santos, A.R., Santos, M., Settani, F., Silva, F.A., Silva, I.T., Souza, T.B., Stabenow, E., Takamori, J.T., Valentim, P.J., Vidotto, A., Xavier, F.C., Yamagushi, F., Cominato, M.L., Correa, P.M., Mendes, G.S., Paiva, R., Ramos, O., Silva, C., Silva, M.J., Tarla, M.V., 2010. Genomics and proteomics approaches to the study of cancer-stroma interactions. BMC Med. Genomics 3, 14.
Sahab, Z.J., Kirilyuk, A., Zhang, L., Khamis, Z.I., Pompach, P., Sung, Y., Byers, S.W., 2012. Analysis of tubulin alpha-1A/1B C-terminal tail post-translational poly-glutamylation reveals novel modification sites. J. Proteome Res. 11, 1913–1923.
Sawicka, D., Car, H., Borawska, M.H., Niklinski, J., 2012. The anticancer activity of propolis. Folia Histochem. Cytobiol. 50, 25–37.
Seda Vatansever, H., Sorkun, K., Ismet Deliloglu Gurhan, S., Ozdal-Kurt, F., Turkoz, E., Gencay, O., Salih, B., 2010. Propolis from Turkey induces apoptosis through activating caspases in human breast carcinoma cell lines. Acta Histochem. 112, 546–556.
Semenza, G.L., 2008. Tumor metabolism: cancer cells give and take lactate. J. Clin. Invest. 118, 3835–3837.
Sforcin, J.M., 2007. Propolis and the immune system: a review. J. Ethnopharmacol. 113, 1–14.
Sforcin, J.M., Bankova, V., 2011. Propolis: is there a potential for the development of new drugs? J. Ethnopharmacol. 133, 253–260.
Shen, K., Rice, S.D., Gingrich, D.A., Wang, D., Mi, Z., Tian, C., Ding, Z., Brower, S.L., Ervin Jr., P.R., Gabrin, M.J., Tseng, G., Song, N., 2012. Distinct genes related to drug response identified in ER positive and ER negative breast cancer cell lines. PLoS One 7, e40900.
Silva, M.S.e.C., 2007. Estudo do efeito da fração BRVD obtida a partir da própolis brasileira tipificada, na proliferação de células tumorais. Universidade de São Paulo, São Paulo.
Siqueira, A.B., Gomes, B.S., Cambuim, I., Maia, R., Abreu, S., Souza-Motta, C.M., de Queiroz, L.A., Porto, A.L., 2009. Trichophyton species susceptibility to green and red propolis from Brazil. Lett. Appl. Microbiol. 48, 90–96.
Smith-Pearson, P.S., Kooshki, M., Spitz, D.R., Poole, L.B., Zhao, W., Robbins, M.E., 2008. Decreasing peroxiredoxin II expression decreases glutathione, alters cell cycle distribution, and sensitizes glioma cells to ionizing radiation and H(2)O(2). Free Radic. Biol. Med. 45, 1178–1189.
Soini, Y., Kallio, J.P., Hirvikoski, P., Helin, H., Kellokumpu-Lehtinen, P., Kang, S.W., Tammela, T.L., Peltoniemi, M., Martikainen, P.M., Kinnula, V.L., 2006. Oxidative/nitrosative stress and peroxiredoxin 2 are associated with grade and prognosis of human renal carcinoma. APMIS 114, 329–337.
Sonveaux, P., Vegran, F., Schroeder, T., Wergin, M.C., Verrax, J., Rabbani, Z.N., De Saedeleer, C.J., Kennedy, K.M., Diepart, C., Jordan, B.F., Kelley, M.J., Gallez, B., Wahl, M.L., Feron, O., Dewhirst, M.W., 2008. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. J. Clin. Invest. 118, 3930–3942.
Stresing, V., Baltziskueta, E., Rubio, N., Blanco, J., Arriba, M., Valls, J., Janier, M., Clézardin, P., Sanz-Pamplona, R., Nieva, C., Marro, M., Dmitri, P., Sierra, A., 2012.

Peroxiredoxin 2 specifically regulates the oxidative and metabolic stress response of human metastatic breast cancer cells in lungs. Oncogene 32, 724–735.
Sugasawa, K., Akagi, J., Nishi, R., Iwai, S., Hanaoka, F., 2009. Two-step recognition of DNA damage for mammalian nucleotide excision repair: directional binding of the XPC complex and DNA strand scanning. Mol. Cell 36, 642–653.
Umthong, S., Phuwapraisirisan, P., Puthong, Songchan, Chanchao, Chanpen, 2011. In vitro antiproliferative activity of partially purified Trigona laeviceps propolis from Thailand on human cancer cell lines. BMC Complement Altern. Med. 11, 37.
Valente, M.J., Baltazar, A.F., Henrique, R., Estevinho, L., Carvalho, M., 2011. Biological activities of Portuguese propolis: protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth in vitro. Food Chem. Toxicol. 49, 86–92.
Vangestel, C., Van de Wiele, C., Mees, G., Peeters, M., 2009. Forcing cancer cells to commit suicide. Cancer Biother. Radiopharm. 24, 395–407.
Wang, T., Tamae, D., LeBon, T., Shively, J.E., Yen, Y., Li, J.J., 2005. The role of peroxiredoxin II in radiation-resistant MCF-7 breast cancer cells. Cancer Res. 65, 10338–10346.
Wang, M., Wey, S., Zhang, Y., Ye, R., Lee, A.S., 2009. Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders. Antioxid. Redox. Signal 11, 2307–2316.
Watanabe, M.A., Amarante, M.K., Conti, B.J., Sforcin, J.M., 2011. Cytotoxic constituents of propolis inducing anticancer effects: a review. J. Pharm. Pharmacol. 63, 1378–1386.
Wilmet, J.P., Tastet, C., Desruelles, E., Ziental-Gelus, N., Blanckaert, V., Hondermarck, H., Le Bourhis, X., 2011. Proteome changes induced by overexpression of the p75 neurotrophin receptor (p75NTR) in breast cancer cells. Int. J. Dev. Biol. 55, 801–809.
Zhang, J., Jiang, Y., Jia, Z., Li, Q., Gong, W., Wang, L., Wei, D., Yao, J., Fang, S., Xie, K., 2006. Association of elevated GRP78 expression with increased lymph node metastasis and poor prognosis in patients with gastric cancer. Clin. Exp. Metastasis 23, 401–410.
Zhang, H., Cao, R., Lee, W.N., Deng, C., Zhao, Y., Lappe, J., Recker, R., Yen, Y., Wang, Q., Tsai, M.Y., Go, V.L., Xiao, G.G., 2010. Inhibition of protein phosphorylation in MIA pancreatic cancer cells: confluence of metabolic and signaling pathways. J. Proteome Res. 9, 980–989.
Zhou, H., Zhang, Y., Fu, Y., Chan, L., Lee, A.S., 2011. Novel mechanism of anti-apoptotic function of 78-kDa glucose-regulated protein (GRP78): endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BCL-2) from BCL-2-interacting killer (BIK). J. Biol. Chem. 286, 25687–25696.

CAPÍTULO 2

LC-MS analysis of Hep-2 and Hek-293 cell lines treated with Brazilian red propolis reveals differences in protein expression

Artigo publicado na revista *Journal of Pharmacy and Pharmacology*

JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP
Journal of Pharmacy
And Pharmacology
Research Paper

LC-MS analysis of Hep-2 and Hek-293 cell lines treated with Brazilian red propolis reveals differences in protein expression

Caroline O. da Silva Frozza[a], Emyle da Silva Brum[a], Anjali Alving[b], Sidnei Moura[c], João A. P. Henriques[a] and Mariana Roesch-Ely[a]

[a]Laboratory of Genomics, Proteomics and DNA Repair, Biotechnology Institute, University of Caxias do Sul, Caxias do Sul, RS, Brazil, [b]Bruker Daltonics, Billerica, MA, USA and [c]Laboratory of Natural and Synthetic Products, Biotechnology Institute, University of Caxias do Sul, Caxias do Sul, RS, Brazil

Keywords
cancer; proteomics; red propolis

Correspondence
Mariana Roesch-Ely, Universidade de Caxias do Sul. Rua Francisco Getúlio Vargas 1130, 95070-560, Caxias do Sul, RS, Brasil.
E-mail: mrely@ucs.br

Received November 4, 2015
Accepted April 30, 2016

doi: 10.1111/jphp.12577

Abstract

Objective Red propolis, an exclusive variety of propolis found in the northeast of Brazil has shown to present antitumour activity, among several other biological properties. This article aimed to help to evaluate the underlying molecular mechanisms of the potential anticancer effects of red propolis on tumour, Hep-2, and non-tumour cells, Hek-293.

Methods Differentially expressed proteins in human cell lines were identified through label-free quantitative MS-based proteomic platform, and cells were stained with Giemsa to show morphological changes.

Key findings A total of 1336 and 773 proteins were identified for Hep-2 and Hek-293, respectively. Among the proteins here identified, 16 were regulated in the Hep-2 cell line and 04 proteins in the Hek-293 line. Over a total of 2000 proteins were identified under MS analysis, and approximately 1% presented differential expression patterns. The GO annotation using Protein Analysis THrough Evolutionary Relationships classification system revealed predominant molecular function of catalytic activity, and among the biological processes, the most prominent was associated to cell metabolism.

Conclusion The proteomic profile here presented should help to elucidate further molecular mechanisms involved in inhibition of cancer cell proliferation by red propolis, which remain unclear to date.

Introduction

Propolis is a resinous substance produced by honeybees by mixing their salivated secretions with resins collected from the cracks of the tree bark.[1] The red propolis variety is obtained exclusively in the northeast of Brazil. Its botanical origin is *Dalbergia ecastophyllum* (L) Taud. (Leguminosae)[2] and presents two molecules responsible for its pigments: retusapurpurin A and B.[3]

For the last decade, red propolis has been attributed as a source of potential anticancer activity product.[1,4] This is characterized by a complex mixture of molecules including flavonoids, chalcones and isoflavonoids.[5] Liquiritigenin, formononetin and biochanin A are some examples of important target compounds present in red propolis extracts that were identified previously by our group.[6] Some of these compounds have been cited by the literature as natural anticancer products. Isoflavones participate in cell death induction patterns using culture models, and biochanin A is attributed to decrease invasive activity of glioblastoma lines.[7,8] Formononetin and liquiritigenin also showed inhibition of tumour growth in osteosarcoma and HeLa cancer cell lines, respectively.[9,10]

In vitro studies have shown that red propolis extract is effective against several types of cancer including leukaemia,[11] pancreatic,[12] breast,[13] bladder,[14] laryngeal epidermoid carcinoma and cervical adenocarcinoma.[6] An increase of reports dealing with red propolis and its effects in cancer cell metabolism has arisen; however, the molecular mechanisms in which red propolis act are yet not fully

© 2016 Royal Pharmaceutical Society,

understood. Recently, laryngeal carcinoma tissue has been submitted to proteomic analysis to screen potential diagnostic and therapeutic targets compared to normal tissue phenotype.[15] Some of these proteins identified are listed here and modulated after red propolis extract treatment, suggesting that red propolis extract is interacting in a direct or indirect way with cancer cell metabolism.

Proteomics is a great tool to investigate protein expression and modifications due to altered physiological conditions in biological systems. This technology has been used to investigate cellular effects of general drug treatment and development of drug resistance.[16] Here, a comparative proteomic analysis combined with high-resolution mass spectrometer (HRMS) and gel-free quantification was performed to identify and determine the profiles of proteins in the Hep-2 and Hek-293 cell lines treated or not with total extract of red propolis. So far, there are no reports on proteomics comparing tumour and normal cell lines treated with red propolis extracts through LC-MS analysis. The different compounds presented in red propolis extract can act alone or combined, generating synergistic effects. Therefore, further studies from our group are also focusing in evaluating the effects of fractions and isolated compounds in cancer cell metabolism.

Materials and Methods

Chemicals

Dulbecco's modified eagle's medium–high glucose (DMEM), trypsin–EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Giemsa, biochanin A, formononetin, and liquiritigenin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA); ethanol was from Merck (Darmstadt, Hesse, Germany). Heat-inactivated fetal bovine serum (FBS) was purchased from Cultilab Lab Inc. (Campinas, SP, Brazil) and penicillin–streptomycin was purchased from GIBCO™ Invitrogen (Grand Island, NY, USA). All other chemicals were of ultrapure grade and obtained from Sigma-Aldrich. For chemical analysis, ethanol was purchased by Tedia (Fairfield, OH, USA) and was used water prepared in MilliQ ® Eppendorf.

Red Propolis Sample

Red propolis was collected in the Capivara Apiary in Brejo Grande, state of Sergipe located in the northeast of Brazil (10° 28′ 25″ S and 36° 26′ 12″ W). The propolis was protected from light and frozen at 20 °C until extract preparation. The hydroalcoholic extract was processed and characterized as reported by our group [6].

Briefly, red propolis samples were ground to a fine powder. A total of 50 g was mixed with 500 ml of EtOH-H_2O 70–30% (v/v) and shaken at room temperature for 24 h. After extraction, the mixture was filtered and the solvent was evaporated and produced a red fine powder. The dry extract was kept frozen at −20 °C and prepared at two different concentrations with EtOH–H_2O 50% (v/v) and finally filtered through a 0.22 μm polyethersulfone membrane (TPP; Techno Plastic Products, Switzerland).

Chemical Composition – LC-MS-MS

The extract was dissolved in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid, which was filtered on a nylon membrane (0.45 μm). The solution was infused directly or with UFLC (Shymadzu) assistance into the ESI source. For direct infusion was used a syringe pump (Harvard Apparatus) at a flow rate of 10 μl/min. The UFLC separation was performed with a 5 μm LiChrospher RP18 column at a flow rate of 0.6 ml/min according to Ku-Cükboyaci *et al.*[17]. The compounds have been identified by HRMS ESI(+)-MS and tandem ESI(+)-MS/MS, which were acquired using a hybrid Orbitrap mass spectrometer (Thermo Fisher Scientific, USA), under the following conditions: capillary and cone voltages were set to +3500 V and +40 V, respectively, with a desolvation temperature of 100 °C. For ESI(+)-MS/MS, the energy for the collision induced dissociations (CID) was optimized for each component. The Xcalibur software was used for data acquisition and processing. The compounds were identified by the comparison of their ESI(+)-MS/MS dissociation patterns with standards or previous studies reported. For compounds quantification, three molecules (biochanin A, formononetin and liquiritigenin) which may be considered as potential interfering agents of cancer progression were further investigated using ultra-fast liquid chromatography (UFLC) and compared to their commercial standards.

Cell Culture and Treatment

Hep-2 (human laryngeal epidermoid carcinoma cell) and Hek-293 (human normal epithelial embryonic kidney) were obtained from American Type Culture Collection (ATCC), USA. Cells were grown in Dulbecco's modified eagle's medium–high glucose, supplemented with 10% (w/v) FBS. The cultures were maintained in a humidified atmosphere at 37 °C, 5% CO_2. The study was performed as cells reached 70–80% confluence.

Initially, 2.6×10^6 cells were seeded into 75-cm^2 culture flasks with supplemented culture medium. After 24 h, Hep-2 cells were treated with red propolis extract with 120 μg/ml and Hek-293 cells with 220 μg/ml for 1 h,

followed by 24 h of supplemented culture medium. These treatment concentrations were chosen according to their IC50, determined by previous survival experiments, through MTT assay, reported by our group.[6] The negative control groups were treated with the same amount of hydroalcoholic solution (2.5% ethanol) used for the extracts. Experimental procedures were performed at least in triplicate.

Morphological Examination

Cells were seeded into 24-well plates containing coverslips. After 24-h incubation, Hep-2 and Hek-293 were treated with red propolis hydroalcoholic extract at 120 and 220 μg/ml, respectively, for 1 h, followed by cultivation in extract-free medium for 24 h. The negative control group was treated with EtOH–H_2O 50% (v/v) for the same period. Cells were stained with Giemsa (Accustain; Sigma-Aldrich) according to standard Giemsa procedure established by the supplier. Morphological changes of the cells were observed and photographed under a light microscope at 200× (Olympus).

Protein Extraction and Digestion

Cells were harvested and lysed in 0.5 ml of lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris base pH 8.5, 20 mM Dithiothreitol (DTT), 4% (w/v) CHAPS and 1% (w/v) protease inhibitor). After 30 min at room temperature shaking at 90 rpm, the lysate was scraped, transferred into microtubes and centrifuged at 20,817 × g rpm for 30 min, at 4 °C. The supernatant was collected and frozen at −20 °C. The procedure was repeated three times until a pool was formed.

After protein extraction, samples were dialysed for 24 h, at 4 °C, with gentle agitation on a magnetic stirrer, changing four times Milli-Q water every 6 h. Bradford assay (BioRad Laboratories, Hercules, CA, USA) was used to determine protein quantification, according to the manufacturer's instructions. Aliquots of 0.4 mg of protein were frozen at −80 °C. Protein precipitation was processed with 20% trichloroacetic acid in cold acetone overnight, followed by five washes with cold acetone. Next, pellet received 25 μl ammonium bicarbonate stock solution (100 mM), followed by 25 μl trifluoroethanol denaturation agent and 2.5 μl DTT stock solution (200 mM). The samples were mixed in Vortex and heated (90 °C for 20 min). A total of 10 μl iodoacetamide (IAM) stock solution (200 mM) was added and allowed to stand at room temperature for 1 h in the dark. To eliminate excess of IAM, 2.5 μl DTT stock solution was added, and samples were kept 1 h in the dark. Next, 300 μl of water diluted the denaturant solution and 100 μl ammonium bicarbonate stock was inserted. Fresh stock solution of trypsin was made and added at 1 : 20 enzyme: substrate and further incubated overnight at 37 °C. Finally, 2 μl TFA was used to lower the pH and stop trypsin activity. The samples were frozen at −80 °C and lyophilized (Labconco FreeZone; Labconco Corporation, Kansas, MO, USA). Peptides were then resuspended in 0.1% formic acid for further mass spectrometry analysis.

Protein Composition – LC-MS/MS

For the LC-MS/MS analysis, the tryptic digests were first trapped on a PepMap trapping column (2 cm × 100 μm; Thermo Scientific) and later separated on a PepMap Acclaim UHPLC column (50 cm × 75 μm, 2 μm particles; Thermo Scientific) with a 60 min acetonitrile gradient from 2 to 40% B (mobile phase A : 0.1% formic acid; mobile phase B : 100% acetonitrile in 0.1% formic acid). A CaptiveSpray nanoBooster source, with acetonitrile as a dopant, was used to interface the LC system to the impact HD benchtop UHR-Q-TOF system (Bruker Daltonics, Billerica, MA, USA).

Label-Free Quantitative Analysis of MS Data : Workflow Using ProfileAnalysis™ and ProteinScape™ Software (Bruker Daltonics)

Each LC-MS run was processed with Bruker Daltonics Compass DataAnalysis software to generate a list of molecular features. A molecular feature contains all signals in the LC-MS run which originate from the same peptide. Therefore, a molecular feature corresponds to a peptide. After molecular features of all LC-MS runs were elucidated, they were used to derive peptide quantification ratios in ProfileAnalysis. Using statistical tests, significantly regulated peptides were determined. Peptide regulation ratios were transferred to the ProteinScape database system.

LC-MS/MS analyses were processed in DataAnalysis software. The resulting peak lists were transferred to the ProteinScape database system. Using ProteinScape, MS/MS spectra were searched against a sequence database to identify peptides. A combined protein list based on all acquired MS/MS spectra of the experiment was generated. Identified peptides were linked to their regulation ratios using accurate mass and retention time as assignment criteria. Finally, protein regulations were determined.

Statistical Analysis in ProfileAnalysis ™

To derive peptide regulation ratios in ProfileAnalysis, an MS-*T*-Test model was calculated. The result of the MS-*T*-Test model calculation is reported in two tables: the Bucket Table and the *T*-Test Result Table. Each bucket is characterized by a retention time and MH+ value and corresponds to a peptide. Each bucket defines a row in the

Bucket Table and in the *T*-Test Result Table. A value specified for a particular bucket in a column is the intensity of a peptide measured in the corresponding LC-MS run. For each bucket, the average intensity is calculated per group attribute. As we have one group defined with two attributes, we have two average intensities for each peptide. The ratio of the average intensities is the regulation ratio of a bucket. It is reported in the *T*-Test Result Table in the column Average Ratio. The *T*-Test Result Table was transferred to ProteinScape where quantification and identification of peptides were combined to a final protein list showing regulation ratios for each protein.

Protein Identification in ProteinScape™

LC-MS/MS data were processed with Bruker's ProteinScape™ bioinformatics software suite. Database searches were performed with Mascot 2.4 triggered by ProteinScape. The parameters used in Mascot searches were as follows: carbamidomethylation of cysteine was set as a fixed modification and variable modifications was the Acetyl (N-term), Gln → pyro-Glu (N-term Q), Glu → pyro-Glu (N-term E) and Oxidation (M). Trypsin was specified as the proteolytic enzyme, and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.5 Da and peptide charges were set at +1,+ 2, and + 3. Mascot results were filtered with Mascot Percolator to improve the accuracy and sensitivity of the peptide identification. The accepted peptide false discovery rate was set to <1%.

Results

The chemical composition of the red propolis extract from Sergipe state (Brejo Grande) was previously analyzed by our group trough HRMS.[6] Among the compounds identified, most are of phenolic acids and flavonoids: 6-acetyl-2,2-dimethyl-3-hydroxychroman (m/z 221.1204); hydroxychroman-2-hydroxy-4-methoxychalcone (m/z 255. 1022); liquiritigenin (m/z 257.0811); formononetin (m/z 269.0820); medicarpin (m/z 271.0975); biochanin A (m/z 285.0765); retusapurpurin B (m/z 523.1751); and hesperetin-7-rhamnoglucoside (m/z 611.1965) (Figure 1). Three of the compounds identified by Frozza *et al.*[6] are of great interest in cancer research investigation and were further quantified through UFLC (Figure 2). The available standards were biochanin A, formononetin and liquiritigenin and presented r^2 of 0.9987; 0.9986; 0.9998, respectively. Sergipe red propolis extract showed biochanin A 0.41 ± 0.007 mg/g; formononetin 2.37 ± 0.010 mg/g and liquiritigenin 1.54 ± 0.015 mg/g. The remaining compounds were not quantified, only qualified by MS/MS as previously reported.[6]

Red propolis extract rich in phenolic acids and flavonoids was initially tested through cytotoxic essays. Afterwards, cells were treated with different concentrations of extract and compared to the control untreated group: Hep-2 with 120 μg/ml and Hek-293 with 220 μg/ml, concentrations that correspond to the IC50 found in the previous MTT analysis. Cell morphology was further investigated using Giemsa staining that revealed altered morphological structure after treatment (Figure 3). The cells in the negative control group that received hydroalcoholic solution of 2.5% (v/v) presented a rounded-like morphology, surrounded by abundant, defined and intact cytoplasm (Figure 3 a, c). Cells that received treatment with red propolis extracts – IC50 from each cell line – presented detachment and shrinkage, indicating several biological changes upon extract activity (Figure 3b, d).

For protein profile analysis, total soluble proteins were extracted from three independent biological replicate samples and exposed to nano-LC-MS/MS analysis. A total of 2109 proteins were identified, 1336 in Hep-2 and 773 in Hek-293 group (Table S1 and S2, respectively). Proteins were identified with at least two unique peptide matches ($P < 0.05$). Identification was made in triplicate and reproducibility was secured. For better visualization, the double fold change for the log2 values derived from the quotient (treated/control) is presented in Figure 4 as a Volcano plot, statistically restricted by a 0.01 *P*-value line, at 2.0 (*y* axis-log10).

Considering that most peptides identified were taken from the tumour line sample, proteins exclusively from these samples were further analyzed and organized according to biological processes. From the 1336 Hep-2 proteins identified, approximately 15% were present in all triplicates in the control group (207) and treated group (208) (Table S3 and S4, respectively). Comparing the proteins identified in the two groups of Hep-2 (control and treated) analyzed, 110 proteins (Table S5) were in accordance for both groups (Figure 5a).

To better understand their biological functions, proteins were further functionally classified using the Protein Analysis THrough Evolutionary Relationships (PANTHER, v. 9.0) bioinformatics tool (http://www.pantherdb.org). The matching proteins ($n = 110$) were classified according to the GO annotations 'biological process' (Figure 5b) where 30% were associated to metabolism, followed by cellular (20%), developmental (14%) and biogenesis (12%).

Proteins identified in both cell lines were also classified and are presented in Figure 6 by percentage of their 'molecular function'. Similar molecular function profiles of identified proteins were observed for both tumour and non-tumour lines. Predominant functions for both groups were of catalytic activity (36%), followed by protein binding properties (33%) and structural molecule activity (13%

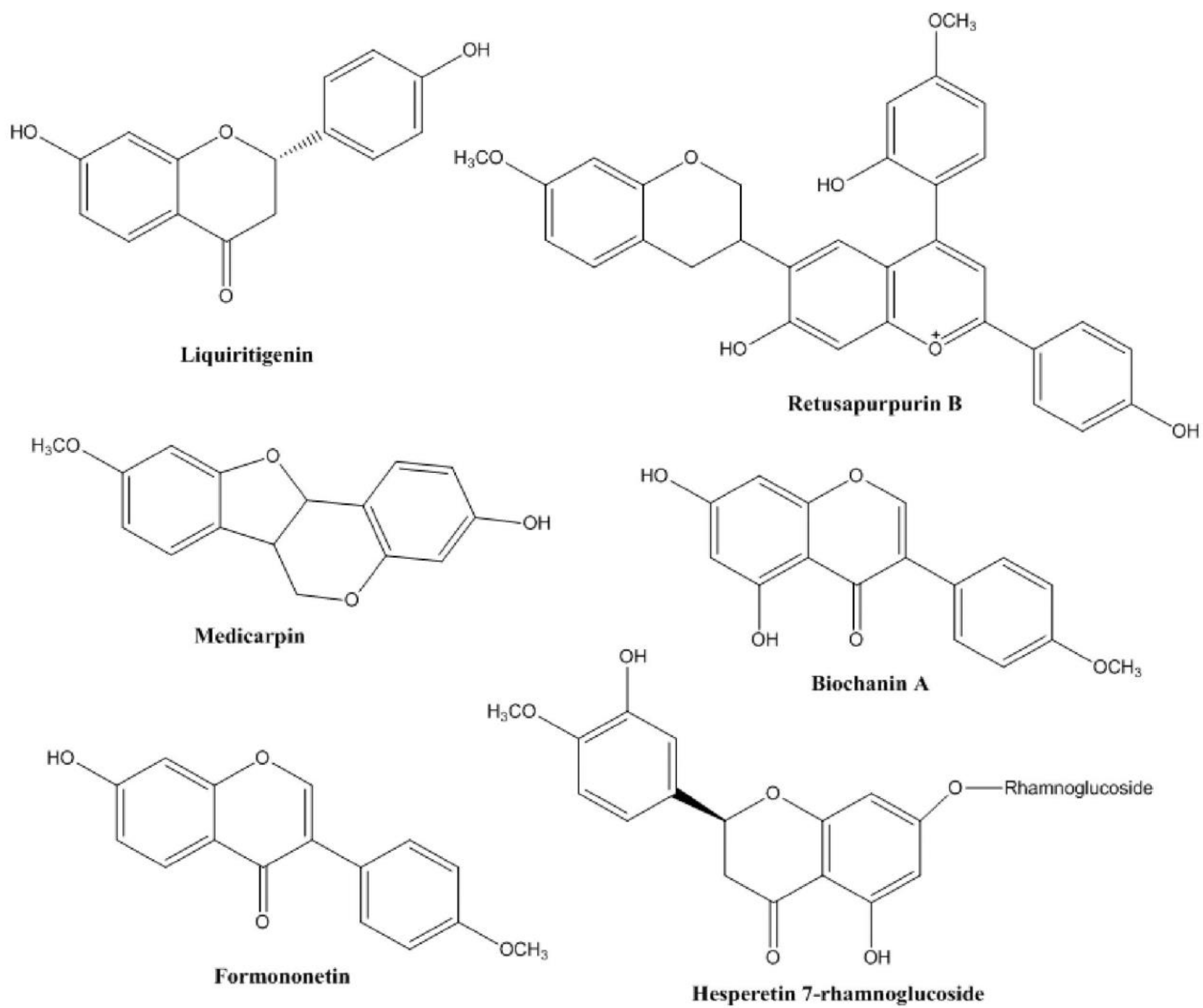


Figure 1 Chemical structures of the identified compounds found in red propolis through high-resolution mass spectrometer (HRMS).[6]

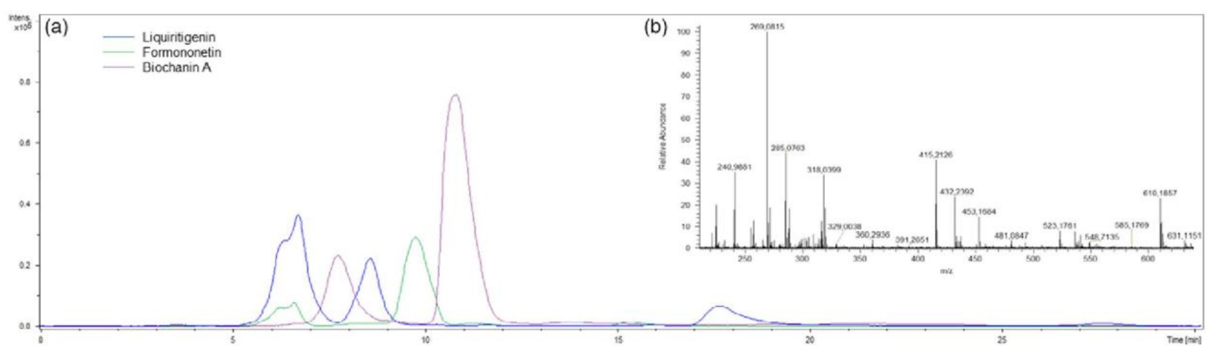


Figure 2 (a) Chromatographic profile of liquiritigenin, formononetin and biochanin A; and (b) Full high-resolution mass spectrometer (HRMS) of Sergipe red propolis extract.

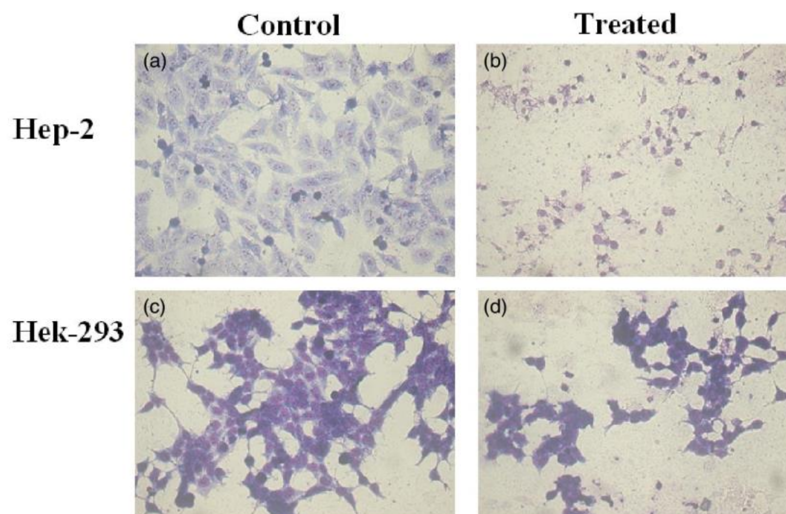


Figure 3 Morphological changes of Hep-2 and Hek-293 cell lines by Giemsa staining. (a) Hep-2 control, (b) Hep-2 treated with 120 μg/ml of red propolis extract, (c) Hek-293 control, (d) Hek-293 treated with 220 μg/ml of red propolis extract.

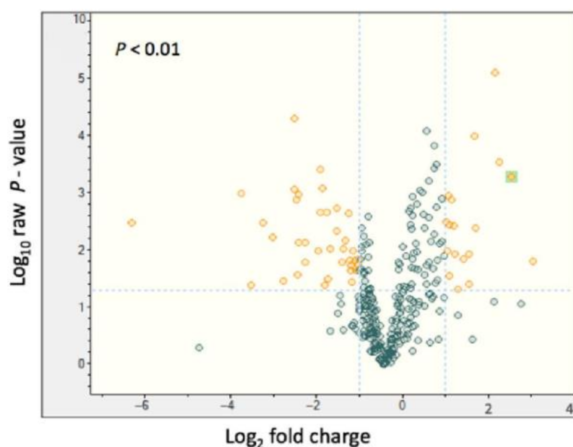


Figure 4 Volcano plot of peptide areas, extracted by ProfileAnalysis software from triplicate LC–MS/MS data set associated with t-test. Each point represents the difference in expression (fold-change) plotted against the level of statistical significance, with double fold change for the log2 values derived from the quotient (treated/control) of Hep-2 cell line. There are two areas of interest in the plot – those points that are found towards the top of the plot are far to either the left- or the right-hand side. These represent values that display large magnitude fold changes (hence being left or right of centre) as well as high statistical significance (hence being towards the top).

and 14%). Functions as enzymatic regulatory, nucleic acid binding transcription factor, transporter and translation regulator activity were also observed in less proportions for both groups.

Comparing Hep-2 cell line treated with red propolis with the control group, 16 proteins were regulated (Table 1), two of them were upregulated and the majority downregulated. Hek-293 cell line presented 04 proteins downregulated (Table 1). Both of the analysis considered $P < 0.05$.

Discussion

Proteomics offers a powerful platform for identifying protein expression using *in vitro* cell models. Accurate proteomic studies can provide a representation of cellular signalling mechanisms, which can be used to the development of drugs that target multiple cellular components. So far, there are no reports on proteomics comparing tumour and normal cell lines treated with Brazilian red propolis total extracts through LC-MS analysis.

The mechanism involved in proliferation inhibition of cancer cells upon red propolis activity is still unclear, thereby proteomics to globally search for differentially expressed proteins in Hep-2 and Hek-293 cells affected by red propolis extract may help to clarify some of the molecular mechanisms underlying carcinogenesis. Functional category analysis suggests that the alterations in protein expression after red propolis treatment are associated with different biological processes and molecular functions. Most of the proteins here identified in the tumour sample that presented significant differential expression are reported in literature and involved in regulation of cancer cell metabolism. These proteins can be modulated in a variety of ways, depending on the experimental design, if samples are taken from tissue or *in vitro* models, treated or not with total extracts, fractions or isolated compounds. Tumour cell lines submitted to a particular

 © 2016 Royal Pharmaceutical Society,

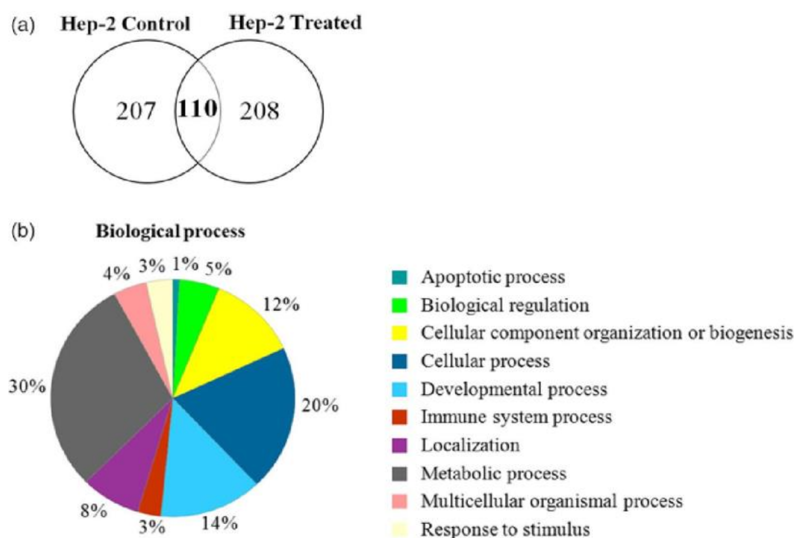


Figure 5 (a) Venn Diagram representing proteins presented in all triplicate of the tumour Hep-2 line groups analyzed. A total of 110 proteins were in accordance for both groups. (b) 110 proteins were classified according to the GO annotations 'biological process', using the PANTHER classification system.

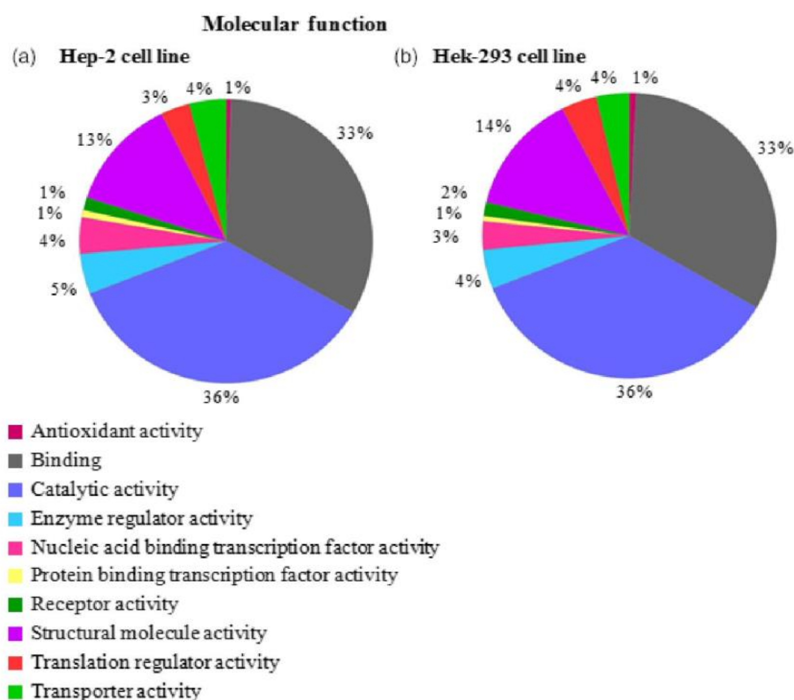


Figure 6 Molecular function of identified proteins from Hep-2 ((a) – 1336 proteins) and Hek-293 ((b) – 773 proteins) cell lines analysed with PANTHER tool (http://www.pantherdb.org). Similar molecular function profiles of identified proteins were observed for both tumour and non-tumour lines.

treatment will present several effects related to protein cell modulation when compared to the untreated group, as observed in this study. It is also important to highlight that the tumour samples analyzed here revealed a larger panel of regulated proteins compared to the non-tumour sample.

Table 1 Differentially expressed protein identified by MS analysis in Hep-2 and Hek-293 cells treated with 120 and 220 μg/ml of red propolis, respectively

Protein ID	Protein name	MW (kDa)	Meta score	Pepti	SC (%)	RMS90 (ppm)	Regulation	*P*-value	Fold change	Protein class*	Subcellular location
Hep-2 cell line											
PDIA3_HUMAN	Protein disulfide-Isomerase A3	56.7	491.2	14	31.5	6.50	Down	0.0069	1.6	Isomerase	Endoplasmic reticulum
AN32E_HUMAN	Acidic leucine-rich nuclear phosphoprotein	30.7	170.6	4	19.4	6.01	Down	0.0032	2.04	Enzyme modulator	Cytoplasm nucleus
NP1L4_HUMAN	Nucleosome assembly protein 1-like 4	42.8	158.4	4	13.3	3.05	Down	0.0068	1.9	Enzyme modulator	Nucleus
TLN1_HUMAN	Talln-1	269.6	1055.9	18	13.9	5.32	Down	0.0036	2.16	Cell adhesion molecule cytoskeletal protein	Cytoplasm
EPIPL_HUMAN	Epiplakin	555.3	622.6	10	3.6	5.01	Down	0.0013	2.2	n.a.	Cytoplasm
FSCN1_HUMAN	Fascin	54.5	585.2	13	34.7	4.99	Down	0.0144	1.76	Cytoskeletal protein	Cytoplasm
VINC_HUMAN	Vinculin	123.7	567.5	13	15.9	6.10	Down	0.0006	1.55	Cell adhesion molecule cytoskeletal protein	Cytoplasm
APEX1.HUMAN	DNA-<apurinic or apyrimidinic site) lyase	35.5	273.8	7	28.3	6.00	Down	0.0157	1.54	n.a.	Nucleus cytoplasm endoplasmic reticulum
PLP2_HUMAN	Proteolipid protein 2	16.7	33.2	1	8.6	3.89	Down	0.0278	2.14	n.a.	Membrane
R0A1_HUMAN	Heterogeneous nuclear ribonucleo protein A1	387	605.7	8	28.0	5.41	Down	0.004	3.25	Nucleic acid binding	Nucleus cytoplasm
RL7_HUMAN	60S ribosomal protein	29.2	217.9	4	23.8	6.80	Down	0.0011	2.1	Nucleic acid binding	Nucleus cytoplasm
YB0X1_HUMAN	Nuclease-sensitive element-binding protein 1	35.9	564.1	9	61.1	5.90	Down	0.0005	5.83	n.a.	Cytoplasm nucleus
HNRH1_HUMAN	Heterogeneous nuclear ribonucleo protein H	49.2	579.5	7	28.5	6.24	Down	0.0011	1.19	nucleic acid binding	Nucleus
SMD1.HUMAN	Small nuclear ribonucleo protein Sm D1	13.3	171.7	2	27.7	8.24	Down	0.0037	2.29	Nucleic acid binding	Cytoplasm nucleus
U2AF1.HUMAN	Splicing factor U2AF 35 kDa subunit	27.9	117.6	4	23.3	4.81	Up	0.0174	−2.04	Nucleic acid binding	Nucleus
FETUA.HUMAN	AJpha-2-HS-glycoprotein	39.3	174.1	3	5.4	8.07	Up	0.0021	−3.35	Enzyme modulator extracellular matrix protein	Extracellular
Hek-293 eel l line											
TBA1B.HUMAN	Tubulin alpha-IB chain	50.1	448.2	12	33.9	5.49	Down	0.0318	8.61	Cytoskeletal protein	Cytoskeleton
ACTG.HUMAN	Actin, cytoplasmic 2	41.8	304.6	11	31.5	5.98	Down	0.0096	7.15	Cytoskeletal protein	Cytoskeleton
ACTB_HUMAN	Actin. cytoplasmic 1	41.7	315.4	12	31.5	6.06	Down	0.0096	7.15	Cytoskeletal protein	Cytoskeleton
TXND5_HUMAN	Thioredoxin domain-containing protein 5	47.6	114	4	13.4	6.68	Down	0.0086	5.11	Isomerase	Endoplasmic reticulum lumen

n.a., not available. *Protein class was made using PANTHER tool (http://www.pantherdb.org).

Among the proteins identified in the normal Hek-293 cell line, 04 proteins were downregulated, most of them are from the cytoskeleton compartment, TBA1B, ACTG, ACTB. Exception was for thioredoxin domain-containing protein 5 (TXNDC5), which belongs to the disulphide isomerase family. The endoplasmic reticulum (ER) presented in eukaryotic cells is a specialized organelle that is the central site of synthesis and folding of proteins. The disproportion between the amount of protein and folding capacity cause ER stress, which is involved in many pathologies such as cardiovascular disease, diabetes, neurodegenerative diseases and cancer.[18] In the deficiency to reestablish ER homoeostasis, the apoptotic pathways can be activated. Protein disulphide isomerases (PDI) has been associated with the regulation of apoptotic signalling events.[18] Thioredoxin domain-containing protein 5 (TXNDC5) that belongs to the PDI family and plays several functions, also involved in protein folding and chaperone activity. Recently, this multifunctional protein has been considered as a biomarker for cancer,[19] and in this study showed to be downregulated in the treated group.

In contrast, among the proteins identified in the tumour Hep-2 cell line, 14 were downregulated and 02 upregulated. Protein disulphide isomerase A3 (PDIA3) showed similar function to the TXNDC5 found in Hek-293 cell line. It has been reported to protect against oxidative stress-induced apoptosis as it was upregulated in a disease associated to the conjunctiva, generating a benign growth of these tissue.[20] The expression of PDIA3 protein was also reported to be upregulated in melanomas that subsequently metastasized.[21]

From the cytoskeleton compartment and organization, 04 downregulated proteins were identified (TLN1, EPIPL, FSCN1, vinculin) in the tumour Hep-2 line. The talin 1 (TLN1) regulates integrin adhesion and was found overexpressed in prostate cancer cell line, promoting active survival signal, adhesion, migration and invasion.[22] Zhang *et al.*[23] found that talin 1 can be considered a biomarker for the diagnosis and prognosis of hepatocellular carcinoma as it may be related in the process of carcinogenesis, infiltration and metastasis. Lai *et al.*[24] found this protein upregulated in oral squamous cell carcinoma, correlating the TLN1 level with early tumour stages and poor clinical response. Tang *et al.*[25] reported that the TLN1 expression was decreased in ovarian serous carcinoma by miR-9 as tumour suppressor agent.

Also as part of cytoskeleton compartment, Epiplakin (EPIPL) belongs to the plakin family and it maintains the integrity of keratin intermediate filament networks in epithelial cells.[26] This protein was found upregulated in oral squamous cell carcinoma.[27] It was also upregulated in buccal mucosa tissue 2 days postchemotherapy in acute myelogenous leukaemia patients.[28]

Fascin is a protein with 55 kDa that bundle the actin and plays a role in regulation, conservation and support of parallel bundles of filamentous actin in various cellular conditions. Here, cytoskeleton protein fascin 1 (FSCN1) was downregulated, playing an important role in multiple epithelial tumours development, invasion and metastasis.[29, 30] Tan *et al.*[30] found that fascin 1 expression could be considered a biomarker or therapeutic target onto several carcinomas. Finally, vinculin is a protein found in large quantities associated with cell–cell and cell–matrix interactions. Vinculin has been reported to suppress tumour by supporting anchorage-dependent cell growth and to repress metastasis by diminishing cell motility.[31]

Here, a protein related to replication, recombination and repair pathway was found downregulated. DNA-(apurinic or apyrimidinic site) lyase (APEX1) is a protein with several functions involved in base excision repair of DNA damage and has been considered an attractive molecular target in the diagnosis, treatment and prevention of breast cancer.[32] Its overexpression in osteosarcoma was correlated with recurrence, metastasis and survival of patients.[33] Deregulation of APEX1 is reported to be associated to increased risk of head and neck cancer.[34] Soya isoflavones seem to decrease the expression of APE1/Ref-1 *in vitro* and *in vivo*, with a mechanism of cell killing and inhibition of prostate tumour growth.[35]Our group has previously identified several compounds in red propolis total extract, among them, an isoflavone known as formononetin, which may contribute to APEX1 downregulation.[6] Here, we quantified formononetin in red propolis extract from Sergipe state, as well as biochanin A and liquiritigenin. Formononetin showed to be more prevalent (2.37 ± 0.010 mg/g) compared to the other compounds quantified through UFLC. Overall, isoflavone exerts inhibitory effects on carcinogenesis and cancer progression by induction of apoptosis and inhibition of cell proliferation.[8]

Another downregulated protein identified is correlated to chemokine binding and ion transmembrane transport. The expression of proteolipid protein 2 (PLP2), a four-transmembrane protein, was found upregulated in MDA-MB231 breast cancer cells when exposed to cadmium.[36] This protein was downregulated in HepG2 cells treated with radiofrequency electromagnetic fields[37] and in the Y925F-mutated FAK-transfected cells.[38] Sonoda *et al.*[38] have shown that mice injected with PLP2 cells increased risk to metastasis compared to those that received empty vector-transfected cells.

A protein involved in RNA processing and modification heterogeneous nuclear ribonucleoprotein A1 (ROA1) was also found downregulated in this study. ROA1 was upregulated, however, in myeloid leukaemia cells lines after treatment with a potent anticancer molecule, adaphostin.[39]

Other protein from the same protein class, heterogeneous nuclear ribonucleoprotein H (HNRH1), was shown to be downregulated upon coxsackievirus B3 infections in HeLa and HepG2 cell lines[40] and small nuclear ribonucleoprotein Sm D1 (SmD1). Small nuclear ribonucleoproteins are splicing factors and present inhibitory effect in tumour cell.[41]

Two proteins were found upregulated in Hep-2 cells in this study after red propolis treatment. One of them was the protein Alpha-2-HS-glycoprotein (FETUA), involved in diverse physiological and pathological processes, considered an acute phase regulator.[42] Gangneux *et al.*[43] reported upregulation of FETUA that could bring benefits to patient in risk, for example, septic syndrome complicated with multiple organ failure. Upregulation of FETUA was also associated with the development of docetaxel resistance.[44] Another upregulated protein here identified was splicing factor U2AF 35 kDa subunit (U2AF1). U2AF1 was found to be upregulated and related to constitutive and enhancer-dependent splicing by mediating protein–protein interactions and protein–RNA interactions in patients with acute myeloid leukaemia.[45]

Some of the proteins identified here (TLN1, EPIPL, FSCN1, VINC and ROA1) are reported to be upregulated in laryngeal tumour tissue compared to the normal phenotype.[15] In contrast with the results presented by Li *et al.*[15] that compared normal and tumour phenotypes without treatment exposition, the expression of these proteins is shown to be downregulated in our study after red propolis extract treatment. U2AF1 protein also presented a discrepant expression pattern, according to Li *et al.*[15] was downregulated in laryngeal tumour, while our results showed a upregulation of U2AF1 after red propolis exposition. Those conditions reflect that many changes in tumour cell metabolism are processed after propolis exposition and extract incubation may regulate protein levels that are overexpressed in tumour samples back to basal levels.

Changes in protein expression are related to the activity of several molecules found in propolis composition, as many compounds have been reported to present anticancer activities. For example, biochanin A was found to decreased uPAR protein levels considerably in human glioblastoma cell line (U87MG). The overexpression of uPAR levels correlates with tumour invasiveness in this particular cell line [7]. Because red propolis total extract presents a complex chemical composition, which can promote several synergistic effects in cell metabolism, our group is now focusing in fractionating and isolating major compounds. These molecules will be tested in cell lines and further evaluated through proteomic analysis.

Conclusion

Taken together, tumour and non-tumour cells treated with red propolis revealed differentially expressed proteins related to a series of metabolic pathways. Importantly, all regulated proteins here identified have been reported in recent literature and participate, direct or indirectly, of cancer cell signalling mechanisms. Thus, our results provided a comprehensive overview for the understanding of protein expression of red propolis in Hep-2 cells. Those results can easily be compared and complement other proteomic reports, benefiting future clinical application and the development of cancer drugs from natural products. Additional proteomic studies will be necessary to evaluate cell metabolism and if there are synergisms among compounds present in extract or if major activities are promoted by isolated players.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

This work was supported by CNPq (MCTI/CNPq/ Universal 2014-4), CAPES (Auxpe 1608/2011) and FAPERGS (2014-4 – Pesquisador Gaúcho).

References

1. Pinheiro KS *et al.* Modulatory activity of Brazilian red propolis on chemically induced dermal carcinogenesis. *Acta Cir Bras* 2014; 2: 111–117.
2. Daugsch A *et al.* Brazilian red propolis – chemical composition and botanical origin. *Evid Based Complement Alternat Med* 2008; 4: 435–441.
3. Piccinelli AL *et al.* Cuban and Brazilian red propolis: botanical origin and comparative analysis by high-performance liquid chromatography-photodiode array detection/electrospray ionization tandem mass spectrometry. *J Agric Food Chem* 2011; 12: 6484–6491.
4. Watanabe MA *et al.* Cytotoxic constituents of propolis inducing anticancer effects: a review. *J Pharm Pharmacol* 2011; 11: 1378–1386.
5. Freires IA *et al.* A pharmacological perspective on the use of Brazilian Red Propolis and its isolated compounds against human diseases. *Eur J Med Chem* 2016; 110: 267–279.
6. Frozza CO *et al.* Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. *Food Chem Toxicol* 2013; 52: 137–142.
7. Puli S *et al.* Inhibition of matrix degrading enzymes and invasion in human glioblastoma (U87MG) cells by isoflavones. *J Neurooncol* 2006; 79: 135–142.

8. Li Y *et al.* Induction of cancer cell death by isoflavone: the role of multiple signaling pathways. *Nutrients* 2011; 10: 877–896.
9. Liu Y *et al.* Liquiritigenin inhibits tumor growth and vascularization in a mouse model of HeLa cells. *Molecules* 2012; 6: 7206–7216.
10. Liu Y *et al.* The proapoptotic effect of formononetin in human osteosarcoma cells: involvement of inactivation of ERK and Akt pathways. *Cell Physiol Biochem* 2014; 3: 637–645.
11. Franchi GCJr *et al.* Comparison of effects of the ethanolic extracts of Brazilian propolis on human leukemic cells as assessed with the MTT assay. *Evid Based Complement Alternat Med* 2012; 2012: 918956.
12. Awale S *et al.* Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition. *Bioorg Med Chem* 2008; 1: 181–189.
13. Kamiya T *et al.* Ethanol extract of Brazilian red propolis induces apoptosis in human breast cancer MCF-7 cells through endoplasmic reticulum stress. *J Agric Food Chem* 2012; 44: 11065–11070.
14. Begnini KR *et al.* Brazilian red propolis induces apoptosis-like cell death and decreases migration potential in bladder cancer cells. *Evid Based Complement Alternat Med* 2014; 2014: 1–13.
15. Li L *et al.* Quantitative proteomics approach to screening of potential diagnostic and therapeutic targets for laryngeal carcinoma. *PLoS ONE* 2014; 2: e90181.
16. Moseley FL *et al.* The use of proteomics to identify novel therapeutic targets for the treatment of disease. *J Pharm Pharmacol* 2007; 5: 609–628.
17. Ku-Cükboyaci N *et al.* Determination of isoflavone content by hplc-uv method and in vitro antioxidant activity of red clover (trifolium pratense l.). *Turk J Pharm Sci* 2013; 3: 463–472.
18. Grek C, Townsend DM. Protein disulfide isomerase superfamily in disease and the regulation of apoptosis. *Endoplasmic Reticulum Stress Dis* 2014; 1: 4–17.
19. Horna-Terron E *et al.* TXNDC5, a newly discovered disulfide isomerase with a key role in cell physiology and pathology. *Int J Mol Sci* 2014; 12: 23501–23518.
20. Kim SW *et al.* Proteomic analysis in pterygium; upregulated protein expression of ALDH3A1, PDIA3, and PRDX2. *Mol Vis* 2014; 20: 1192–1202.
21. Linge A *et al.* Differential expression of fourteen proteins between uveal melanoma from patients who subsequently developed distant metastases versus those who did not. *Invest Ophthalmol Vis Sci* 2012; 8: 4634–4643.
22. Sakamoto S *et al.* Talin1 promotes tumor invasion and metastasis via focal adhesion signaling and anoikis resistance. *Cancer Res* 2010; 5: 1885–1895.
23. Zhang JL *et al.* Talin1, a valuable marker for diagnosis and prognostic assessment of human hepatocelluar carcinomas. *Asian Pac J Cancer Prev* 2011; 12: 3265–3269.
24. Lai MT *et al.* Talin-1 overexpression defines high risk for aggressive oral squamous cell carcinoma and promotes cancer metastasis. *J Pathol* 2011; 3: 367–376.
25. Tang H *et al.* miR-9 functions as a tumor suppressor in ovarian serous carcinoma by targeting TLN1. *Int J Mol Med* 2013; 2: 381–388.
26. Shimada H *et al.* Epiplakin modifies the motility of the HeLa cells and accumulates at the outer surfaces of 3-D cell clusters. *J Dermatol* 2013; 4: 249–258.
27. Chi LM *et al.* Enhanced interferon signaling pathway in oral cancer revealed by quantitative proteome analysis of microdissected specimens using 16O/18O labeling and integrated two-dimensional LC-ESI-MALDI tandem MS. *Mol Cell Proteomics* 2009; 7: 1453–1474.
28. Brennan MT *et al.* Gene expression changes in buccal mucosa tissue 2 days post-chemotherapy in acute myelogenous leukemia (AML) patients. *J Clin Oncol* 2005; 16S: Abstract 8041.
29. Hanker LC *et al.* Prognostic impact of fascin-1 (FSCN1) in epithelial ovarian cancer. *Anticancer Res* 2013; 2: 371–377.
30. Tan VY *et al.* Association of fascin-1 with mortality, disease progression and metastasis in carcinomas: a systematic review and meta-analysis. *BMC Med* 2013; 11: 52.
31. Goldmann WH *et al.* Vinculin, cell mechanics and tumour cell invasion. *Cell Biol Int* 2013; 5: 397–405.
32. Ali K *et al.* Germline variations of apurinic/apyrimidinic endonuclease 1 (APEX1) detected in female breast cancer patients. *Asian Pac J Cancer Prev* 2014; 18: 7589–7595.
33. Yang J *et al.* APEX1 gene amplification and its protein overexpression in osteosarcoma: correlation with recurrence, metastasis, and survival. *Technol Cancer Res Treat* 2010; 2: 161–169.
34. Mahjabeen I *et al.* Genetic and expressional variations of APEX1 are associated with increased risk of head and neck cancer. *Mutagenesis* 2013; 2: 213–218.
35. Raffoul JJ *et al.* Down-regulation of apurinic/apyrimidinic endonuclease 1/redox factor-1 expression by soy isoflavones enhances prostate cancer radiotherapy in vitro and in vivo. *Cancer Res* 2007; 5: 2141–2149.
36. Longo A *et al.* Effect of transfection with PLP2 antisense oligonucleotides on gene expression of cadmium-treated MDA-MB231 breast cancer cells. *Anal Bioanal Chem* 2013; 6: 1893–1901.
37. Zimmerman JW *et al.* Cancer cell proliferation is inhibited by specific modulation frequencies. *Br J Cancer* 2012; 2: 307–313.
38. Sonoda Y *et al.* Proteolipid protein 2 is associated with melanoma metastasis. *Oncol Rep* 2010; 2: 371–376.
39. Stockwin LH *et al.* Proteomic analysis identifies oxidative stress induction by adaphostin. *Clin Cancer Res* 2007; 12: 3667–3681.
40. Rassmann A *et al.* Proteome alterations in human host cells infected with coxsackievirus B3. *J Gen Virol* 2006; 87: 2631–2638.
41. Li Z, Putzer BM. Spliceosomal protein E regulates neoplastic cell growth by

modulating expression of cyclin E/ CDK2 and G2/M checkpoint proteins. *J Cell Mol Med* 2008; 6A: 2427–2438.
42. Doring Y *et al.* Accelerated evolution of Fetuin-A (FETUA, also AHSG) is driven by positive Darwinian selection, not GC-biased gene conversion. *Gene* 2010; 1–2: 49–55.
43. Gangneux C *et al.* The inflammation-induced down-regulation of plasma Fetuin-A (alpha2HS-Glycoprotein) in liver results from the loss of interaction between long C/EBP isoforms at two neighbouring binding sites. *Nucleic Acids Res* 2003; 20: 5957–5970.
44. Zhao L *et al.* Identification of candidate biomarkers of therapeutic response to docetaxel by proteomic profiling. *Cancer Res* 2009; 19: 7696–7703.
45. Lindberg ML *et al.* Gene Array Biomarkers in Acute Myeloid Leukemia to Predict Remission Duration. American Society of Hematology: ASH Annual Meeting Abstracts, 2009: Abstract 3466.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Total proteins identified in Hep-2 cell line.

Table S2. Total proteins identified in Hek-293 cell line.

Table S3. Proteins identified in all triplicates in the control group of Hep-2 cell line.

Table S4. Proteins identified in all triplicates in the treated group of Hep-2 cell line.

Table S5. Proteins identified in both groups, control and treated, of Hep-2 cell line.

 © 2016 Royal Pharmaceutical Society,

CAPÍTULO 3

Antitumor activity of Brazilian red propolis fractions against Hep-2 cancer cell line

Artigo a ser submetido para a revista *Food and Chemical Toxicology*

Antitumor activity of Brazilian red propolis fractions against Hep-2 cancer cell line

Caroline Olivieri da Silva Frozza[a], Denis Amilton Santos[a], Luciane Corbellini Rufatto[b], Luciane Minetto[b], Fernando Joel Scariot[c], Sergio Echeverrigaray[c,d], Claus Tröger Pich[e], Sidnei Moura[b], Francine Ferreira Padilha[f], Sibele Borsuk[f], Lucielli Savegnago[f], Tiago Collares[g], Fabiana Kömmling Seixas[g], Odir Dellagostin[f], Mariana Roesch-Ely[a,], João Antonio Pêgas Henriques[a,]*

[a]Laboratory of Genomics, Proteomics and DNA Repair, Biotechnology Institute, University of Caxias do Sul, RS, Brazil.

[b]Laboratory of Natural and Synthetic Products, Biotechnology Institute, University of Caxias do Sul, RS, Brazil.

[c]Laboratory of Applied Microbiology, Biotechnology Institute, University of Caxias do Sul, RS, Brazil.

[d]Cytogene Molecular Diagnostics Company[1], RS, Brazil.

[e]NITBIO Federal University of Santa Catarina, Araranguá, SC, Brazil

[f]Biotechnology Unit, Center for Technology Development, Federal University of Pelotas, RS, Brazil

[g]Cancer Biotechnology Laboratory, Research Group on Cellular and Molecular Oncology, Postgraduate Program in Biotechnology, Technology Development Center, Federal University of Pelotas, Pelotas, RS, Brazil

* Corresponding author. Address: Universidade de Caxias do Sul. Rua Francisco Getúlio Vargas 1130, 95070-560, Caxias do Sul, RS, Brasil. Tel: +55-54-3218-2100; fax: +55-54-3218-2664. E-mail address: pegas.henriques@gmail.com (J. A. P. Henriques).

Abbreviations: DAPI (4', 6-diamidino-2-phenylindole); EB (ethidium bromide); ESI – MS/MS (electrospray ionization tandem mass spectrometry); HPLC – High Performance Liquid Chromatography; HBRP (hydroalcoholic Brazilian red propolis); MMP (mitochondrial membrane potential); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide); PI (propidium iodide); ROS (reactive oxygen species)

Abstract

Continuous increases in the rates of tumor diseases have highlighted the need for identification of novel and inexpensive antitumor agents from natural sources. In this study, we investigated the effects of enriched fraction from hydroalcoholic Brazilian red propolis extract against Hep-2 cancer cell line. Initially 201 fractions were arranged in 12 groups according to their chromatographic characteristics (A – L). After an *in vitro* cell viability screening, J and L were further selected as promising enriched fractions for this study. The chemical characterization was performed by LC - ESI(+) – MS/MS and Biochanin A, Formononetin, and Liquiritigenin compounds were quantified. The hydroalcoholic Brazilian red propolis (HBRP) showed the following values 0.277 ± 0.011, 1.52 ± 0.04 and 1.66 ± 0.03 mg/g, respectively. Through MTT viability assay and morphological changes observed by Giemsa and DAPI staining, the results showed that red propolis inhibited cancer cells growth. Flow cytometry results indicated effects that were partly mediated through programmed cell death as confirmed by externalization of phosphatidylserine, DNA cleaved assay, increase at SUB G1-G0 phase in cell cycle analysis and loss of mitochondrial membrane potential. In conclusion, our results demonstrated that red propolis enriched fractions promoted apoptotic effects in human cancer cells through the mechanisms involving mitochondrial perturbation. Therefore, red propolis fractions contain candidate agents for adjuvant cancer treatment, which further studies should elucidate the comprehensive mechanistic pathways.

Keywords: Red Propolis, Cancer, Apoptosis, Hep-2, Fractions

1. Introduction

Cancer is a worldwide major public health problem (Siegel *et al.*, 2016). The number of new estimated cases for 2020, excluding non-melanoma skin cancer, will reach more than 17 million and cancer deaths more than 10 million until 2020 (WHO 2016). This reinforces the need for anticancer compounds screening from natural sources, which have been used for centuries to treat a variety of diseases.

Propolis is a natural resinous product made by melliferous bees, used in traditional medicine all over the world. This resin is known to have chemical composition, which presents biological properties such as anti-inflammatory, antifungal, antibacterial, antioxidative and anticancer (Frozza *et al.*, 2013; Kamiya *et al.*, 2012a). Brazilian red propolis was found at mangrove regions of northeastern Brazilian states, which the major botanical source is *Dalbergia ecastophyllum* (Daugsch *et al.*, 2007). The propolis chemical composition has been characterized and varies according to its source (Machado *et al.*, 2016). With the improvement of purification process and instrumental analysis, most compounds can be further qualified and quantified. However, few studies have identified and characterized chemical composition and activity from red propolis enriched fraction (de Mendonca *et al.*, 2015; Novak *et al.*, 2014), which is considering the first step toward discovery unknown bioactive compounds (Hayacibara *et al.*, 2005).

Various studies have shown the anticancer properties of Brazilian red propolis and its compounds, which are effective in reducing cancer cell survival by affecting various growth-promoting and apoptotic pathways (Begnini *et al.*, 2014; de Mendonca *et al.*, 2015; Frozza *et al.*, 2014). The aim of this present study was to analyze the chemical profile of enriched fractions of hydroalcoholic Brazilian red propolis (HBRP) extract, and to demonstrate its antiproliferative effect on laryngeal cancer cells. In addition, to understand the antitumor benefits of red propolis, some parameters related to apoptosis as ROS

accumulation, mitochondrial membrane potential, were analyzed to help to elucidate cell signaling involved in propolis mechanism of action.

2. Materials and methods

2.1 Chemicals

Dulbecco's modified eagle's medium–high glucose (DMEM), trypsin–EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Biochanin A, Liquiritigenin, Formononetin and Giemsa were purchased from Sigma–Aldrich (St. Louis, MO, USA); ethanol was from Merck (Darmstadt, Hesse, GE). Heat-inactivated fetal bovine serum (FBS) was purchased from Cultilab Lab Inc. (Campinas, SP, BR) and penicillin–streptomycin were purchased from GIBCO™ Invitrogen (Grand Island, NY, USA). All other chemicals were of ultrapure grade and obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2 Red propolis sample

Red propolis was collected in 2013 in state of Alagoas located in the northeast of Brazil. The propolis was protected from light and frozen at -20 °C until extract preparation was proceeded. Samples of red propolis were ground to a fine powder and 50 g of red propolis powder was mixed with 500 mL of EtOH-H_2O 70% - 30% (v/v) and shaken at room temperature for 24 h. After extraction, the mixture was filtered and the solvent was evaporated (37 °C) with subsequent lyophilization. The dry extract was kept frozen at -20 °C and prepared at different concentrations with EtOH - H_2O 50% (v/v) (HBRP), and finally filtered through a 0.22 µm polyethersulfone membrane (TPP, Techno Plastic Products, Switzerland).

2.3 Fractionation

The dry extract of red propolis (5 g) was fractionated on a silica gel column (4 × 45 cm). Chloroform (90 to 100%), methanol (0 to 10%) and 0.1% acetic acid; ethanol (100%) and Milli-Q® water were used for the mobile phase in sequence. The fractions were analyzed by Thin Liquid Chromatography (TLC) using chloroform: methanol: acetic acid (95:4:1) as mobile phase. The plates were revealed with vanillin:chloridric acid:sulphuric acid:ethanol (1:4:5:90) and chromatograms were evaluated under 254 and 365 nm yielding 12 fractions (A – L) (Figure 1). After, the solvent was removed under reduced pressure, and all the fractions were individually stored in amber vials at -20 °C.

2.4 Chemical characterization by ESI – MS/MS and quantification

After an in vitro cell viability screening, J and L were further selected as promising enriched fractions for this study therefore the dry samples (J and L fractions and HBRP) were dissolved in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid for ESI(+). The solutions were infused individually into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 10μL/min. ESI(+)-MS were acquired using a hybrid high-resolution and high accuracy (5μL/L) microTOF-QII mass spectrometer (Bruker® Daltonics) under the following conditions: capillary and cone voltages were set to +3500 eV and +40 eV, respectively, with a de-solvation temperature of 100 °C. Diagnostic ions were identified by the comparison of exact *m/z* with compounds determined in previous studies (Table 1). Hystar software (Bruker® Daltonics) was used for data acquisition and processing. The data were collected in the *m/z* range of 70–800 at the speed of two scans per

second, providing the resolution of 50.000 (FWHM) at *m/z* 200. No important ions were observed below *m/z* 150 or above *m/z* 800.

The quantification was performed by HPLC-UV and ESI-MS in a Quadrupole Time–of–Flight (Q–TOF) mass spectrometry (MicrOTOF-QII Bruker) in a positive mode. The following conditions were used: the TOF capillary voltage was set at 4000 eV for 30 min at a flow rate of 0.5 mL/min. The nebulizer gas pressure was 2 Bar, and the N_2 drying gas was maintained at a flow rate of 8 L/min at 200 °C. MS spectra were collected over the range of 50–800 *m/z* and then processed using Compass Data Analysis 4.0 software.

The linearity was determined by the analysis of three authentic curves, constructed with sample solutions, in five levels of concentration ranged from 0.001 to 1.0 µg/mL. The curves were constructed by plotting the average values of the areas according to the concentration. The results were analyzed by calculating the linear regression in order to define the determination coefficient (R^2). The calibration curves of standards solutions (Biochanin A, Liquiritigenin, Formononetin) were used if the correlation coefficient was 0.99 or higher.

2.5 Cell culture

Hep-2 (human laryngeal epidermoid carcinoma cell) was obtained by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM supplemented with 10% (w/v) heat-inactivated FBS and 1% (w/v) penicillin–streptomycin. The cultures were maintained in a humidified atmosphere at 37 ºC, 5% CO_2 and 95% air. The study was performed as cells reached 70–80% confluence.

2.6 Cytotoxic assay

Cell viability was measured using the MTT assay (Mosmann, 1983). Briefly, cells were seeded into the 96-well plates at a density of 5×10^4 cells/mL. After 24 h, cells were treated with different concentrations (5 – 175 μg/mL) of HBRP and fractions and incubated for 24 h and 48 h. Negative controls were treated with the same amounts of hydroalcoholic solution. The medium was removed and 1 mg/mL MTT dye in serum-free medium was added to the wells. Plates were incubated at 37 °C for 2 h in humidified 5% CO_2 atmosphere. Subsequently, the MTT solution was removed and the obtained formazan violet product was dissolved in 100 μL DMSO. Absorbance was measured using a microplate reader (Spectra Max 190, Molecular Devices, USA) at 570 nm. All readings were compared with the control, which represented 100% viability. The IC50 (concentration μg/mL that inhibits cell growth by 50%) ratio of cancerous cell was also calculated. Each experiment was performed in triplicate and independently repeated at least four times.

The fractions that yielded best results after a screening with MTT assay, J and L fractions, were used in the following tests. For the cells treatment the dry extract and the fractions were prepared at different concentrations with EtOH-H2O 50% (v/v), and filtered through a 0.22 μm polyethersulfone membrane (TPP, Techno Plastic Products, Switzerland).

2.7 Morphological examination of cancer cells

Cells were seeded into 24-well plates containing coverslips. After 24 h incubation, the cells were treated with red propolis hydroalcoholic extract and fractions at 50 and 75 μg/mL (concentrations close to the IC50 values) for 24 h. The negative control group was treated with EtOH–H_2O 2.5% (v/v) instead of extract for the same period. Then they were stained with Giemsa (Accustain®– Sigma–Aldrich), according to standard Giemsa procedure established by the supplier. Morphological changes of the cells were observed and photographed under a light microscope at 200 x (Olympus®).

In DAPI (4', 6-diamidino-2-phenylindole) staining, after 24h extract treatment, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature, and then washed again with PBS. The fixed cells were incubated with 1 mg/mL DAPI solution for 5 min at room temperature in the dark. After washing twice with PBS, cells undergoing apoptosis, represented by the morphological changes of apoptotic nuclei, were examined under an inverted fluorescence microscope. The apoptotic cells were identified by the presence of highly condensed chromatin or fragmented nuclei.

2.8 Flow Cytometry

Cell cycle, apoptosis, mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) were measured by flow cytometry. An amount of 2.6×10^6 cells were seeded into 75 cm^2 culture flasks with supplemented culture medium. After 24 h, tumor cells were treated with 50 µg/mL and 75 µg/mL for 24 h. The negative control groups were treated with the same amount of hydroalcoholic solution used for the extracts. The intensity of fluorescence from 10,000 cells were quantified by a BD FACSCalibur four colors flow cytometer (Becton Dickinson LTDA). Data were collected by CellQuest Pro software (BD Biosciences) and analyzed using FlowJo (TreeStar, Inc). Experimental procedures were performed at least in triplicate.

2.8.1 Annexin-V/PI Assay

The procedure was conducted according to methodology provided in Annexin V-FITC Apoptosis Detection Kit (catalog number APOAF, Sigma, USA). All adhering and floating cells were harvested and washed twice with PBS before being transferred into sterile centrifuge tube. The cell pellet was then suspended in 100 µL of binding buffer (10 mM HEPES/NaOH pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$). This cell suspension was transferred to a microtube, to which 5 µL of Annexin V-FITC conjugate and 10 µL of

propidium iodide were added. The cells were incubated in the dark for 15 minutes at room temperature. The fluorescence of the cells was determined by flow cytometry using FL1 (488/533) and FL3 (488/670) filters.

2.8.2 Mitochondrial Membrane Potential Analysis

Changes in the MMP as a result of mitochondrial depolarization were measured using the method of incorporation of 3,3'-dihexiloxacarbocianina iodate (DioC6(3); Molecular Probes Inc., USA) according to Nocentini *et al.,* (1997). Briefly, treated and untreated Hep-2 cells were harvested by trypsin, washed once in PBS, and then stained with DioC6(3) (175 nM) 30 min and analyzed by flow cytometry using FL1 filter.

2.8.3 ROS Analysis

Measurement of intracellular levels of ROS in Hep-2 cells were stained by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) and by dihydrorhodamine 123 (DHR123) according to Shan *et al.,* (2016) methodology with modifications. DCFH-DA penetrates into the intracellular matrix of cells to be oxidized by ROS to fluorescent dichlorofluorescein (DCF) and DHR123 is oxidized to rhodamine 123. Fluorescent dye solutions (10 µM) was added to the cells and incubated for 30 min at 37 °C in the dark. The levels of ROS were analyzed at an excitation 488 nm and emission 533 nm.

2.8.4 Cell Cycle Analysis

Hep-2 cells were cultured with or without red propolis or vehicle (ethanol 2.5%) for 24 h. Following treatment, cells were washed in PBS, trypsinized and collected for centrifugation (1800 rpm for 7 min) in PBS. Cell pellets were gently resuspended, fixed in ice-cold 70% ethanol and stored overnight at −20 °C (Wlodkowic *et al.*, 2009). Prior to analysis, cells were again collected by centrifugation to remove ethanol, gently resuspended in PBS and re-pelleted. Cells were then resuspended and permeabilized in Triton X-100 in PBS and incubated with RNAse A and propidium iodide in the dark for 30 min at room

temperature. All cell samples were immediately analyzed by flow cytometry for DNA content.

2.9 DNA cleavage assay

To assess the nuclease activity of the crude extract and the L and J fractions of red propolis, samples, according to Biscaro *et al.*, (2013) method, in concentrations of 0 (negative control), 5, 15, 25, 35, 50 and 75 µg/ml were incubated in 20 µL of a water solution containing pBSKII plasmid DNA (300 ng) and 10 mM of HEPES buffer (pH 7.4) for 16 h at 37 ºC. Subsequently, these solutions were loaded onto agarose gels containing ethidium bromide (EB), and after electrophoresis, bands corresponding to supercoiled form (FI), open circular form (FII) and the linear form (FIII) were photodocumented. Fluorescence intensity of EB stained bands was measured with ImageJ® and a correction factor of 1.47 was applied to FI according to a previous study (Biscaro *et al.*, 2013). The data were plotted in form of tables and graphics.

2.10 Statistical analysis

Results were expressed as mean ± standard deviation obtained from three independent experiments. Statistical significance was evaluated using t-test and one way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Tukey test) to assess statistical differences in normal distribution. Significance was accepted at p lower than 0.05 using the Statistical Package for Social Sciences (SPSS, version 19.0) for Windows.

3. Results and Discussion

3.1 Chemical characterization and quantification

Chemical composition of Brazilian red propolis extracts has been characterized by our study group (Frozza *et al.*, 2013). In this work, the chemical composition was determined using ESI(+)-MS in a Q-TOF (Q-TOF-II Bruker® Daltonics), which allows to analyze a wide range of compounds at the same time.

The exact mass, the isotopic ratio and the fragmentation pathway were used in order to distinguish the molecules. The identified compounds are listed in Table 1, accepted accuracy threshold for confirmation of elemental compositions of 5 ppm. Many other compounds are presented in red propolis, however it was not possible to identify all of them. All of the identified compounds have been found in Brazilian red propolis from different regions (Awale *et al.*, 2008; Bueno-Silva *et al.*, 2013; Trusheva *et al.*, 2006). The chemical structures of all them are presented in the Figure 2.

In fact, the set of information generated from High Resolution Mass Spectrometry (HRMS) in tandem (MS-MS) assisted chemical characterization. From the fragmentation pathways it was possible the differentiation of isobars Cis-asarone or Trans-isoelemicin from Elemicin (m/z 209.1180) by the presence of ion m/z 194.0969 ($[M - CH_3]^+$), as well as, Vestitol or Isovestitol from Neovestitol by the presence of ions m/z 149.0562 and 123.0482 which are referents at $[M - C_7H_7O]^+$ $[M - C_9H_9O]^+$ respectively.

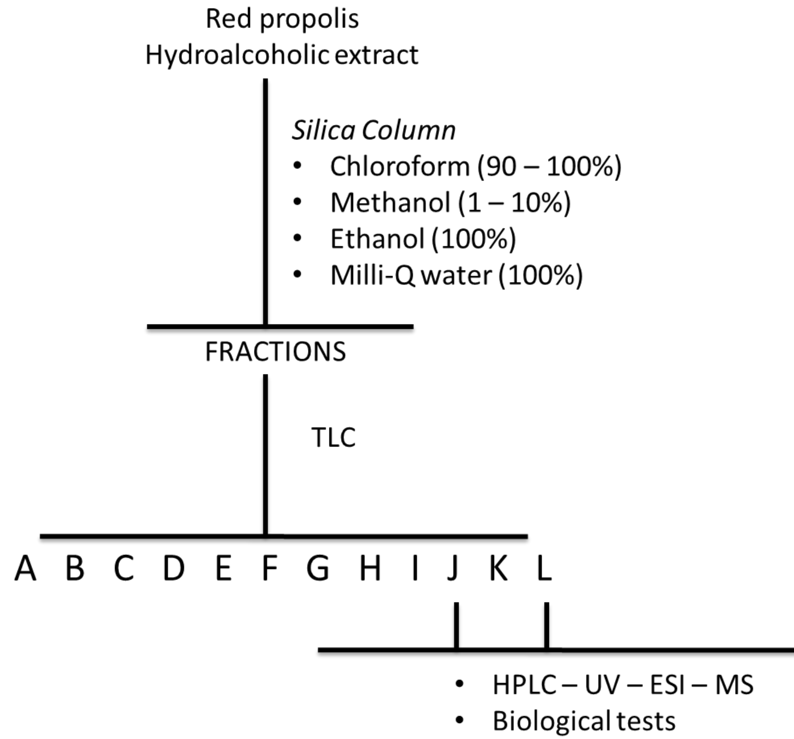


Figure 1. Workflow of fractionation by solvents and column chromatography. 12 fractions (A – L) were collected out of 201 samples, which were grouped according to chemical similarities.

Table 1. The major compounds in red propolis hydroalcoholic extract and their fractions.

Entry	Identification	Elem. Comp. $(M+H)^+$	Precursor ion *m/z*	Diff. ppm	Fragmentation ions (%) [MS-MS]	Fragmentation pathways	Ref.
HBRP	Benzoic acid (**1**)	$C_7H_7O_2$	123.0450	3.215			(Alencar *et al.*, 2007)
HBRP	2H-1-Benzopyran-7-ol (**2**)	$C_9H_9O_2$	149.0607	2.99			(Alencar *et al.*, 2007)
HBRP	Methoxyeugenol (**3**)	$C_{11}H_{15}O_3$	195.1016	2.662			(Alencar *et al.*, 2007; Righi *et al.*, 2011)
HBRP	Cis-asarone (**4a**); or Trans-isoelemicin (**4b**)	$C_{12}H_{17}O_3$	209.1180	1.103	209.1180 (14); 194.0969 (45); 178.0982 (22); 168.0783 (100); 153.0571 (37).	$[M - CH_3]^+$; $[M - CH3O]^+$; $[M - C_3H_5]^+$; $[M - C_4H_8]^+$	(Righi *et al.*, 2011; Trusheva *et al.*, 2006)
HBRP	Liquiritigenin (**5**)	$C_{15}H_{13}O_4$	257.0810	1.49	257.0810 (10); 153.0177 (24); 147.0448 (33); 137.0216 (100); 131.0450 (12); 123.0483 (4); 119.0471 (13).	$[M - C_8H_8]^+$; $[M - C_6H_6O_2]^+$; $[M - C_8H_8O]^+$; $[M - C_6H_6O_3]^+$; $[M - C_8H_8O_2]^+$; $[M - C_7H_6O_3]^+$.	(Piccinelli *et al.*, 2011)
HBRP	Formononetin (**6**)	$C_{16}H_{13}O_4$	269.0816	0.8	269.0816 (33); 254.0636 (45); 253.0512 (51); 237.0592 (43); 226.0692 (100); 225.0571 (40); 213.0974 (40); 197.0666 (92); 137.0216 (17); 133.0615 (9); 118.0434 (29).	$[M - CH_3]^+$; $[M - CH_4]^+$; $[M - CH_4O]^+$; $[M - C_2H_3O]^+$; $[M - C_2H_4O]^+$; $[M - C_2O_2]^+$; $[M - C_3H_4O_2]^+$; $[M - C_9H_8O]^+$; $[M - C_7H_4O_3]^+$; $[M - C_8H_7O_3]^+$.	(Piccinelli *et al.*, 2011)
HBRP	Medicarpin (**7**)	$C_{16}H_{15}O_4$	271.0970	3.563	271.0970 (73); 243.1078 (9); 177.0561 (8); 163.0391	$[M - CO]^+$; $[M - C_6H_6O]^+$; $[M - C_7H_8O]^+$; [M -	(Piccinelli *et al.*,

					(12); 161.0643 (10); 151.0422 (40); 149.0562 (6); 147.0448 (10); 137.0514 (70); 131.0523 (13); 123.0412 (11).	$C_6H_6O_2]^+$; $[M - C_8H_8O]^+$; $[M - C_7H_6O_2]^+$; $[M - C_7H_8O_2]^+$; $[M - C_7H_8O_3]^+$; $[M - C_9H_8O_2]^+$.	2011)
HBRP	Naringenin **(8)**	$C_{15}H_{13}O_5$	273.0771	2.935	273.0771 (16); 255.0881 (3); 163.0797 (32); 153.0177 (15); 149.0640 (56); 147.0448 (8); 137.0588 (100); 123.0412 (93); 121.0674 (7).	$[M - C_5H_2O_3]^+$; $[M - C_8H_8O]^+$; $[M - C_6H_4O_3]^+$; $[M - C_6H_6O_3]^+$; $[M - C_7H_4O_3]^+$; $[M - C_8H_6O_3]^+$; $[M - C_7H_4O_4]^+$.	(Piccinelli *et al.*, 2011)
HBRP	Vestitol **(9a)**; or Isovestitol **(9b)**	$C_{16}H_{17}O_4$	273.1136	3.354	273.1136 (73); 163.0716 (19); 149.0562 (22); 137.0588 (95); 123.0482 (71).	$[M - C_6H_6O_2]^+$; $[M - C_7H_6O_2]^+$; $[M - C_8H_4O_3]^+$; $[M - C_9H_{10}O_2]^+$.	(Awale *et al.*, 2008; Bueno-Silva *et al.*, 2013)
HBRP	Biochanin A **(10)**	$C_{16}H_{13}O_5$	285.0768	1.76	285.0768 (63); 270.0559 (100); 253.0512 (32); 242.0581 (16); 229.0905 (29); 225.0570 (61); 214.0637 (33); 213.0603 (31); 137.0666 (17); 137.0216 (48).	$[M - CH_3]^+$; $[M - CH_4O]^+$; $[M - C_2H_3O]^+$; $[M - C_2O_2]^+$; $[M - C_2H_4O_2]^+$; $[M - C_3H_3O_2]^+$; $[M - C_3H_4O_2]^+$; $[M - C_9H_8O_2]^+$.	(Piccinelli *et al.*, 2011)
HBRP	(3S)-Vestitone **(11)**	$C_{16}H_{15}O_5$	287.0920	0.18	287.0920 (68); 269.0955 (10); 241.0897 (12); 167.0331 (28); 163.0716 (31); 153.0570 (24); 137.0588 (100).	$[M - CH_2O_2]^+$; $[M - C_8H_6O]^+$; $[M - C_6H_4O_3]^+$; $[M - C_8H_6O]^+$; $[M - C_8H_9O_2]^+$.	(Awale *et al.*, 2008)
HBRP	(3S)-Ferreirin **(12)**	$C_{16}H_{15}O_6$	303.0871	0.78	303.0871 (71); 285.0746 (36); 193.0972 (10); 167.0825 (44); 123.0412 (30).	$[M - OH_2]^+$; $[M - C_7H_7O_2]^+$; $[M - C_9H_8O_4]^+$.	(Awale *et al.*, 2008)
HBRP	(3S)-Mucronulatol	$C_{17}H_{19}O_5$	303.1226	2.140	303.1226 (100); 285.0745	$[M - C_9H_9O_2]^+$; [M -	(Awale *et*

	(13)				(38); 257.1129 (13); 225.0570 (9); 183.0276 (7); 177.0561 (10); 167.0742 (56); 161.0643 (10); 153.0570 (18); 151.0187 (8); 149.0640 (7); 137.0588 (10); 123.0412 (33).	$H_{10}O_3]^+$; $[M - C_9H_{10}O_3]^+$; $[M - C_{10}H_{12}O_3]^+$.	*al.*, 2008; Piccinelli *et al.*, 2011)
HBRP	Guttiferone E **(14a)** or Xanthochymol **(14b)**	$C_{38}H_{51}O_6$	603.3706	3.374	603.3706 (100); 585.3631 (11); 479.2531 (16); 467.2620 (31); 411.1820 (60); 343.1205 (35); 137.1333 (17).	$[M - H_2O]^+$; $[M - C_5H_{16}O_3]^+$; $[M - C_6H_{16}O_3]^+$.	(Piccinelli *et al.*, 2011)
J Fraction	Liquiritigenin (**5**)	$C_{15}H_{13}O_4$	257.0810	1.49	257.0810 (10); 153.0177 (24); 147.0448 (33); 137.0216 (100); 131.0450 (12); 123.0483 (4); 119.0471 (13).	$[M - C_8H_8]^+$; $[M - C_6H_6O_2]^+$; $[M - C_8H_8O]^+$; $[M - C_6H_6O_3]^+$; $[M - C_8H_8O_2]^+$; $[M - C_7H_6O_3]^+$.	(Piccinelli *et al.*, 2011)
J Fraction	Formononetin (**6**)	$C_{16}H_{13}O_4$	269.0816	0.8	269.0816 (33); 254.0636 (45); 253.0512 (51); 237.0592 (43); 226.0692 (100); 225.0571 (40); 213.0974 (40); 197.0666 (92); 137.0216 (17); 133.0615 (9); 118.0434 (29).	$[M - CH_3]^+$; $[M - CH_4]^+$; $[M - CH_4O]^+$; $[M - C_2H_3O]^+$; $[M - C_2H_4O]^+$; $[M - C_2O_2]^+$; $[M - C_3H_4O_2]^+$; $[M - C_9H_8O]^+$; $[M - C_7H_4O_3]^+$; $[M - C_8H_7O_3]^+$.	(Piccinelli *et al.*, 2011)
J Fraction	Medicarpin (**7**)	$C_{16}H_{15}O_4$	271.0970	3.563	271.0970 (73); 243.1078 (9); 177.0561 (8); 163.0391 (12); 161.0643 (10); 151.0422 (40); 149.0562 (6); 147.0448 (10); 137.0514 (70); 131.0523 (13); 123.0412 (11).	$[M - CO]^+$; $[M - C_6H_6O]^+$; $[M - C_7H_8O]^+$; $[M - C_6H_6O_2]^+$; $[M - C_8H_8O]^+$; $[M - C_7H_6O_2]^+$; $[M - C_7H_8O_2]^+$; $[M - C_7H_8O_3]^+$; $[M - C_9H_8O_2]^+$.	(Piccinelli *et al.*, 2011)
J Fraction	Vestitol (**9a**); or	$C_{16}H_{17}O_4$	273.1136	3.354	273.1136 (73); 163.0716	$[M - C_6H_6O_2]^+$; [M -	(Awale *et*

	Isovestitol **(9b)**				(19); 149.0562 (22); 137.0588 (95); 123.0482 (71).	$C_7H_6O_2]^+$; $[M - C_8H_4O_3]^+$; $[M - C_9H_{10}O_2]^+$.	*al.*, 2008; Bueno-Silva *et al.*, 2013)
J Fraction	(3S)-Ferreirin (**12**)	$C_{16}H_{15}O_6$	303.0871	0.78	303.0871 (71); 285.0746 (36); 193.0972 (10); 167.0825 (44); 123.0412 (30).	$[M - OH_2]^+$; $[M - C_7H_7O_2]^+$; $[M - C_9H_8O_4]^+$.	(Awale *et al.*, 2008)
L Fraction	Liquiritigenin (**5**)	$C_{15}H_{13}O_4$	257.0810	1.49	257.0810 (10); 153.0177 (24); 147.0448 (33); 137.0216 (100); 131.0450 (12); 123.0483 (4); 119.0471 (13).	$[M - C_8H_8]^+$; $[M - C_6H_6O_2]^+$; $[M - C_8H_8O]^+$; $[M - C_6H_6O_3]^+$; $[M - C_8H_8O_2]^+$; $[M - C_7H_6O_3]^+$.	(Piccinelli *et al.*, 2011)

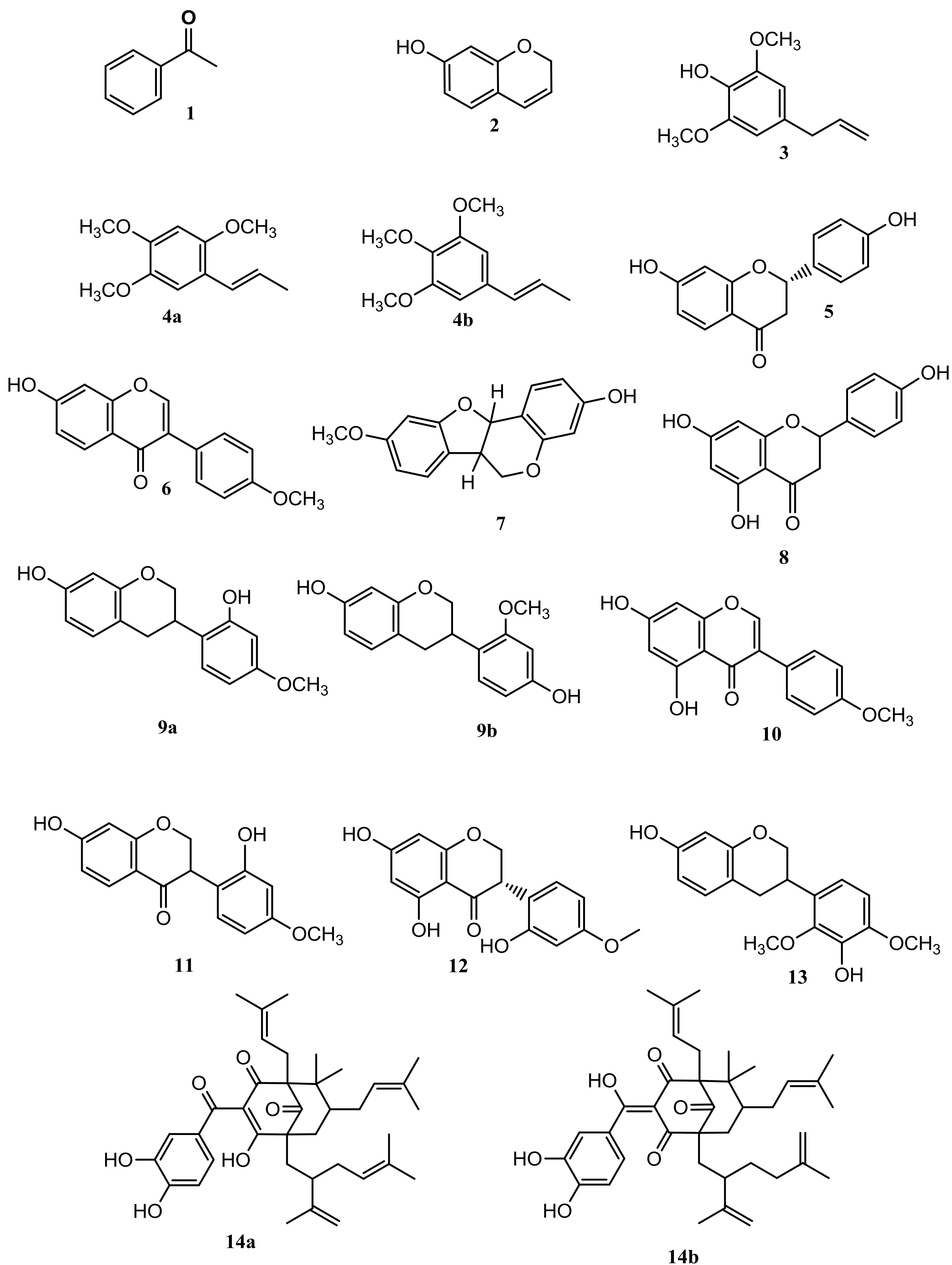


Figure 2. Structures of compounds 1-14 detected in red propolis sample (HBRP) and fractions.

Our group has identified and quantified previously (Frozza *et al.*, 2013; Frozza *et al.*, 2016) typical compounds present in red propolis extracts as Liquiritigenin, Formononetin and Biochanin A, which were further analyzed in this work. The MS fingerprint of HBRP showing the isoflavones is presented in Figure 3. Formononetin has recognized pro-apoptotic activity in different cancer cells (Hu & Xiao, 2015; Zhang *et al.*, 2014) as well as Biochanin A (Bhardwaj *et al.*, 2014). Liquiritigenin has also been investigated with potential activity against cancer (Sareddy & Vadlamudi, 2015).

In order to quantify, was developed a method using HPLC-UV and ESI-MS, which was applied Liquiritigenin, Formononetin and Biochanin A standards. Limits of quantification (LOQ) and limits of detection (LOD) were estimated for each analyte from the regression analysis using the standard deviation of the intercept and the slope. The R^2 values were ≥ 0.997 for all analytes, indicating a good linear relationship.

Table 2 shows the quantified compounds and the respective detection and quantification limits were 0.3 ppb and 1ppb, respectively. Linear working range was from 1 to 1000 ppb; for Biochanin A, $y = 1606.558027x + 3930.724446$, $R = 0.999096$; for Formononetin, $y = 3849.958134x + 4377.617848$, $R = 0.997997$ and for Liquiritigenin, $y = 648.050189x + 1827.463225$, $R = 0.999283$.

The quantified compounds were isoflavonoids that could be potentially responsible to antitumor activity. These results are different from previous studies that evaluated the compositions of Brazilian red propolis especially for Formononetin which presented a smaller value from others studies, however, account should be taken of the diversify composition of samples (Table 3).

Table 2. Biochanin A, Formononetin and Liquiritigenin's composition in HBRP and its active fractions (J and L) at mg/1 g of extract. Results are expressed as mean ± standard deviations of three determinations.

Sample	Biochanin A (mg/g)	Formononetin (mg/g)	Liquiritigenin (mg/g)
J Fraction	n.d.	0.09 ± 0.004	11.65 ± 0.450
L Fraction	n.d.	n.d.	0.04 ± 0.006
HBRP	0.28 ± 0.011	1.52 ± 0.040	1.66 ± 0.030

n.d.; not detected

Table 3. Biochanin A, Formononetin and Liquiritigenin's composition in HBRP from other studies. Results are expressed as mg/1g of extract.

Ref. and Method	Origin of Brazilian Red propolis	Biochanin A	Formononetin	Liquiritigenin
Daugsch *et al.,* (2007) RPHPLC / RPHPTLC	Northeast	0.5 mg/g	10.2 mg/g	1.8 mg/g
Franchi *et al.,* (2012) RPHPLC	Northeast	1.5 mg/g	19.5 mg/g	7.1 mg/g
Neves *et al.,* (2016) HPLC	Pernambuco (2 samples)	0.56 mg/g 0.17 mg/g	2.86 mg/g 1.71 mg/g	n.d
Cavendish *et al.,* (2015) HPLC	Sergipe	n.d	21.62 mg/g	n.d
Ribeiro *et al.,* (2015) HPLC	Sergipe	0.67 mg/g	23.29 mg/g	n.d
Frozza *et al.,* (2016) UFLC	Sergipe	0.41 mg/g	2.37 mg/g	1.54 mg/g
This work	Alagoas	0.27 mg/g	1.52 mg/g	1.66 mg/g

n.d.; not determinated

3.2 Viability and morphological changes

The ability of the crude extract (HBRP) and their fractions (J and L) to inhibit growth of laryngeal cancer cell was determined by MTT assay. According to the IC50 results from cell viability after 24 h and 48 h extract incubation (Table 3), the HBRP and fractions demonstrated potent cytotoxic activity. Moreover, both fractions, J and L, exerted a stronger effect compared to the crude extract. The J fraction presented the most cytotoxic effect against Hep-2 cells. This suggests that the cell line was more sensitive to the fractions than to HBRP and the increased sensitivity may be correlated with the purification of the sample. Fractionation decreases the number of compounds in the treatment, and may increase the concentration of active compounds when compared to exposure of the crude extract. By the way, some of these compounds have been cited in the literature as natural anticancer products. According to Novak *et al.,* (2014), which have tested red propolis fraction in others cell lines, the antiproliferative and cytotoxic activities of Brazilian red propolis fractions on cancer cells may not correspond singularly to the concentration of a unique component, but instead arise from the synergistic antiproliferative activity of the individual compounds and further isolation may be necessary to fully determine all of the active components.

In order to assess whether fractions induce any morphological changes, cells were treated for 24 h, colored with Giemsa and then observed under a microscope. As shown in Figure 4 red propolis treatment changed considerably the cellular morphology. The examination of cancer cells through Giemsa staining revealed altered morphological structure dependent of the concentration of sample used. The cells in the negative control group that received hydroalcoholic solution of 2.5% (v/v) showed a regular appearance, intensive growth, surrounded by abundant cytoplasm, represented as healthy cells (Figure 4A). Different morphological patterns were observed in the cell

culture that received J and L fraction (50 µg/mL) treatment (Figure 4 B and D). The cells that received higher concentrations (75 µg/mL) (Figure 4 C and E) exhibited decreased growth, loss of volume, cytoplasmic shrinkage, nuclear condensation and deformation to a round appearance indicating several biological changes upon extract cytotoxic activity. To confirm the condensation and fragmentation of nuclear DNA was performed a more specific assay, DAPI. In the control group, the nuclei were roundish and homogeneously stained with DAPI (Figure 5A). In contrast, the nuclei in the treated Hep-2 cells were irregularly shaped, small, detached, presenting condensed chromatin and had apoptotic bodies that are typical of the early and late stages of apoptosis (Figure 5B).

Table 3. IC50 results from cell viability after 24 h and 48 h extract incubation. The values represent the media and standard deviation of at least triplicates.

Red propolis samples	24 h treatment IC50 (µg/mL)	48 h treatment IC50 (µg/mL)
J Fraction	60.96 ± 4.06^{b}	30.71 ± 3.54^{b}
L Fraction	74.60 ± 2.39^{c}	43.73 ± 2.84^{c}
HBRP	145.40 ± 6.56^{a}	57.54 ± 0.98^{a}

Different superscript letters within the same column indicate significant differences between tested samples ($p < 0.05$, ANOVA One-way).

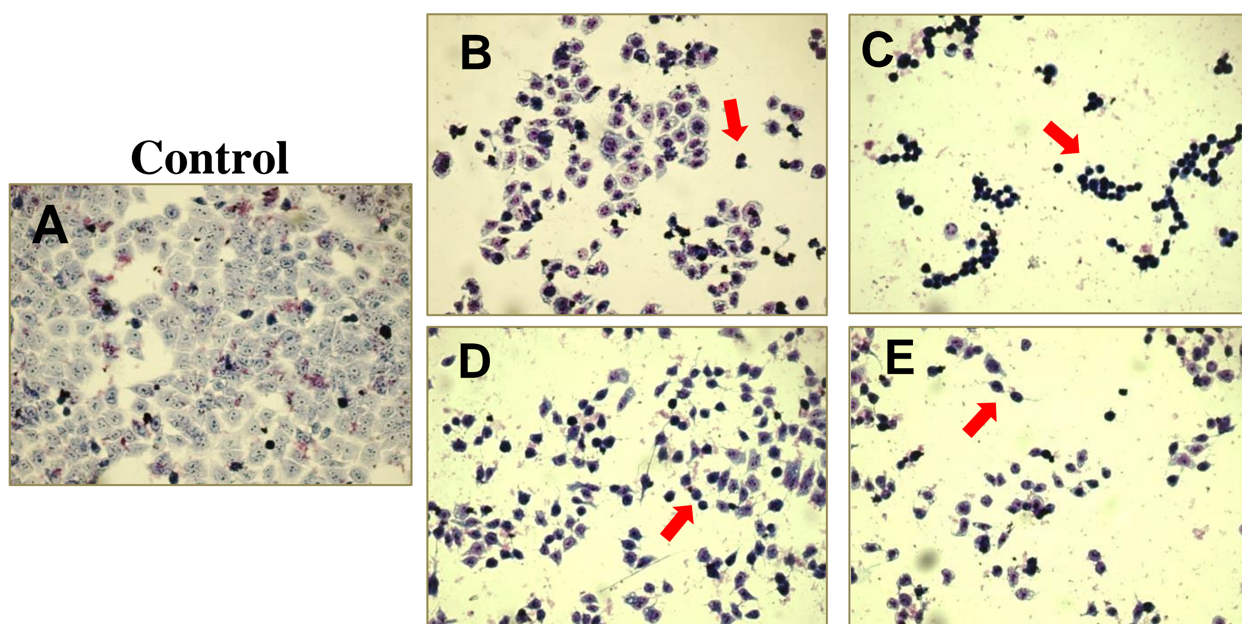


Figure 4. Morphological structure of Hep-2 cancer cells through Giemsa staining after 24 h of treatment. (A) control, (B) J fraction (50 µg/mL), (C) J fraction (75 µg/mL), (D) L fraction (50 µg/mL) and (E) L fraction (75 µg/mL). Arrows indicate nuclear condensation.

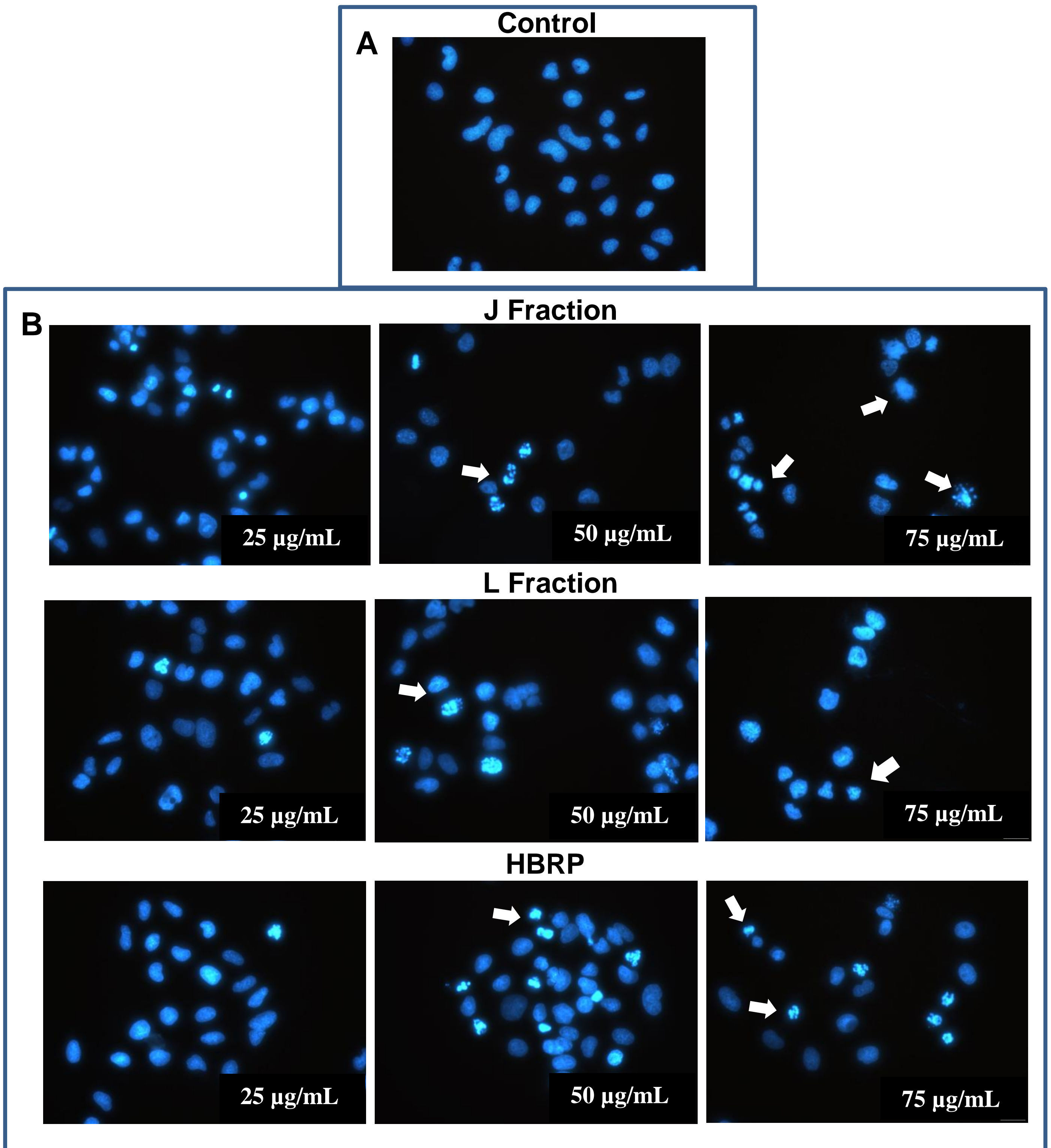


Figure 5. Nuclear morphological changes in Hep-2 cells after treatment with red propolis samples for 24 hours followed by DAPI staining. (A) Control cells treated with media with 2.5% ethanol; (B) Treated groups with 25, 50 and 75 µg/mL of J and L

Fractions and HBRP. Arrows indicate apoptotic bodies of nuclear fragmentation observed at 40× magnification under an inverted fluorescence microscope.

3.3 Flow Cytometry

3.3.1 Annexin/PI

Cells were exposed to different concentrations of red propolis fractions revealed that the percentages of late apoptosis cells were gradually increased (Figure 6). According to our previous studies with samples from Sergipe (Frozza *et al.*, 2014), red propolis extract induced apoptosis in Hep-2 cancer cells in a dose-dependent manner and the signaling to late apoptosis/necrosis increased in a significant way in the cells treated with higher concentrations comparing with control group. Begnini *et al.,* (2014) also presented that red propolis extract induced in 5637 cells a higher percentage of late apoptosis at 100 μg/mL concentration compared to the control. Note that most cells exposed to enriched fractions presented a late stage of apoptosis phenotype compared to the crude extract (HBRP). The low quantity of apoptotic cells were not eminent in the HBRP once the concentrations used were below the IC50 for 24 hours of treatment.

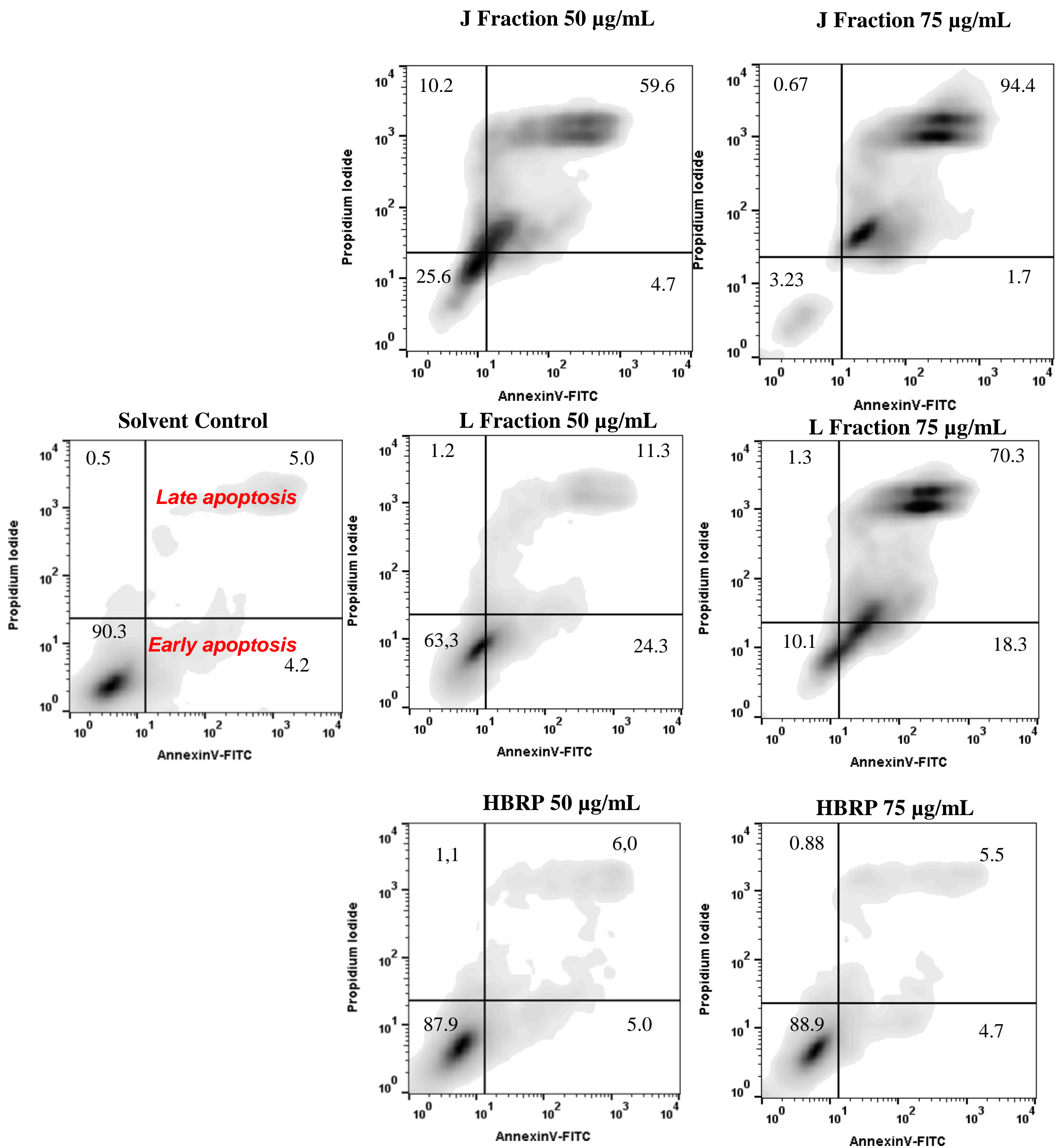


Figure 6. Representative data after 24 h red propolis treatment incubation in Hep-2 cell line. The percentage of Annexin V-positive and PI-negative cells in early stages of apoptosis (bottom right) and Annexin V-positive and PI positive cells that were dead or in late-stages of apoptosis (top right), are presented in each quadrant.

3.3.2 Mitochondrial Membrane Potential and ROS measurement

Loss of mitochondrial membrane potential is an important event during the mitochondrial pathway of apoptosis, so we investigated whether red propolis could induce the loss of MMP in Hep-2 cells. As shown in Figure 7, the percentages of cells with loss of MMP increased in cells treated with red propolis fractions at 50 and 75 µg/mL, respectively (Figure 7 A and B). This profile was not representative for HBRP (Figure 7 C). These data provide evidence that red propolis triggers an intrinsic mitochondrial apoptotic pathway in Hep-2 cells.

On the other hand, ROS generation is also linked to mitochondria. Fluorescence probe DCFH-DA and DHR123 was used to determine the levels of ROS production in Hep-2 cells. Levels of ROS in control and red propolis-treated cells are depicted in Figure 8. Considering the damage to the cells upon treatment over the range of high concentrations of red propolis in fractions J and L, ROS decreased levels in Hep-2 cells, evidenced by the decreased 2–7-diacetyl dichlorofluorescein fluorescence. Kamiya et al., (2012a) showed that red propolis has inhibitory effect on CdCl2-induced cytotoxicity associated by the reduction of intracellular ROS accumulation.

Mitochondrial Membrane Potential

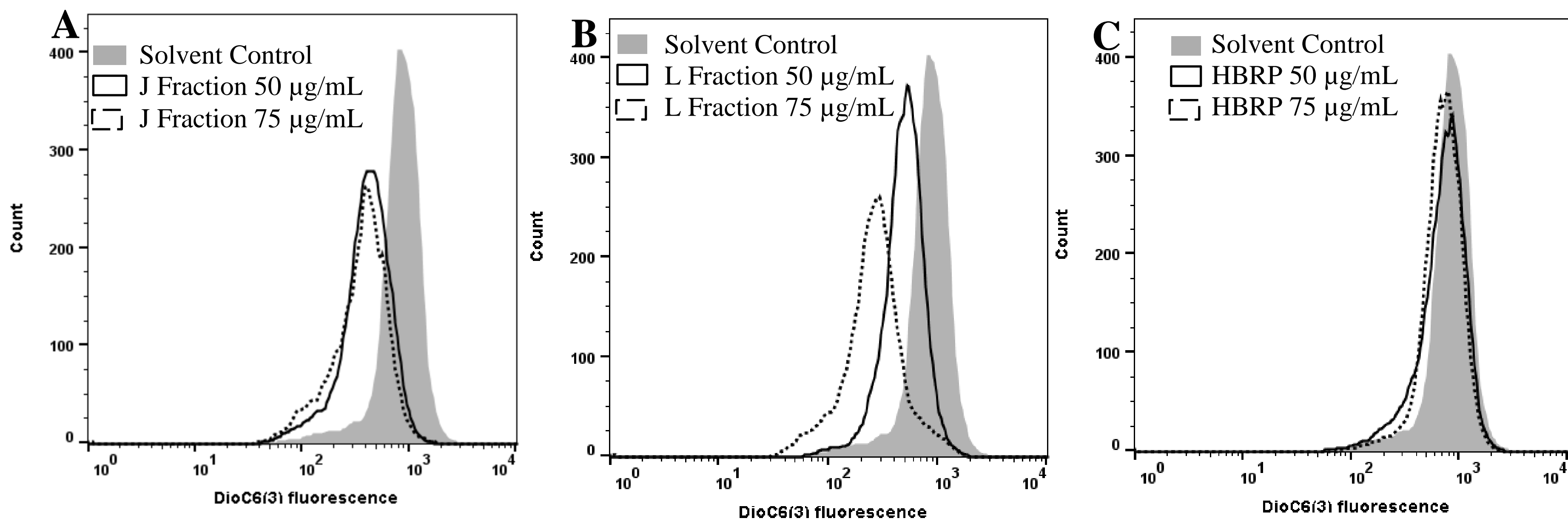


Figure 7. Effects of red propolis samples in MMP determined by fluorescence intensity. A, B – Red propolis fractions (J and L) and C – Crude extract (HBRP).

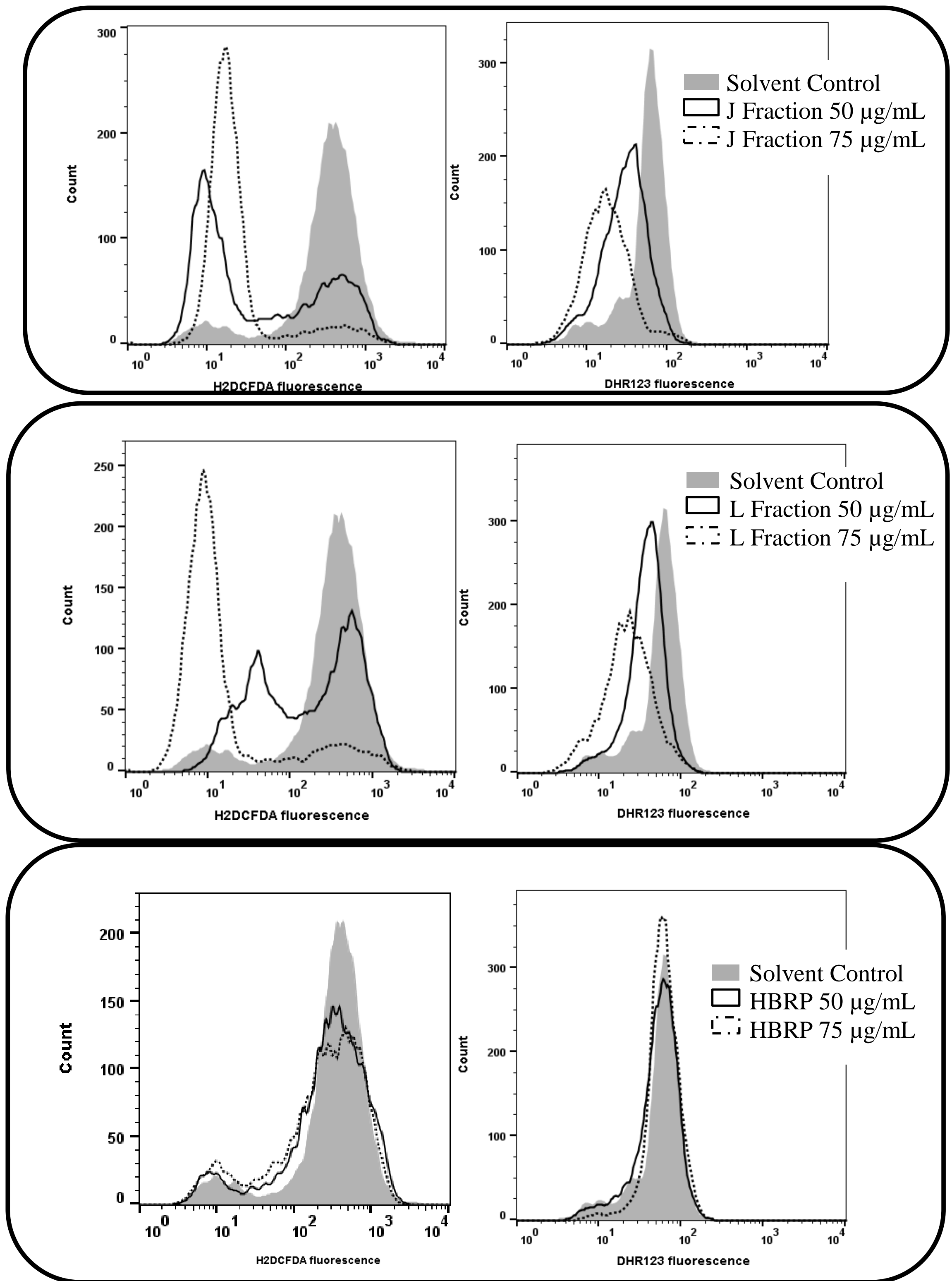
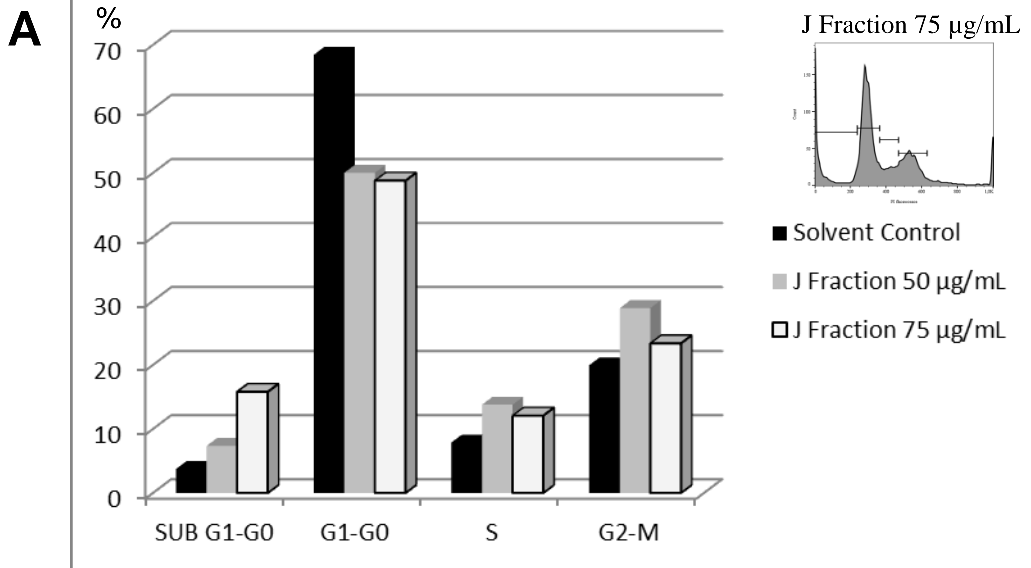


Figure 8. Effects of red propolis samples on intracellular ROS which were determined by fluorescence intensity. A, B – red propolis fractions (J and L) and C – crude extract (HBRP).

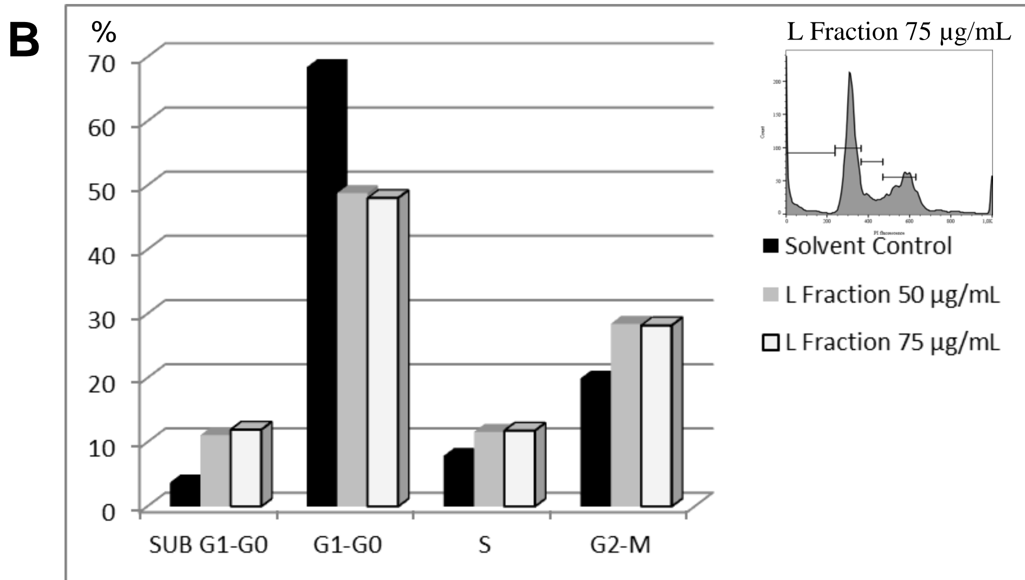
3.3.3 Cell Cycle Analysis

To determine whether the cytotoxic effect of the samples on Hep-2 cells was associated with an induction of cell cycle arrest, the distribution of cells in various phases of the cycle was analyzed using flow cytometry. The treated groups showed an accumulation of cells in SUB G1-G0, S and G2-M phase (Figure 9). This increase in SUB G1-G0 phase can be considered as reflecting the induction of cell death. It has been generally accepted that the increase of cells having SUB G1-G0 DNA content is a marker of apoptotic cell death (Mojzisova *et al.*, 2016). In cell treatments a gradual decrease in the G1-G0 phase was observed. Novak *et al.,* (2014) demonstrated that treated B16F10 cells with a fraction (IV) of Brazilian red propolis for 48 h revealed a significant increase of DNA fragmented (SUB G1-G0 phase) compared to control group.

A



B



C

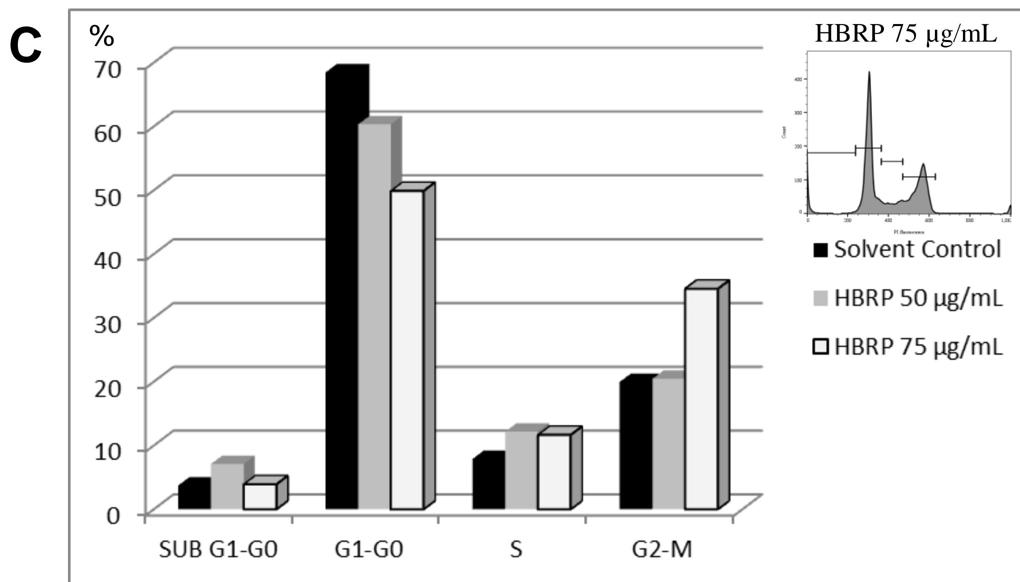


Figure 9. Cell cycle analysis of Hep-2 cells treated with red propolis samples (50 µg/mL or 75 µg/mL) or vehicle (2.5 % ethanol) for 24 h. Cells were stained with propidium iodide and analyzed by flow cytometry to determine the percentage of cells in each stage of the cell cycle. Representative histograms (panels A, B and C) are shown for red propolis treatment with the quantification of the percentage of cells.

3.1 DNA cleavage assay

To investigate the precise mechanism of the cell death induced by samples we examined the mode of cell death by DNA cleavage assay. The fractions J and L presented a stronger activity showing, respectively, a fast complete transformation of the FI form to FII form trough single strand breaks (Figure 10 A) or even double strand breaks with the formation of linear form (FIII) (Figure 10 B). The crude extract, however, presented no significant cleavage activity on plasmid DNA, but a slight reduction of the supercoiled form (FI) with the corresponding increase in the open circular form (FII) (Figure 10 C). These results indicate that there are probably antagonist substances in the crude extract or that the substances present in the fractions only reaches the DNA molecule when purified.

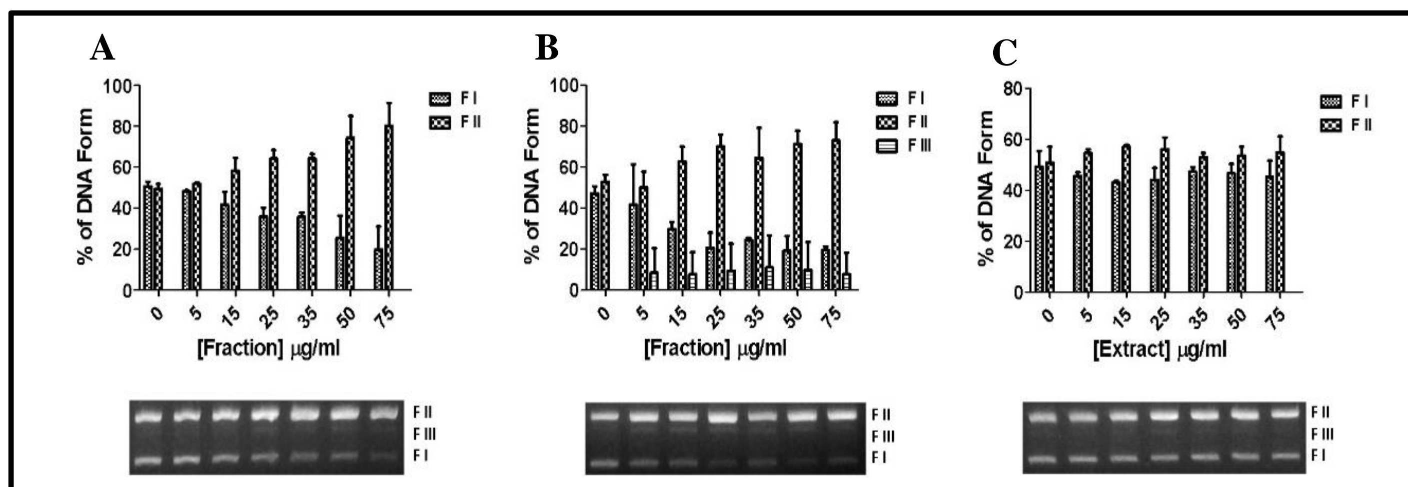


Figure 10. Graphics and photos showing the cleavage activity of J fraction (A), L fraction (B) and of crude extract (C) on plasmid DNA. It can be observed that the activity is higher in the fractions than in the crude extract and the fraction L present the higher activity of all.

According to Kamiya et al (2012b) DNA fragmentation was observed in MCF-7 cells treated with ethanol extracts of Brazilian red propolis but the same was not seen in

human Fibroblast cells. They indicated that endoplasmic reticulum stress plays a major role in the ethanol extract of Brazilian red propolis, which triggers apoptosis.

4. Conclusion

In conclusion, our findings indicate that Brazilian red propolis fractions were most effective against Hep-2 cancer cells *in vitro* when compared to crude extract. Chemical characterization demonstrated that each fraction presents compounds that act distinctly on cell metabolism bringing different responses of the cell signaling.

It is well known that single parameters will not define responses of a role system, therefore combination of methods for reliable detection of apoptosis should be considered. In this study, we found that fractions induced apoptosis in Hep-2 cells, as verified by the formation of apoptotic nuclei that are characterized by chromatin condensation and DNA fragmentation and these results are consistent with an accumulation of cells in the sub-G1 apoptotic phase. However these fractions are enriched with a group of compounds which needs to be isolated and tested individually in further studies. In order to elucidate whether these molecules respond best as isolated compound or generate increase activity in combination within enriched fraction, further studies are mandatory.

Acknowledgements

This work was supported by CAPES, CNPq and FAPERGS.

5. References

1. Alencar, S.M.; Oldoni, T.L.; Castro, M.L.; Cabral, I.S.; Costa-Neto, C.M.; Cury, J.A.; Rosalen, P.L.; Ikegaki, M., (2007). Chemical composition and biological activity of a new type of Brazilian propolis: red propolis. **J Ethnopharmacol** 113, 278-283.
2. Awale, S.; Li, F.; Onozuka, H.; Esumi, H.; Tezuka, Y.; Kadota, S., (2008). Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition. **Bioorg Med Chem** 16, 181-189.
3. Begnini, K.R.; Moura de Leon, P.M.; Thurow, H.; Schultze, E.; Campos, V.F.; Martins Rodrigues, F.; Borsuk, S.; Dellagostin, O.A.; Savegnago, L.; Roesch-Ely, M.; Moura, S.; Padilha, F.F.; Collares, T.; Pegas Henriques, J.A.; Seixas, F.K., (2014). Brazilian red propolis induces apoptosis-like cell death and decreases migration potential in bladder cancer cells. **Evid Based Complement Alternat Med** 2014, 639856.
4. Bhardwaj, V.; Tadinada, S.M.; Jain, A.; Sehdev, V.; Daniels, C.K.; Lai, J.C.; Bhushan, A., (2014). Biochanin A reduces pancreatic cancer survival and progression. **Anticancer Drugs** 25, 296-302.
5. Biscaro, F.; Parisotto, E.B.; Zanette, V.C.; Gunther, T.M.; Ferreira, E.A.; Gris, E.F.; Correia, J.F.; Pich, C.T.; Mattivi, F.; Filho, D.W.; Pedrosa, R.C., (2013). Anticancer activity of flavonol and flavan-3-ol rich extracts from Croton celtidifolius latex. **Pharm Biol** 51, 737-743.
6. Bueno-Silva, B.; Alencar, S.M.; Koo, H.; Ikegaki, M.; Silva, G.V.; Napimoga, M.H.; Rosalen, P.L., (2013). Anti-inflammatory and antimicrobial evaluation of neovestitol and vestitol isolated from Brazilian red propolis. **J Agric Food Chem** 61, 4546-4550.
7. Cavendish, R.L.; de Souza Santos, J.; Belo Neto, R.; Oliveira Paixao, A.; Valeria Oliveira, J.; Divino de Araujo, E.; Berretta, E.S.A.A.; Maria Thomazzi, S.; Cordeiro Cardoso, J.; Zanardo Gomes, M., (2015). Antinociceptive and anti-inflammatory effects of Brazilian red propolis extract and formononetin in rodents. **J Ethnopharmacol** 173, 127-133.

8. Daugsch, A.; Moraes, C.S.; Fort, P.; Park, Y.K., (2007). Brazilian red propolis--chemical composition and botanical origin. **Evid Based Complement Alternat Med** 5, 435-441.
9. de Mendonca, I.C.; Porto, I.C.; do Nascimento, T.G.; de Souza, N.S.; Oliveira, J.M.; Arruda, R.E.; Mousinho, K.C.; dos Santos, A.F.; Basilio-Junior, I.D.; Parolia, A.; Barreto, F.S., (2015). Brazilian red propolis: phytochemical screening, antioxidant activity and effect against cancer cells. **BMC Complement Altern Med** 15, 357.
10. Franchi, G.C., Jr.; Moraes, C.S.; Toreti, V.C.; Daugsch, A.; Nowill, A.E.; Park, Y.K., (2012). Comparison of effects of the ethanolic extracts of brazilian propolis on human leukemic cells as assessed with the MTT assay. **Evid Based Complement Alternat Med** 2012, 918956.
11. Frozza, C.O.; Garcia, C.S.; Gambato, G.; de Souza, M.D.; Salvador, M.; Moura, S.; Padilha, F.F.; Seixas, F.K.; Collares, T.; Borsuk, S.; Dellagostin, O.A.; Henriques, J.A.; Roesch-Ely, M., (2013). Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. **Food Chem Toxicol** 52, 137-142.
12. Frozza, C.O.; Ribeiro Tda, S.; Gambato, G.; Menti, C.; Moura, S.; Pinto, P.M.; Staats, C.C.; Padilha, F.F.; Begnini, K.R.; de Leon, P.M.; Borsuk, S.; Savegnago, L.; Dellagostin, O.; Collares, T.; Seixas, F.K.; Henriques, J.A.; Roesch-Ely, M., (2014). Proteomic analysis identifies differentially expressed proteins after red propolis treatment in Hep-2 cells. **Food Chem Toxicol** 63, 195-204.
13. Frozza, C.O.d.S.; Brum, E.d.S.; Alving, A.; Moura, S.; Henriques, J.A.; Roesch-Ely, M., (2016). LC-MS analysis of Hep-2 and Hek-293 cell lines treated with Brazilian red propolis reveals differences in protein expression. **J Pharm Pharmacol** 68, 1073-1084.
14. Hayacibara, M.F.; Koo, H.; Rosalen, P.L.; Duarte, S.; Franco, E.M.; Bowen, W.H.; Ikegaki, M.; Cury, J.A., (2005). In vitro and in vivo effects of isolated fractions of Brazilian propolis on caries development. **J Ethnopharmacol** 101, 110-115.

15. Hu, W.; Xiao, Z., (2015). Formononetin induces apoptosis of human osteosarcoma cell line U2OS by regulating the expression of Bcl-2, Bax and MiR-375 in vitro and in vivo. **Cell Physiol Biochem** 37, 933-939.
16. Kamiya, T.; Izumi, M.; Hara, H.; Adachi, T., (2012a). Propolis suppresses CdCl(2)-induced cytotoxicity of COS7 cells through the prevention of intracellular reactive oxygen species accumulation. **Biol Pharm Bull** 35, 1126-1131.
17. Kamiya, T.; Nishihara, H.; Hara, H.; Adachi, T., (2012b). Ethanol extract of Brazilian red propolis induces apoptosis in human breast cancer MCF-7 cells through endoplasmic reticulum stress. **J Agric Food Chem** 60, 11065-11070.
18. Machado, C.S.; Mokochinski, J.B.; de Lira, T.O.; de Oliveira Fde, C.; Cardoso, M.V.; Ferreira, R.G.; Sawaya, A.C.; Ferreira, A.G.; Pessoa, C.; Cuesta-Rubio, O.; Monteiro, M.C.; de Campos, M.S.; Torres, Y.R., (2016). Comparative Study of Chemical Composition and Biological Activity of Yellow, Green, Brown, and Red Brazilian Propolis. **Evid Based Complement Alternat Med** 2016, 6057650.
19. Mojzisova, G.; Kello, M.; Pilatova, M.; Tomeckova, V.; Vaskova, J.; Vasko, L.; Bernatova, S.; Mirossay, L.; Mojzis, J., (2016). Antiproliferative effect of beta-escin - an in vitro study. **Acta Biochim Pol** 63, 1013.
20. Mosmann, T., (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J Immunol Methods** 65, 55-63.
21. Neves, M.V.d.; da Silva, T.M.; Lima Ede, O.; da Cunha, E.V.; Oliveira Ede, J., (2016). Isoflavone formononetin from red propolis acts as a fungicide against Candida sp. **Braz J Microbiol** 47, 159-166.
22. Nocentini, S.; Moreno, G.; Petit, P.X.; Guggiari, M.; Salet, C.; Demerseman, P.; Dodin, G., (1997). Induction of mitochondrial dysfunction and apoptosis in HeLa cells by bis-pyridinium oximes, a newly synthesized family of lipophilic biscations. **Biochem Pharmacol** 53, 1543-1552.
23. Novak, E.M.; Costa Silva, M.S.e.; Marcucci, M.C.; Sawaya, A.C.H.F.; López, B.G.; Fortes, M.A.H.Z.; Giorgi, R.R.; Marumo, K.T.; Rodrigues, R.F.; Maria, D.A., (2014). Antitumoural activity of Brazilian red propolis fraction enriched

with xanthochymol and formononetin: an in vitro and in vivo study. **J Funct Foods** 2, 91-102.

24. Piccinelli, A.L.; Lotti, C.; Campone, L.; Cuesta-Rubio, O.; Campo Fernandez, M.; Rastrelli, L., (2011). Cuban and Brazilian red propolis: botanical origin and comparative analysis by high-performance liquid chromatography-photodiode array detection/electrospray ionization tandem mass spectrometry. **J Agric Food Chem** 59, 6484-6491.
25. Ribeiro, D.R.; Alves, A.V.; dos Santos, E.P.; Padilha, F.F.; Gomes, M.Z.; Rabelo, A.S.; Cardoso, J.C.; Massarioli, A.P.; de Alencar, S.M.; de Albuquerque-Junior, R.L., (2015). Inhibition of DMBA-induced Oral Squamous Cells Carcinoma Growth by Brazilian Red Propolis in Rodent Model. **Basic Clin Pharmacol Toxicol** 117, 85-95.
26. Righi, A.A.; Alves, T.R.; Negri, G.; Marques, L.M.; Breyer, H.; Salatino, A., (2011). Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. **J Sci Food Agric** 91, 2363-2370.
27. Sareddy, G.R.; Vadlamudi, R.K., (2015). Cancer therapy using natural ligands that target estrogen receptor beta. **Chin J Nat Med** 13, 801-807.
28. Shan, F.; Shao, Z.; Jiang, S.; Cheng, Z., (2016). Erlotinib induces the human non-small-cell lung cancer cells apoptosis via activating ROS-dependent JNK pathways. **Cancer Med** 5, 3166-3175.
29. Siegel, R.L.; Miller, K.D.; Jemal, A., (2016). Cancer statistics, 2016. **CA Cancer J Clin** 66, 7-30.
30. Trusheva, B.; Popova, M.; Bankova, V.; Simova, S.; Marcucci, M.C.; Miorin, P.L.; da Rocha Pasin, F.; Tsvetkova, I., (2006). Bioactive constituents of brazilian red propolis. **Evid Based Complement Alternat Med** 3, 249-254.
31. Wlodkowic, D.; Skommer, J.; Darzynkiewicz, Z., (2009). Flow cytometry-based apoptosis detection. **Methods Mol Biol** 559, 19-32.
32. Zhang, X.; Bi, L.; Ye, Y.; Chen, J., (2014). Formononetin induces apoptosis in PC-3 prostate cancer cells through enhancing the Bax/Bcl-2 ratios and regulating the p38/Akt pathway. **Nutr Cancer** 66, 656-661.

4. DISCUSSÃO

Os benefícios da própolis têm sido amplamente explorados em várias áreas da medicina como um importante recurso na prevenção, gestão e tratamento de doenças bucais e sistêmicas (Freires *et al.*, 2016). O número crescente de publicações sobre as propriedades antiproliferativas da própolis vermelha brasileira e de seus constituintes revela um enorme potencial no desenvolvimento de novos agentes anticancerígenos. A maioria dos estudos com própolis vermelha são baseados em ensaios de citotoxicidade *in vitro* contra uma gama de células tumorais (de Mendonca *et al.*, 2015; Franchi *et al.*, 2012). Além disso, efeitos sobre o perfil de expressão diferencial de proteínas de linhagens de câncer e efeitos moduladores sobre a carcinogênese *in vivo* também têm sido relatados (Freires *et al.*, 2016).

Os primeiros estudos do nosso grupo efetuaram uma abordagem com extratos brutos da própolis vermelha oriunda de Sergipe, mostrando a composição rica em polifenóis (151 mg/g) com capacidade de eliminar radicais livres, o que foi comprovado pela medição do radical livre DPPH•, e inibir o crescimento de células tumorais de forma seletiva em relação às células não tumorais (Frozza *et al.*, 2013). Os efeitos citotóxicos da própolis vermelha neste trabalho foram observados nas linhagens tumorais Hep-2 e HeLa (adenocarcinoma cervical) e na linhagem não tumoral Hek-293 pelos períodos de 1 hora e 24 horas, sendo que a inibição do crescimento celular foi estatisticamente maior nas linhagens tumorais.

O presente estudo investiga a própolis oriunda de Alagoas na sua forma bruta e fracionada e mostra que a identificação química reforça a complexidade de seus compostos, entre eles pode-se citar flavonóides como a Formononetina, Liquiritigenina, Medicarpina e Biochanina A, além da identificação de novas moléculas como a

Narigenina, (3S)–Vestitona, (3S)–Ferreirina, entre outras que não haviam sido identificadas na amostra de Sergipe. Cada amostra de própolis possui composições químicas qualitativamente e quantitativamente diferentes de acordo com a localização de procedência. Estes compostos identificados já foram citados por outros autores e alguns são considerados marcadores da própolis vermelha (Lopez *et al.*, 2014). Muitos outros compostos estão presentes nas amostras de própolis, contudo não é possível identificar todos eles, desta forma, neste trabalho, a massa exata, a razão isotópica as vias de fragmentação foram utilizadas para identificar e distinguir o maior número de moléculas possível. O conhecimento do perfil químico das amostras de própolis faz com que seja possível entender melhor suas promissoras propriedades terapêuticas, sendo que esta identificação e caracterização podem ser efetuadas através da espectrometria de massas com ionização por *electronspray*, considerada uma técnica analítica rápida e confiável para a análise direta de extratos hidroalcoólico de própolis (Buriol et al., 2009).

O fracionamento, apresentado no Capítulo 3, possibilitou a aquisição de frações com composições químicas distintas, o que pode ser comprovado não só pela identificação química como também através dos efeitos distintos observados nos ensaios biológicos. Em relação aos ensaios de citotoxicidade, as frações J e L tiveram um resultado melhor do que ao extrato hidroalcoólico, sendo que a fração J foi a mais citotóxica em ambos os períodos de tratamento (24 h e 48 h) (Tabela 3, Capítulo 3). Da mesma forma, Novak *et al.,* (2014) também realizaram um fracionamento da própolis de Alagoas e testaram a melhor fração (contendo Xantoquimol e Formononetina) em diversas linhagens celulares e em roedores. Os valores de IC50 encontrados por eles também foram inferiores ao extrato hidroalcoólico precursor das frações (Novak *et al.,* 2014). A citotoxicidade elevada das frações em relação aos extratos brutos pode não ser

exclusivamente devido à concentração de um único componente presente na amostra, mas sim, a partir da atividade antitumoral sinérgica de vários compostos, os quais devem ser identificados (Novak *et al.*, 2014).

A quantificação dos compostos Biochanina A, Formononetina e Liquiritigenina realizada neste estudo permite a comparação das duas amostras, de Sergipe e Alagoas (Capítulo 2 e 3). Os valores encontrados para os três compostos analisados nos extratos brutos não foram discrepantes nesta comparação, contudo foi salientada a grande quantidade de Liquiritigenina encontrada na fração J da amostra de Alagoas. Como, ambas as frações, J e L, apresentaram comportamento citotóxico na linhagem tumoral, pode-se esperar um sinergismo de compostos que atuem inibindo o crescimento celular, já que a grande quantidade deste composto na fração J não refletiu na sua atividade biológica na mesma proporção.

Com relação ao mecanismo de ação, a indução de apoptose tem sido um dos fatores propostos para os efeitos terapêuticos antitumorais da própolis, contudo o mecanismo exato de atuação permanece obscuro, uma vez que cada amostra possui particularidades únicas. A escolha de parâmetros individuais não pode definir a resposta de um sistema por inteiro, sendo assim, uma combinação de métodos deve ser considerada para uma detecção fiel de apoptose, tendo em vista a quantidade de tipos de morte celulares existentes (Galluzzi *et al.*, 2012). A morte celular está intrinsicamente relacionada ao tratamento do câncer, servindo como função efetora principal de muitas terapias antitumorais, sendo assim, as vias mitocondriais, associada com a morte celular por apoptose, tem sido considerada um alvo terapêutico para o câncer (Lopez & Tait, 2015).

Neste estudo, conforme apresentado nos Capítulos 1 e 3, verificou-se que as amostras induziram, ao menos em parte, apoptose em células Hep-2. As colorações por

Giemsa (Fig. 4, Capítulo 1; Fig. 3, Capítulo 2 e Fig. 4, Capítulo 3), as quais demostraram o efeito citotóxico tanto do extrato hidroalcoólico quanto das frações sobre a morfologia celular, e por DAPI, onde foi verificada a formação de corpos apoptóticos e a condensação de cromatina, evidenciaram características marcantes de apoptose. A própolis proveniente de Sergipe (Capítulo 1) mostrou aumento de células em apoptose tardia no ensaio de TUNEL e na marcação *in situ* de Anexina-V/PI (Fig. 3 e 5, Capítulo 1), da mesma forma, a própolis proveniente de Alagoas, também apresentou este aumento de células em apoptose tardia, nos grupos tratados com as frações, no ensaio Anexina-V/PI utilizando citometria de fluxo (Fig. 6, Capítulo 3). Além do mais, o aumento do número de células na fase SUB G1-G0 do ciclo celular (Capítulo 3) também é um marcador de morte celular por apoptose (Mojžišová *et al.,* 2016).

Alguns constituintes da própolis já foram descritos como pró-apoptóticos em células cancerosas. O aumentou do índice de células em apoptose foi observado em células cancerosas de pâncreas e de pulmão no tratamento com Biochanina A e Formononetina, respectivamente (Bhardwaj *et al.*, 2014; Yang *et al.*, 2014). Já a Liquiritigenina apresentou efeitos citotóxicos contra células de carcinoma hepatocelular causando alterações apoptóticas mitocondriais (Wang *et al.*, 2014). Como estes flavonoides estão presentes nas amostras, isto pode justificar, em parte, os resultados encontrados.

Os resultados provenientes do ensaio de ROS (Capítulo 3) revelaram que as frações e o extrato hidroalcoólico ocasionaram diminuição na formação de ROS nas células que receberam tratamento. Kamiya *et al.,* (2012a) também relatou esta diminuição causada pelo tratamento com própolis vermelha, o autor acrescenta que as espécies reativas de oxigênio, as quais estão relacionadas a várias doenças, podem ser diminuídas através de produtos naturais. Contudo, a diminuição de ROS encontrada

aqui pode ser justificada pela diminuição do potencial de membrana (Ferranti *et al.*, 2003), pois uma vez a membrana estando exaurida, a mesma fica incapaz de gerar novas espécies reativas. Enquanto que a fragmentação do DNA Plasmidial, observada no Capítulo 3, pode ser corroborada com os estudos de Kamiya *et al.,* (2012b) que também relataram a fragmentação do DNA da linhagem MCF-7 de câncer de mama, tratada com extrato hidroalcoólico de própolis vermelha.

A análise proteômica realizada utilizando as concentrações (IC50) de extrato hidroalcoólico de própolis vermelha em células tumorais Hep-2 e na linhagem Hek-293, não tumoral, pelo período de 1 hora, indicou perfis proteicos diferentes quando comparado ao controle que recebeu a mesma quantidade de solução hidroalcoólica (Capítulo 2). Tomadas em conjunto, as células tumorais e não tumorais tratadas com própolis vermelha apresentaram proteínas diferencialmente expressas relacionadas com uma série de vias metabólicas. Algumas proteínas identificadas (TLN1, EPIPL, FSCN1, VINC e ROA1) apresentam-se em grande quantidade nos tecidos dos tumores de laringe comparado ao fenótipo dos tecidos sem tumor, as quais apresentaram expressão diminuída após tratamento com extratos da própolis (Li *et al.*, 2014). Ainda mais importante, a grande maioria das proteínas que sofreram regulação foram relatadas em literatura recentemente e participam, direta ou indiretamente, dos mecanismos de sinalização celular do câncer (Ali *et al.*, 2014; Sonoda *et al.*, 2010; Tan *et al.*, 2013). Assim, os resultados forneceram uma visão abrangente para a compreensão da expressão proteica após exposição à própolis vermelha, principalmente nas células Hep-2. Esses resultados podem ser facilmente comparados e complementados com outros estudos proteômicos, beneficiando uma futura aplicação clínica e o desenvolvimento de medicamentos contra o câncer.

Os resultados da proteômica comparativa mostram que a própolis interage com um conjunto de eventos intracelulares e, assim, passa a ser uma candidata promissora para inibir o crescimento celular e contribuir para os diferentes passos relacionados com o processo de carcinogênese. Os efeitos sobre algumas proteínas aqui identificadas sugerem outras aplicações como alvos moleculares para intervenção terapêutica em vários tipos tumorais como é o caso da proteína TLN1, a qual está relacionada ao câncer de próstata (Sakamoto *et al.*, 2010), carcinoma hepatocelular (Zhang *et al.*, 2011) e ao carcinoma de células escamosas orais (Lai *et al.*, 2011).

Ensaios baseados em estudos proteômicos são particularmente valiosos na busca de agentes terapêuticos, não somente ao selecionar atividade contra um alvo molecular particular, mas também para outras propriedades desejáveis, tais como as interações proteína-proteína e para permear as células e reter atividade biológica. Embora os mecanismos moleculares pelos quais a própolis vermelha interaja com o metabolismo das células permanecem ainda desconhecidos, estudos adicionais, incluindo testes citogenéticos e moleculares servirão para melhor elucidar a atividade antitumoral aqui observada.

5. CONCLUSÕES

Os dados obtidos neste estudo permitem concluir que:

- Os extratos hidroalcoólicos de própolis vermelha, de Sergipe e de Alagoas, possuem em suas constituições principalmente flavonoides que conferem importantes propriedades biológicas;
- As concentrações de extrato hidroalcoólico de própolis vermelha e as frações utilizadas foram capazes de inibir o crescimento de células tumorais;
- A coloração com DAPI permitiu a visualização de corpos apoptóticos e condensação da cromatina na linhagem Hep-2 tratada com própolis vermelha;
- Tratamentos com própolis vermelha diminuíram a produção de ROS e o potencial de membrana mitocondrial das células Hep-2;
- Tratamentos com as própolis vermelhas, provenientes de Sergipe e de Alagoas, resultaram em aumento de apoptose tardia em células Hep-2;
- O DNA plasmidial apresentou danos após exposição à própolis vermelha;
- A linhagem Hep-2 apresentou perfil proteico distinto na presença ou ausência de extratos da própolis vermelha comparada a linhagem não tumoral;
- Dentre as proteínas identificadas na linhagem Hep-2, após exposição à própolis vermelha, algumas apresentaram expressão diferencial em relação ao grupo controle, as quais estão relacionadas ao processo de carcinogênese.

6. PERSPECTIVAS FUTURAS

Como continuidade deste trabalho seria importante:

- Avaliar e validar a expressão de proteínas diferencialmente expressas na linhagem Hep-2 após tratamento com própolis vermelha através de imunofluorescência indireta e Western blot;
- Avaliar as caspases envolvidas no processo de apoptose nas células Hep-2 tratadas com própolis vermelha;
- Realizar estudos proteômicos a partir de linhagens tratadas com as frações de própolis;
- Realizar isolamento de compostos a partir das frações;
- Testar os compostos obtidos, a partir das frações, em linhagens tumorais *in vitro* em conjunto e isoladamente;
- Realizar ensaios *in vivo* com os compostos mais ativos das frações de própolis vermelha em células tumorais.

7. REFERÊNCIAS BIBLIOGRÁFICAS

Ahmad, A.; Biersack, B.; Li, Y.; Bao, B.; Kong, D.; Ali, S.; Banerjee, S.; Sarkar, F.H., (2013). Perspectives on the role of isoflavones in prostate cancer. **AAPS J** 15, 991-1000.

Alencar, S.M.; Oldoni, T.L.; Castro, M.L.; Cabral, I.S.; Costa-Neto, C.M.; Cury, J.A.; Rosalen, P.L.; Ikegaki, M., (2007). Chemical composition and biological activity of a new type of Brazilian propolis: red propolis. **J Ethnopharmacol** 113, 278-283.

Ali, K.; Mahjabeen, I.; Sabir, M.; Baig, R.M.; Zafeer, M.; Faheem, M.; Kayani, M.A., (2014). Germline variations of apurinic/apyrimidinic endonuclease 1 (APEX1) detected in female breast cancer patients. **Asian Pac J Cancer Prev** 15, 7589-7595.

Archana, M.; Bastian; Yogesh, T.L.; Kumaraswamy, K.L., (2013). Various methods available for detection of apoptotic cells--a review. **Indian J Cancer** 50, 274-283.

Awale, S.; Li, F.; Onozuka, H.; Esumi, H.; Tezuka, Y.; Kadota, S., (2008). Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition. **Bioorg Med Chem** 16, 181-189.

Ayres, D.C.; Marcucci, M.C.; Giorgio, S., (2007). Effects of Brazilian propolis on Leishmania amazonensis. **Mem Inst Oswaldo Cruz** 102, 215-220.

Bankova, V., (2009). Chemical diversity of propolis makes it a valuable source of new biologically active compounds. . **Journal of ApiProduct and ApiMedical Science** 1, 23 – 28.

Barbosa, M.H.; Zuffi, F.B.; Maruxo, H.B.; Jorge, L.L.R., (2009). Ação terapêutica da própolis em lesões cutâneas. **Acta Paul Enferm.** 22, 318-322.

Begnini, K.R.; Moura de Leon, P.M.; Thurow, H.; Schultze, E.; Campos, V.F.; Martins Rodrigues, F.; Borsuk, S.; Dellagostin, O.A.; Savegnago, L.; Roesch-Ely, M.; Moura, S.; Padilha, F.F.; Collares, T.; Pegas Henriques, J.A.; Seixas, F.K., (2014). Brazilian red propolis induces apoptosis-like cell death and decreases migration potential in bladder cancer cells. **Evid Based Complement Alternat Med** 2014, 639856.

Bhardwaj, V.; Tadinada, S.M.; Jain, A.; Sehdev, V.; Daniels, C.K.; Lai, J.C.; Bhushan, A., (2014). Biochanin A reduces pancreatic cancer survival and progression. **Anticancer Drugs** 25, 296-302.

Bianchi, L.; Bruzzese, F.; Leone, A.; Gagliardi, A.; Puglia, M.; Di Gennaro, E.; Rocco, M.; Gimigliano, A.; Pucci, B.; Armini, A.; Bini, L.; Budillon, A., (2011).

Proteomic analysis identifies differentially expressed proteins after HDAC vorinostat and EGFR inhibitor gefitinib treatments in Hep-2 cancer cells. **Proteomics** 11, 3725-3742.

Biscaro, F.; Parisotto, E.B.; Zanette, V.C.; Gunther, T.M.; Ferreira, E.A.; Gris, E.F.; Correia, J.F.; Pich, C.T.; Mattivi, F.; Filho, D.W.; Pedrosa, R.C., (2013). Anticancer activity of flavonol and flavan-3-ol rich extracts from Croton celtidifolius latex. **Pharm Biol** 51, 737-743.

Bueno-Silva, B.; Alencar, S.M.; Koo, H.; Ikegaki, M.; Silva, G.V.; Napimoga, M.H.; Rosalen, P.L., (2013). Anti-inflammatory and antimicrobial evaluation of neovestitol and vestitol isolated from Brazilian red propolis. **J Agric Food Chem** 61, 4546-4550.

Bufalo, M.C.; Candeias, J.M.; Sforcin, J.M., (2009). In vitro cytotoxic effect of Brazilian green propolis on human laryngeal epidermoid carcinoma (HEp-2) cells. **Evid Based Complement Alternat Med** 6, 483-487.

Burdock, G.A., (1998). Review of the biological properties and toxicity of bee propolis (propolis). **Food Chem Toxicol** 36, 347-363.

Cabral, I.S.R.O., Tatiane Luiza Cadorin; Prado, Adna; Bezerra, Rosângela Maria Neves; Alencar, Severino Matias de, (2009). Composição fenólica, atividade antibacteriana e antioxidante da própolis vermelha brasileira **Química Nova** 32, 1523-1527.

Cavendish, R.L.; de Souza Santos, J.; Belo Neto, R.; Oliveira Paixao, A.; Valeria Oliveira, J.; Divino de Araujo, E.; Berretta, E.S.A.A.; Maria Thomazzi, S.; Cordeiro Cardoso, J.; Zanardo Gomes, M., (2015). Antinociceptive and anti-inflammatory effects of Brazilian red propolis extract and formononetin in rodents. **J Ethnopharmacol** 173, 127-133.

Daleprane, J.B.; Abdalla, D.S., (2013). Emerging roles of propolis: antioxidant, cardioprotective, and antiangiogenic actions. **Evid Based Complement Alternat Med** 2013, 175135.

Daugsch, A.; Moraes, C.S.; Fort, P.; Park, Y.K., (2007). Brazilian red propolis--chemical composition and botanical origin. **Evid Based Complement Alternat Med** 5, 435-441.

Daugsch, A.; Moraes, C.S.; Fort, P.; Park, Y.K., (2008). Brazilian red propolis--chemical composition and botanical origin. **Evid Based Complement Alternat Med** 5, 435-441.

de Mendonca, I.C.; Porto, I.C.; do Nascimento, T.G.; de Souza, N.S.; Oliveira, J.M.; Arruda, R.E.; Mousinho, K.C.; dos Santos, A.F.; Basilio-Junior, I.D.; Parolia, A.; Barreto, F.S., (2015). Brazilian red propolis: phytochemical screening, antioxidant activity and effect against cancer cells. **BMC Complement Altern Med** 15, 357.

Dunn, B., (2012). Cancer: Solving an age-old problem. **Nature** 483, S2-6.

Favaloro, B.; Allocati, N.; Graziano, V.; Di Ilio, C.; De Laurenzi, V., (2012). Role of apoptosis in disease. **Aging (Albany NY)** 4, 330-349.

Ferranti, R.; da Silva, M.M.; Kowaltowski, A.J., (2003). Mitochondrial ATP-sensitive K+ channel opening decreases reactive oxygen species generation. **FEBS Lett** 536, 51-55.

Filardi, M.A., 2010. Potencial antitumoral de extratos da própolis Brasileira e de folhas de graviola (Annona muricata): efeito citotóxico sobre células hepatocarcinogênicas HEPG2. . Universidade Federal de Viçosa. Viçosa, Brasil.

Franchi, G.C., Jr.; Moraes, C.S.; Toreti, V.C.; Daugsch, A.; Nowill, A.E.; Park, Y.K., (2012). Comparison of effects of the ethanolic extracts of brazilian propolis on human leukemic cells as assessed with the MTT assay. **Evid Based Complement Alternat Med** 2012, 918956.

Freires, I.A.; de Alencar, S.M.; Rosalen, P.L., (2016). A pharmacological perspective on the use of Brazilian Red Propolis and its isolated compounds against human diseases. **Eur J Med Chem** 110, 267-279.

Frozza, C.O.; Garcia, C.S.; Gambato, G.; de Souza, M.D.; Salvador, M.; Moura, S.; Padilha, F.F.; Seixas, F.K.; Collares, T.; Borsuk, S.; Dellagostin, O.A.; Henriques, J.A.; Roesch-Ely, M., (2013). Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. **Food Chem Toxicol** 52, 137-142.

Frozza, C.O.; Ribeiro Tda, S.; Gambato, G.; Menti, C.; Moura, S.; Pinto, P.M.; Staats, C.C.; Padilha, F.F.; Begnini, K.R.; de Leon, P.M.; Borsuk, S.; Savegnago, L.; Dellagostin, O.; Collares, T.; Seixas, F.K.; Henriques, J.A.; Roesch-Ely, M., (2014). Proteomic analysis identifies differentially expressed proteins after red propolis treatment in Hep-2 cells. **Food Chem Toxicol** 63, 195-204.

Frozza, C.O.d.S.; Brum, E.d.S.; Alving, A.; Moura, S.; Henriques, J.A.; Roesch-Ely, M., (2016). LC-MS analysis of Hep-2 and Hek-293 cell lines treated with Brazilian red propolis reveals differences in protein expression. **J Pharm Pharmacol** 68, 1073-1084.

Galluzzi, L.; Vitale, I.; Abrams, J.M.; Alnemri, E.S.; Baehrecke, E.H.; Blagosklonny, M.V.; Dawson, T.M.; Dawson, V.L.; El-Deiry, W.S.; Fulda, S.; Gottlieb, E.; Green, D.R.; Hengartner, M.O.; Kepp, O.; Knight, R.A.; Kumar, S.; Lipton, S.A.; Lu, X.; Madeo, F.; Malorni, W.; Mehlen, P.; Nunez, G.; Peter, M.E.; Piacentini, M.; Rubinsztein, D.C.; Shi, Y.; Simon, H.U.; Vandenabeele, P.; White, E.; Yuan, J.; Zhivotovsky, B.; Melino, G.; Kroemer, G., (2012). Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. **Cell Death Differ** 19, 107-120.

Hassan, M.; Watari, H.; AbuAlmaaty, A.; Ohba, Y.; Sakuragi, N., (2014). Apoptosis and molecular targeting therapy in cancer. **Biomed Res Int** 2014, 150845.

Hayacibara, M.F.; Koo, H.; Rosalen, P.L.; Duarte, S.; Franco, E.M.; Bowen, W.H.; Ikegaki, M.; Cury, J.A., (2005). In vitro and in vivo effects of isolated fractions of Brazilian propolis on caries development. **J Ethnopharmacol** 101, 110-115.

Hu, W.; Xiao, Z., (2015). Formononetin induces apoptosis of human osteosarcoma cell line U2OS by regulating the expression of Bcl-2, Bax and MiR-375 in vitro and in vivo. **Cell Physiol Biochem** 37, 933-939.

Huang, Z.; Ma, L.; Huang, C.; Li, Q.; Nice, E.C., (2016). Proteomic profiling of human plasma for cancer biomarker discovery. **Proteomics**.

INCA/MS, 2016. Instituto Nacional de Câncer. Ministério da Saúde. Disponível (on line): http://www.inca.gov.br/estimativa/2016/index.asp?ID=2. (17 de outubro).

Inokuchi, Y.; Shimazawa, M.; Nakajima, Y.; Suemori, S.; Mishima, S.; Hara, H., (2006). Brazilian green propolis protects against retinal damage in vitro and in vivo. **Evid Based Complement Alternat Med** 3, 71-77.

INPI, 2016. Instituto Nacional de Propriedade Industrial. Disponível (on line): http://www.inpi.gov.br/menu-servicos/indicacao-geografica/arquivos/lista_com_as_indicacoes_geograficas_concedidas_-_10-03-2015.pdf/view (17 de outubro).

Jacob, A.; Parolia, A.; Pau, A.; Davamani Amalraj, F., (2015). The effects of Malaysian propolis and Brazilian red propolis on connective tissue fibroblasts in the wound healing process. **BMC Complement Altern Med** 15, 294.

Jain, A.; Lai, J.C.; Bhushan, A., (2015). Biochanin A inhibits endothelial cell functions and proangiogenic pathways: implications in glioma therapy. **Anticancer Drugs** 26, 323-330.

Johnson, T.L.; Lai, M.B.; Lai, J.C.; Bhushan, A., (2010). Inhibition of Cell Proliferation and MAP Kinase and Akt Pathways in Oral Squamous cell Carcinoma by Genistein and Biochanin A. **Evid Based Complement Alternat Med** 7, 351-358.

Kamiya, T.; Izumi, M.; Hara, H.; Adachi, T., (2012a). Propolis suppresses CdCl(2)-induced cytotoxicity of COS7 cells through the prevention of intracellular reactive oxygen species accumulation. **Biol Pharm Bull** 35, 1126-1131.

Kamiya, T.; Nishihara, H.; Hara, H.; Adachi, T., (2012b). Ethanol extract of Brazilian red propolis induces apoptosis in human breast cancer MCF-7 cells through endoplasmic reticulum stress. **J Agric Food Chem** 60, 11065-11070.

Khalid, E.B.; Ayman, E.E.; Rahman, H.; Abdelkarim, G.; Najda, A., (2016). Natural products against cancer angiogenesis. **Tumour Biol**.

Khalil, M.L., (2006). Biological activity of bee propolis in health and disease. **Asian Pac J Cancer Prev** 7, 22-31.

Kim, H.K.; Wilson, E.G.; Choi, Y.H.; Verpoorte, R., (2010). Metabolomics: a tool for anticancer lead-finding from natural products. **Planta Med** 76, 1094-1102.

Kroemer, G.; Galluzzi, L.; Vandenabeele, P.; Abrams, J.; Alnemri, E.S.; Baehrecke, E.H.; Blagosklonny, M.V.; El-Deiry, W.S.; Golstein, P.; Green, D.R.; Hengartner, M.; Knight, R.A.; Kumar, S.; Lipton, S.A.; Malorni, W.; Nunez, G.; Peter, M.E.; Tschopp, J.; Yuan, J.; Piacentini, M.; Zhivotovsky, B.; Melino, G.; Nomenclature Committee on Cell, D., (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. **Cell Death Differ** 16, 3-11.

Kuropatnicki, A.K.; Szliszka, E.; Krol, W., (2013). Historical aspects of propolis research in modern times. **Evid Based Complement Alternat Med** 2013, 964149.

Lai, M.T.; Hua, C.H.; Tsai, M.H.; Wan, L.; Lin, Y.J.; Chen, C.M.; Chiu, I.W.; Chan, C.; Tsai, F.J.; Jinn-Chyuan Sheu, J., (2011). Talin-1 overexpression defines high risk for aggressive oral squamous cell carcinoma and promotes cancer metastasis. **J Pathol** 224, 367-376.

Li, F.; Awale, S.; Tezuka, Y.; Kadota, S., (2008). Cytotoxic constituents from Brazilian red propolis and their structure-activity relationship. **Bioorg Med Chem** 16, 5434-5440.

Li, L.; Zhang, Z.; Wang, C.; Miao, L.; Zhang, J.; Wang, J.; Jiao, B.; Zhao, S., (2014). Quantitative proteomics approach to screening of potential diagnostic and therapeutic targets for laryngeal carcinoma. **PLoS One** 9, e90181.

Liu, C.; Wang, Y.; Xie, S.; Zhou, Y.; Ren, X.; Li, X.; Cai, Y., (2011). Liquiritigenin induces mitochondria-mediated apoptosis via cytochrome c release and caspases activation in HeLa Cells. **Phytother Res** 25, 277-283.

Liu, Y.; Xie, S.; Wang, Y.; Luo, K.; Wang, Y.; Cai, Y., (2012). Liquiritigenin inhibits tumor growth and vascularization in a mouse model of HeLa cells. **Molecules** 17, 7206-7216.

Lopez, B.G.; de Lourenco, C.C.; Alves, D.A.; Machado, D.; Lancellotti, M.; Sawaya, A.C., (2015). Antimicrobial and cytotoxic activity of red propolis: an alert for its safe use. **J Appl Microbiol** 119, 677-687.

Lopez, B.G.; Schmidt, E.M.; Eberlin, M.N.; Sawaya, A.C., (2014). Phytochemical markers of different types of red propolis. **Food Chem** 146, 174-180.

Lopez, J.; Tait, S.W., (2015). Mitochondrial apoptosis: killing cancer using the enemy within. **Br J Cancer** 112, 957-962.

Lustosa, S.R.; Galindo, A.B.; Nunes, L.C.C.; Randau, K.P.; Neto, P.J.R., (2008). Própolis: atualizações sobre a química e a farmacologia. **Braz. J. Pharmacog.** 18, 447-454.

Machado, C.S.; Mokochinski, J.B.; de Lira, T.O.; de Oliveira Fde, C.; Cardoso, M.V.; Ferreira, R.G.; Sawaya, A.C.; Ferreira, A.G.; Pessoa, C.; Cuesta-Rubio, O.; Monteiro, M.C.; de Campos, M.S.; Torres, Y.R., (2016). Comparative Study of Chemical Composition and Biological Activity of Yellow, Green, Brown, and Red Brazilian Propolis. **Evid Based Complement Alternat Med** 2016, 6057650.

Mallick, P.; Kuster, B., (2010). Proteomics: a pragmatic perspective. **Nat Biotechnol** 28, 695-709.

Misra, A.; Rai, S.; Misra, D., (2016). Functional role of apoptosis in oral diseases: An update. **J Oral Maxillofac Pathol** 20, 491-496.

Mojzisova, G.; Kello, M.; Pilatova, M.; Tomeckova, V.; Vaskova, J.; Vasko, L.; Bernatova, S.; Mirossay, L.; Mojzis, J., (2016). Antiproliferative effect of beta-escin - an in vitro study. **Acta Biochim Pol** 63, 1013.

Moon, Y.J.; Brazeau, D.A.; Morris, M.E., (2007). Effects of flavonoids genistein and biochanin a on gene expression and their metabolism in human mammary cells. **Nutr Cancer** 57, 48-58.

Moraes, C.S.; Daugsch, A.; Li, H.; Rhim, J.S.; Park, Y.K., (2010). Comparative antiproliferation of human prostate cancer cells by ethanolic extracts of two groups of Brazilian propolis. **Ciênc. Tecnol. Aliment.** 30, 539-543.

Mosmann, T., (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J Immunol Methods** 65, 55-63.

Najafi, M.F.; Vahedy, F.; Seyyedin, M.; Jomehzadeh, H.R.; Bozary, K., (2007). Effect of the water extracts of propolis on stimulation and inhibition of different cells. **Cytotechnology** 54, 49-56.

Neves, M.V.d.; da Silva, T.M.; Lima Ede, O.; da Cunha, E.V.; Oliveira Ede, J., (2016). Isoflavone formononetin from red propolis acts as a fungicide against Candida sp. **Braz J Microbiol** 47, 159-166.

Newman, D.J.; Cragg, G.M., (2016). Natural Products as Sources of New Drugs from 1981 to 2014. **J Nat Prod** 79, 629-661.

Nobili, S.; Lippi, D.; Witort, E.; Donnini, M.; Bausi, L.; Mini, E.; Capaccioli, S., (2009). Natural compounds for cancer treatment and prevention. **Pharmacol Res** 59, 365-378.

Nocentini, S.; Moreno, G.; Petit, P.X.; Guggiari, M.; Salet, C.; Demerseman, P.; Dodin, G., (1997). Induction of mitochondrial dysfunction and apoptosis in HeLa cells by bis-pyridinium oximes, a newly synthesized family of lipophilic biscations. **Biochem Pharmacol** 53, 1543-1552.

Novak, E.M.; Costa Silva, M.S.e.; Marcucci, M.C.; Sawaya, A.C.H.F.; López, B.G.; Fortes, M.A.H.Z.; Giorgi, R.R.; Marumo, K.T.; Rodrigues, R.F.; Maria, D.A., (2014). Antitumoural activity of Brazilian red propolis fraction enriched with xanthochymol and formononetin: an in vitro and in vivo study. **J Funct Foods** 2, 91-102.

Ohta, T.; Kunimasa, K.; Kobayashi, T.; Sakamoto, M.; Kaji, K., (2008). Propolis suppresses tumor angiogenesis by inducing apoptosis in tube-forming endothelial cells. **Biosci Biotechnol Biochem** 72, 2436-2440.

OMS, 2016. Organização Mundial da Saúde (2016). Em inglês World Health Organization. **Disponível (on line)**: http://www.who.int/mediacentre/factsheets/fs297/en/ (19 de outubro).

Oršolić, N., (2010). A review of propolis antitumour action in vivo and in vitro. **JAAS** 2, 1-20.

Patel, S.; Goyal, A., (2012). Recent developments in mushrooms as anti-cancer therapeutics: a review. **3 Biotech** 2, 1-15.

Piccinelli, A.L.; Lotti, C.; Campone, L.; Cuesta-Rubio, O.; Campo Fernandez, M.; Rastrelli, L., (2011). Cuban and Brazilian red propolis: botanical origin and comparative analysis by high-performance liquid chromatography-photodiode array detection/electrospray ionization tandem mass spectrometry. **J Agric Food Chem** 59, 6484-6491.

Pietta, P.G.; Gardana, C.; Pietta, A.M., (2002). Analytical methods for quality control of propolis. **Fitoterapia** 73 Suppl 1, S7-20.

Pinheiro, K.S.; Ribeiro, D.R.; Alves, A.V.; Pereira-Filho, R.N.; Oliveira, C.R.; Lima, S.O.; Reis, F.P.; Cardoso, J.C.; Albuquerque-Junior, R.L., (2014). Modulatory activity of Brazilian red propolis on chemically induced dermal carcinogenesis. **Acta Cir Bras** 29, 111-117.

Pinheiro, M.S., 2009. Avaliação da atividade antimicrobiana e citoprotetora gástrica dos extratos de mangaba, caju e própolis vermelha. **Dissertação de mestrado.** Universidade Tiradentes, Aracaju, Brasil.

Pippi, B.; Lana, A.J.; Moraes, R.C.; Guez, C.M.; Machado, M.; de Oliveira, L.F.; Lino von Poser, G.; Fuentefria, A.M., (2015). In vitro evaluation of the acquisition of resistance, antifungal activity and synergism of Brazilian red propolis with antifungal drugs on Candida spp. **J Appl Microbiol**. doi: 10.1111/jam.12746.

Ribeiro, D.R.; Alves, A.V.; dos Santos, E.P.; Padilha, F.F.; Gomes, M.Z.; Rabelo, A.S.; Cardoso, J.C.; Massarioli, A.P.; de Alencar, S.M.; de Albuquerque-Junior, R.L., (2015). Inhibition of DMBA-induced Oral Squamous Cells Carcinoma Growth by Brazilian Red Propolis in Rodent Model. **Basic Clin Pharmacol Toxicol** 117, 85-95.

Righi, A.A.; Alves, T.R.; Negri, G.; Marques, L.M.; Breyer, H.; Salatino, A., (2011). Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. **J Sci Food Agric** 91, 2363-2370.

Sakamoto, S.; McCann, R.O.; Dhir, R.; Kyprianou, N., (2010). Talin1 promotes tumor invasion and metastasis via focal adhesion signaling and anoikis resistance. **Cancer Res** 70, 1885-1895.

Salatino, A.; Fernandes-Silva, C.C.; Righi, A.A.; Salatino, M.L., (2011). Propolis research and the chemistry of plant products. **Nat Prod Rep** 28, 925-936.

Sareddy, G.R.; Vadlamudi, R.K., (2015). Cancer therapy using natural ligands that target estrogen receptor beta. **Chin J Nat Med** 13, 801-807.

Sawicka, D.; Car, H.; Borawska, M.H.; Niklinski, J., (2012). The anticancer activity of propolis. **Folia Histochem Cytobiol** 50, 25-37.

Sehdev, V.; Lai, J.C.; Bhushan, A., (2009). Biochanin A Modulates Cell Viability, Invasion, and Growth Promoting Signaling Pathways in HER-2-Positive Breast Cancer Cells. **J Oncol** 2009, 121458.

Sforcin, J.M., (2016). Biological Properties and Therapeutic Applications of Propolis. **Phytother Res** 30, 894-905.

Shan, F.; Shao, Z.; Jiang, S.; Cheng, Z., (2016). Erlotinib induces the human non-small-cell lung cancer cells apoptosis via activating ROS-dependent JNK pathways. **Cancer Med** 5, 3166-3175.

Siegel, R.L.; Miller, K.D.; Jemal, A., (2016). Cancer statistics, 2016. **CA Cancer J Clin** 66, 7-30.

Silva-Carvalho, R.; Baltazar, F.; Almeida-Aguiar, C., (2015). Propolis: A Complex Natural Product with a Plethora of Biological Activities That Can Be Explored for Drug Development. **Evid Based Complement Alternat Med** 2015, 206439.

Silva, B.B.; Rosalen, P.L.; Cury, J.A.; Ikegaki, M.; Souza, V.C.; Esteves, A.; Alencar, S.M., (2008). Chemical composition and botanical origin of red propolis, a new type of brazilian propolis. **Evid Based Complement Alternat Med** 5, 313-316.

Silva, M.S.C., (2007). Estudo do efeito da fração BRVD obtida a partir da própolis brasileira tipificada, na proliferação de células tumorais. **Dissertação de mestrado.** Universidade de São Paulo. São Paulo, Brasil.

Singh, M.; Arseneault, M.; Sanderson, T.; Murthy, V.; Ramassamy, C., (2008). Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. **J Agric Food Chem** 56, 4855-4873.

Siqueira, A.B.; Gomes, B.S.; Cambuim, I.; Maia, R.; Abreu, S.; Souza-Motta, C.M.; de Queiroz, L.A.; Porto, A.L., (2009). Trichophyton species susceptibility to green and red propolis from Brazil. **Lett Appl Microbiol** 48, 90-96.

Sonoda, Y.; Warita, M.; Suzuki, T.; Ozawa, H.; Fukuda, Y.; Funakoshi-Tago, M.; Kasahara, T., (2010). Proteolipid protein 2 is associated with melanoma metastasis. **Oncol Rep** 23, 371-376.

Souza, P.Z., 2010. Dinâmica espaço-temporal de Dalbergiaecastophyllum (L.) Taub. em restinga no sul do Brasil., Florianópolis, Brasil. 2010. **Dissertação de mestrado.** Universidade Federal de Santa Catarina, Florianópolis, Brasil.

Sun, T.; Wang, J.; Huang, L.H.; Cao, Y.X., (2013). Antihypertensive effect of formononetin through regulating the expressions of eNOS, 5-HT2A/1B receptors and alpha1-adrenoceptors in spontaneously rat arteries. **Eur J Pharmacol** 699, 241-249.

Szliszka, E.; Czuba, Z.P.; Domino, M.; Mazur, B.; Zydowicz, G.; Krol, W., (2009). Ethanolic extract of propolis (EEP) enhances the apoptosis- inducing potential of TRAIL in cancer cells. **Molecules** 14, 738-754.

Tan, V.Y.; Lewis, S.J.; Adams, J.C.; Martin, R.M., (2013). Association of fascin-1 with mortality, disease progression and metastasis in carcinomas: a systematic review and meta-analysis. **BMC Med** 11, 52.

Teixeira, E.W.; Negri, G.; Meira, R.M.; Message, D.; Salatino, A., (2005). Plant Origin of Green Propolis: Bee Behavior, Plant Anatomy and Chemistry. **Evid Based Complement Alternat Med** 2, 85-92.

Tinari, A.; Giammarioli, A.M.; Manganelli, V.; Ciarlo, L.; Malorni, W., (2008). Analyzing morphological and ultrastructural features in cell death. **Methods Enzymol** 442, 1-26.

Toreti, V.C.; Sato, H.H.; Pastore, G.M.; Park, Y.K., (2013). Recent progress of propolis for its biological and chemical compositions and its botanical origin. **Evid Based Complement Alternat Med** 2013, 697390.

Trusheva, B.; Popova, M.; Bankova, V.; Simova, S.; Marcucci, M.C.; Miorin, P.L.; da Rocha Pasin, F.; Tsvetkova, I., (2006). Bioactive constituents of brazilian red propolis. **Evid Based Complement Alternat Med** 3, 249-254.

Tsao, R., (2010). Chemistry and biochemistry of dietary polyphenols. **Nutrients** 2, 1231-1246.

Vural, F.; Cebesoy, S.; Karakas, M., (2013). Classification of Cell Death. **Journal of Entomology and Zoology Studies** 1, 120-126.

Walsh, G.M.; Rogalski, J.C.; Klockenbusch, C.; Kast, J., (2010). Mass spectrometry-based proteomics in biomedical research: emerging technologies and future strategies. **Expert Rev Mol Med** 12, e30.

Wang, D.; Lu, J.; Liu, Y.; Meng, Q.; Xie, J.; Wang, Z.; Teng, L., (2014). Liquiritigenin induces tumor cell death through mitogen-activated protein kinase- (MPAKs-) mediated pathway in hepatocellular carcinoma cells. **Biomed Res Int** 2014, 965316 ID, 1-11.

Watanabe, M.A.; Amarante, M.K.; Conti, B.J.; Sforcin, J.M., (2011). Cytotoxic constituents of propolis inducing anticancer effects: a review. **J Pharm Pharmacol** 63, 1378-1386.

Wlodkowic, D.; Skommer, J.; Darzynkiewicz, Z., (2009). Flow cytometry-based apoptosis detection. **Methods Mol Biol** 559, 19-32.

Yang, Y.; Zhao, Y.; Ai, X.; Cheng, B.; Lu, S., (2014). Formononetin suppresses the proliferation of human non-small cell lung cancer through induction of cell cycle arrest and apoptosis. **Int J Clin Exp Pathol** 7, 8453-8461.

Zhang, J.L.; Qian, Y.B.; Zhu, L.X.; Xiong, Q.R., (2011). Talin1, a valuable marker for diagnosis and prognostic assessment of human hepatocelluar carcinomas. **Asian Pac J Cancer Prev** 12, 3265-3269.

Zhang, S.P.; Zhou, Y.J.; Liu, Y.; Cai, Y.Q., (2009). Effect of liquiritigenin, a flavanone existed from Radix glycyrrhizae on pro-apoptotic in SMMC-7721 cells. **Food Chem Toxicol** 47, 693-701.

Zhang, X.; Bi, L.; Ye, Y.; Chen, J., (2014). Formononetin induces apoptosis in PC-3 prostate cancer cells through enhancing the Bax/Bcl-2 ratios and regulating the p38/Akt pathway. **Nutr Cancer** 66, 656-661.

Zhou, M.; Higo, H.; Cai, Y., (2010). Inhibition of hepatoma 22 tumor by Liquiritigenin. **Phytother Res** 24, 827-833.

Zhou, R.; Xu, L.; Ye, M.; Liao, M.; Du, H.; Chen, H., (2014). Formononetin inhibits migration and invasion of MDA-MB-231 and 4T1 breast cancer cells by suppressing MMP-2 and MMP-9 through PI3K/AKT signaling pathways. **Horm Metab Res** 46, 753-760.

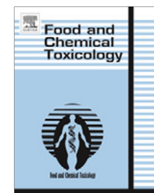
8. ANEXO

Food and Chemical Toxicology 52 (2013) 137–142

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox



Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis

Caroline Olivieri da Silva Frozza [a], Charlene Silvestrin Celi Garcia [a], Gabriela Gambato [a], Marcia Denize Oliveira de Souza [b], Mirian Salvador [b], Sidnei Moura [c], Francine Ferreira Padilha [d], Fabiana Kömmling Seixas [e], Tiago Collares [e], Sibele Borsuk [e], Odir Antonio Dellagostin [e], João Antonio Pêgas Henriques [a], Mariana Roesch-Ely [a,*]

[a] Laboratory of Genomics, Proteomics and DNA Repair, University of Caxias do Sul, RS, Brazil
[b] Laboratory of Oxidative Stress and Antioxidants, University of Caxias do Sul, RS, Brazil
[c] Laboratory of Essential Oils, University of Caxias do Sul, RS, Brazil
[d] Program on Health and Environment, Tiradentes University, Aracaju, SE, Brazil
[e] Biotechnology Unit, Center for Technology Development, Federal University of Pelotas, Pelotas, RS, Brazil

ARTICLE INFO

Article history:
Received 24 August 2012
Accepted 8 November 2012
Available online 19 November 2012

Keywords:
Propolis
Chemical characterization
Antioxidants
Cell line

ABSTRACT

Propolis is known for a long time for its health benefits and biological activities. Here, the red variety from the northeast of Brazil was chemically analyzed and extracts were investigated regarding their antioxidant and antitumor activity. Hydroalcoholic extracts, obtained from the red propolis, revealed polyphenol content, 2,2-diphenyl-1-picrylhydrazyl scavenging potential and enzymatic activities for catalase-like and superoxide dismutase-like. Cytotoxic activity was evaluated for human laryngeal epidermoid carcinoma cell (Hep-2), human cervical adenocarcinoma (HeLa) and human normal epithelial embryonic kidney (Hek-293). Survival analysis for non-tumor cell line showed greater IC50 compared to tumor cell lines, suggesting an increased sensitivity that may correlate with the higher proliferative index of the tumor vs. normal cells. Our results indicate that the Brazilian red propolis is capable of inhibiting cancer cell growth and constitutes an excellent source of antioxidant and antitumor natural agent.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Propolis is a natural resinous product of honey bees that has been used for centuries in traditional medicine all over the world (Burdock, 1998; Castaldo and Capasso, 2002; Daugsch et al., 2008). More than 300 compounds have been identified so far from different propolis samples. The composition of propolis varies according to its botanical origin; generally it contains resin and vegetable balsams, wax, essential and aromatic oils, pollens, and others (Lustosa et al., 2008; Sforcin, 2007).

In the last 30 years, different sort of propolis became subject of intense pharmacological and chemical studies to improve health and prevent illnesses (Li et al., 2008; Lustosa et al., 2008; Sforcin, 2007). Brazilian propolis was classified into 12 groups based on their physicochemical characteristics (Park, 2000). The propolis type most commercialized is known as “green propolis” and it has been extensively studied and used in foods and beverages (Moraes et al., 2010). However 13th type named red propolis has been an important source of investigation since 2007 by local and international research groups. Beside differences in chemical composition, Franchi et al. (2012) reported that red propolis is more cytotoxic than the green sort in cell lines of leukemia.

The red propolis variety is obtained in the northeast of Brazil. This unique propolis composition has not been found elsewhere out from this country. Its botanical origin is *Dalbergia ecastophyllum* (L) Taud. (Leguminosae), responsible for the red color of the propolis (Daugsch et al., 2008; Franchi et al., 2012; Piccinelli et al., 2011; Silva et al., 2008; Silva, 2007), with the presence of two pigments: retusapurpurin A and B (Piccinelli et al., 2011).

Red propolis has been shown to present several biological activities such as: antibacterial (Cabral et al., 2009; Righi et al., 2011), antifungal (Siqueira et al., 2009), anti-inflammatory (Barreto, 2008), antiulcerative (Pinheiro, 2009), antioxidant (Cabral et al., 2009; Righi et al., 2011), antitumor (Alencar et al., 2007; Li et al., 2008), among others. Antioxidants are known to protect against oxidative stress, which has been involved in the development of several chronic diseases, including cardiovascular disease and cancer (Da Costa et al., 2012). They can prevent the formation of reactive species, scavenge, neutralize and remove reactive species,

* Corresponding author. Address: Universidade de Caxias do Sul, Rua Francisco Getúlio Vargas 1130, 95070-560 Caxias do Sul, RS, Brazil. Tel.: +55 54 218 2100; fax: +55 54 218 2664.
E-mail address: mrely@ucs.br (M. Roesch-Ely).

0278-6915/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2012.11.013

beside inhibiting oxidative chain reactions, chelating reactive metals, and repairing damage to biological molecules (Da Costa et al., 2012). The antitumor activity of propolis has been widely studied (Valente et al., 2011).

The aim of this study was to investigate the major compounds presented in the hydroalcoholic red propolis extracts from the northeast of Brazil, Sergipe. Moreover, this study also evaluated the antioxidant activity and the cytotoxic effect of red propolis extract against well-known classic tumor cancer cell lines (Hep-2, HeLa), which was compared with as the non-malignant one (Hek-293), all lines presenting the same epithelial morphology.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified eagle's medium–high glucose (DMEM), trypsin–EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide (MTT), Folin–Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH·), dimethyl sulfoxide (DMSO) and crystal violet were purchased from Sigma–Aldrich (St. Louis, MO, USA); ethanol was from Merck (Darmstadt, Hesse, GE). Heat-inactivated fetal bovine serum (FBS) was purchased from Cultilab Lab Inc. (Campinas, SP, BR) and penicillin–streptomycin were purchased from GIBCO™ Invitrogen (Grand Island, NY, USA). All other chemicals were of ultrapure grade and obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Red propolis sample

Red propolis was collected in the year 2010, in the Capivara Apiary in Brejo Grande, state of Sergipe in the northeast of Brazil (10°28′25″S and 36°26′12″W). The propolis was protected from light and frozen at −20 °C until extract preparation was proceeded.

2.3. Preparation of red propolis extract

Propolis was ground to a fine powder and 1 g (dry weight) was mixed with10 mL of EtOH–H_2O 70% (v/v) and shaken at room temperature for 24 h. After extraction, the mixture was filtered and the solvent was evaporated and produced a red fine powder. The dry extract was kept frozen at −20 °C and prepared at different concentrations with EtOH–H_2O 50% (v/v), and finally filtered through a 0.22 μm polyethersulfone membrane (TPP, Techno Plastic Products, Switzerland).

2.4. Chemical characterization

The propolis dry extract was dissolved in a solution of 70% (v/v) chromatographic grade ethanol (Tedia, Fairfield, OH, USA), 30% (v/v) deionized water and 0.1% formic acid. The propolis solution was infused directly into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 μL min^{-1}. ESI(+)-MS and tandem ESI(+)-MS/MS were acquired using a hybrid high-resolution and high accuracy (5 μL/L) Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) under the following conditions: capillary and cone voltages were set to +3500 V and +40 V, respectively, with a desolvation temperature of 100 °C. For ESI(+)-MS/MS, the energy for the collision induced dissociations (CID) was optimized for each component. Diagnostic ions in the different propolis samples were identified by the comparison of their ESI(+)-MS/MS dissociation patterns with compounds identified in previous studies (indicated in Table 1). For data acquisition and processing, Xcalibur software (Thermo Fisher Scientific, USA) was used. The data were collected in the *m/z* range of 70–700 at the speed of two scans per second, providing the resolution of 50.000 (FWHM) at *m/z* 200. No important ions were observed below *m/z* 200 or above *m/z* 650, therefore ESI(+)-MS data is shown in the *m/z* 200–650 range.

2.5. Determination of total phenolic content

Total phenolic content of the extract was measured by using the Folin–Ciocalteu colorimetric method of Singleton and Rossi (1965) with slight modifications. Briefly, 100 μL of the hydroalcoholic extract (1 mg/mL) was mixed with 400 μL of sodium carbonate (7.5%, w/v) and 500 μL of Folin–Ciocalteu reagent. After 30 min in the dark, the absorbance was measured at 765 nm in a spectrophotometer (model UV-1700 spectrophotometer, Shimadzu, Kyoto, Japan). Gallic acid was used as standard to produce the calibration curve. The total phenolic content was expressed in mg of gallic acid equivalents/g of dry extract.

2.6. Antioxidant activity assays

2.6.1. DPPH· radical scavenging assay

DPPH· assay was carried out using a modified Yamaguchi et al. (1998) method. Briefly, the extract was diluted at different concentrations (25–750 μg/mL) and added to Tris–HCl buffer (100 mM, pH 7.0) containing 250 μmol L^{-1} DPPH· dissolved in ethanol. The tubes were stored in the dark for 20 min and absorbance was measured at 517 nm. Results were expressed as IC50 (amount of the extract needed to scavenger 50% of DPPH·).

2.6.2. SOD-like and CAT-like assays

To evaluate enzyme-like activities, red propolis extracts were prepared in a concentration of 100 μg/mL. SOD-like assay was done by measuring the inhibition of self-catalytic adrenochrome formation rate at 480 nm, in a reaction medium containing 60 mM/L of adrenaline (pH 2.0), and 50 mM/L of glycine (pH 10.2). This reaction was performed at 30 °C for 3 min (Bannister and Calabrese, 1987) with different volumes of propolis extract (50, 25 and 10 μL). The result was expressed in units of Sod-like.

CAT-like assay was performed by determining hydrogen peroxide (H_2O_2) decomposition rate at 240 nm (Aebi, 1984). This reaction, containing 2910 μL of phosphate buffer (pH 7.0), 70 μL of H_2O_2 (0.3 M) and 20 μL of red propolis extract, was performed at 30 °C for 3 min. Results were expressed as millimoles of H_2O_2 decomposed/min.

2.7. Cell culture

Hep-2 (human laryngeal epidermoid carcinoma cells) and HeLa (human cervical adenocarcinoma) cancer cell lines and Hek-293 (human normal epithelial embryonic kidney) non-tumor cell line were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, and 1% penicillin–streptomycin. Cells were maintained in a humidified atmosphere at 37 °C, in 5% CO_2, and 95% air. The cytotoxicity study was performed when the cells reached 70–80% confluence.

2.8. Cytotoxic assay

2.8.1. MTT assay

Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). Briefly, cells were seeded into the 96-well plates at a density of 5.0×10^4 - cells/mL, in a volume of 100 μL of supplemented culture media. After 24 h, cells were treated with different concentrations (50–150 μg/mL) of red propolis extract and incubated at 37 °C in 5% CO_2 for 1 or 24 h. Negative controls were treated with the same amounts of hydroalcoholic solution. Cells treated for 1 h with propolis extract were subsequently incubated in fresh medium for 24 h. The medium was removed and 1 mg/mL MTT dye in serum-free medium was added to the wells. Plates were incubated at 37 °C for 2 h in humidified 5% CO_2 atmosphere. Subsequently, the MTT solution was removed and the obtained formazan violet product was dissolved in 100 μL DMSO. Absorbance was measured using a microplate reader (Spectra Max 190, Molecular Devices, USA) at 570 nm. All readings were compared with the control, which represented 100% viability. The percentage growth inhibition was calculated using the equation developed by Monks et al. (1991): cell viability (%) = (absorbance of experimental wells/absorbance of control wells) × 100. The IC50 (concentration μg/mL that inhibits cell growth by 50%) ratio of cancerous (HeLa and Hep-2) and non-tumor (Hek-293) cell was also calculated. Each experiment was performed in triplicate and independently repeated at least four times.

2.8.2. Clonogenic assay

The clonogenic assay is based on the ability of cell to form colonies after the extract treatment (Mirabelli et al., 1985). After trypsinization, 200 Hep-2 cells were added to each 06-well microtiter plate and incubated overnight at 37 °C in 5% CO_2 atmosphere. The supernatant was discarded and the cells were exposed to different concentrations (0.01–10 μg/mL) of the hydroalcoholic extracts for 24 h. Afterward, the medium was replaced with a fresh one. Colonies were allowed to grow for 1 week at 37 °C, 5% CO_2. The medium was then removed, the colonies were fixed in methanol and stained with 0.1% crystal violet and then counted. The tolerance limit was determined by the ability of a cell to form a colony containing more than 50 cells.

2.9. Statistical analysis

Results were expressed as mean ± standard deviation obtained from three independent experiments. Statistical significance was evaluated using *t*-test and one-way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Tukey test) to assess statistical differences in case of normal distribution. Significance was accepted at *p* lower than 0.05 using the Statistical Package for Social Sciences (SPSS, version 19.0) for Windows.

Table 1
ESI(+)-MS/MS data for major compounds found in red propolis from Sergipe.

Entry	Precursor ion *m/z* (%)	Identification	Elem. comp.	Diff. (ppm)	Fragment ions MS/MS (%)[MS2]{MS3}	Fragmentation pathways	Ref.
1	221.1204 (25)	6-Acetyl-2,2-dimethyl-3-hydroxychroman	$C_{13}H_{17}O_3$	2.392	206.0969(100)[196.5332(100); 137.1431(80)].	206.0969 [M−CH_3]	Valcic et al. (1999)
2	255.1022 (8)	2-Hydroxy-4-methoxychalcon	$C_{16}H_{15}O_3$	0.316			Righi et al. (2011)
3	257.0811(9)	Liquiritigenin	$C_{15}H_{13}O_4$	1.068	242.0582(20); 239.0700(100) [211.0755(100) {(196.5468(80); 183.0809(15); 137.177(100)}; 196.5520(20); 137.1649(20)]; 229.0868(25); 211.0755(30); 178.9466(15); 163.0888(7); 147.0441(100); 137.0236(100).	239.0708 [M−OH]; 211.0759 [M−OH−CO); 196.5468(M−OH−CH_2); 147.0441[M−$C_6H_6O_2$]; 137.0236[$C_8H_9O_2$]$^+$	Piccinelli et al. (2011)
4	269.0820 (100)	Formononetin	$C_{16}H_{13}O_4$	0.265	254.0575(100) [237.0552(100); 226.0624(30); 136.0155(10)]; 241.0865(15); 237.0552(60) [209.0604(100)]; 213.0917(100) [198.0680(100); 195.0810(70); 182.0725(20)]; 107.0492(15).	254.0575[M−CH_3]; 237.0552[M−OH−CH_3]; 136.0155[M−CH_3−PhOH−C_2H_2]	Piccinelli et al. (2011)
5	271.0975 (16)	Medicarpin	$C_{16}H_{15}O_4$	0.125	253.0858(30) [225.0909(100); 196.5134(20); 137.1273(20)]; 239.0700(5); 229.0868(5); 177.0546(7); 163.0388(7); 151.0394(100) [137.0600(10); 131.0494(100)]; 137.0600(5); 131.0494(30).	253.0858[M−OH]; 239.0700[M−OCH_3]; 151.0394[$C_8H_7O_3$]$^+$	Piccinelli et al. (2011)
6	285.0765 (44)	Biochanin A	$C_{16}H_{13}O_5$	0.707	270.0534(100) [253.0493(60); 242.0582(20); 214.0621(30); 137.0236(100)]; 257.0811(25); 253.0493(60) [225.0552(100) {197.0599(100); 137.1576(100}]; 229.0868(30) [211.0755(90); 197.0599(100); 183.0809(20)]; 225.0552(20); 152.0104(7); 137.0236(25).	270.0534[M−CH_3]; 257.0811[M−CO]; 253.0493[M−OCH_3]; 137.0236[$C_7H_5O_3$]$^+$	Piccinelli et al. (2011)
7	523.1751 (8)	Retusapurpurin B	$C_{32}H_{27}O_7$	0	399.1220(50) [387.1244(100); 373.1066(20)]; 387.1244(100) [371.0919(100); 355.0979(30); 279.0645(30); 196.5436(5)]; 386.1164(30); 371.0919(15).	399.1220 [M−Ph−OH−OCH3]; 387.1244 [M−Ph−OCH_3−OCH_2]; 386.1164 [$C_{24}H_{18}O_5$]$^+$; 371.0919 [$C_{23}H_{15}O_5$]$^+$	Piccinelli et al. (2011)
8	611.1965 (10)	Hesperetin 7-rhamnoglucoside	$C_{28}H_{35}O_{15}$	1.792			Sulaiman et al. (2011)

3. Results and discussion

3.1. Chemical characterization

Red propolis was collected from a geographic region on northeast of Brazil known as Brejo Grande, situated in the state of Sergipe. To our knowledge, this is the first report that investigates the major compounds presented in the hydroalcoholic red propolis extracts from Sergipe by ESI-MS and ESI-MS/MS techniques and confirms its antioxidant activity and cytotoxic effect against cancer cell lines. In this study is shown that the red Brazilian propolis extract from Sergipe is characterized by a complex mixture of interesting chemical compounds, similar to the red propolis found in other regions of the northeast of our country. Chemical composition of Brazilian red propolis extracts from the northeast region has been extensively characterized by several recent studies, most of them describe similar components, including the major composts here investigated and listed in Table 1. The red propolis presents high concentration of phenolic acids and flavonoids such as formononetin, isoliquiritigenin, liquiritigenin, medicarpin, and biochanin A (Awale et al., 2008; Daugsch et al., 2008; Franchi et al., 2012; Li et al., 2008; Moraes et al., 2010; Piccinelli et al., 2011). These compounds have been associated with a variety of health benefits (Li et al., 2008).

Considering the complex constituents of the red propolis sample, we used ESI-MS and ESI-MS/MS techniques, which are important tools to characterize and identify metabolites. They have been applied for the rapid analysis of natural products, such as flavonoids (Vessecchi et al., 2011) and terpenoids (Yang et al., 2007). The measurements were done in high-resolution direct-infusion mass spectrometry (HR-DIMS) Orbitrap (Thermo Fisher Scientific, USA). Formic acid was introduced into the sample solution and the system operated in positive mode. The instruments accurate mass measurement gives the elemental composition of parent and fragment ions. For direct comparison with different propolis samples reported in the literature, the majority of compounds could be detected in the positive ion mode (Table 1). Since the widely accepted accuracy threshold for confirmation of elemental compositions was established as 5 ppm (Lacorte and Fernandez-Alba, 2006), this usually provides highly reliable identification of the target compounds. In addition, mass measurement accuracy is also easily obtained for all the characteristic fragment ions, thus providing two sets of important information for unequivocal identification, being able to differentiate also isobaric interferences. Thus, the main chemical compounds are shown in Table 1.

The full mass spectrum in Fig. 1 shows the isoflavones formononetin (*m/z* 269.0820) and biochanin A (*m/z* 285.0785) as the main components. They have been described in most articles identifying propolis chemical compositions (Awale et al., 2008; Franchi et al., 2012; Piccinelli et al., 2011). In the same way, the hesperetin-7-rhamnglucoside, a sugar group substituted for some of the hydrogen atoms of the flavones, also has been reported (Gómez-Romero et al., 2011; Lu et al., 2004; Sulaiman et al., 2011). The structural elucidation of other product ions in MS spectrum is not trivial and the deduction needs further confirmation.

The fragmentor voltage usually cannot be fixed for each compound independently due to the proximity of other targets, and the appropriate collision energy was changed in accordance. From these fragmentation pathways we can infer the presence of liquiritigenin (*m/z* 257.0811), instead of isoliquiritigenin (*m/z* 257.0811) (Fig. 2). Because of its structural difference, the loss of carbon monoxide (—CO) would not be seen in isoliquiritigenin.

Li et al. (2008) investigated the *in vitro* cytotoxic activities of several Brazilian red propolis isolated compounds. Among the mol-

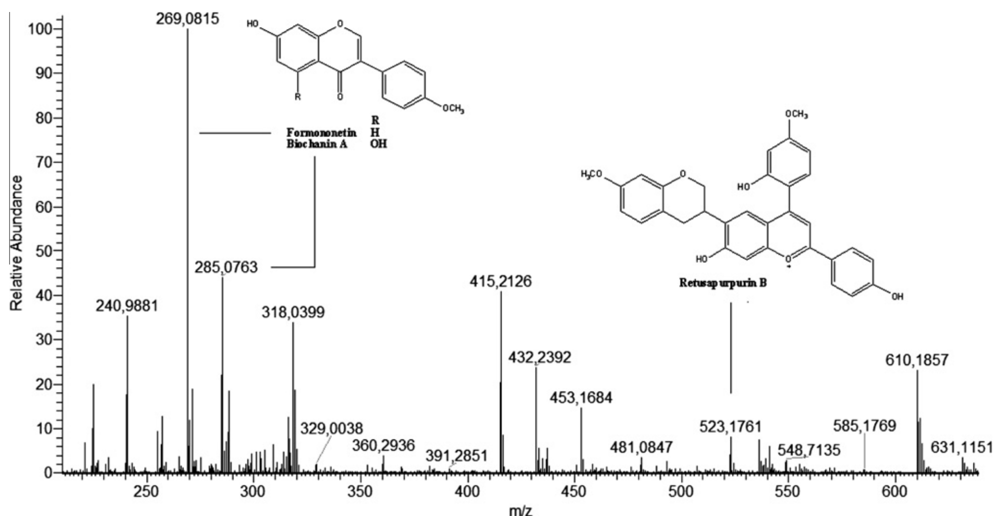


Fig. 1. ESI(+)-MS fingerprint for red propolis from Sergipe.

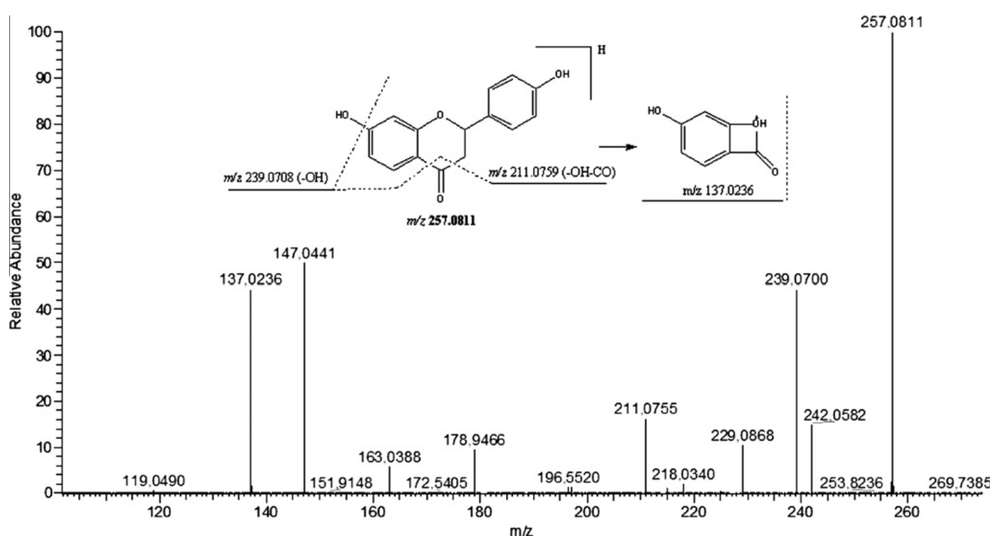


Fig. 2. ESI(+)-MS/MS of liquiritigenin (*m*/*z* 257.0811).

ecules investigated, isoliquiritigenin and medicarpin showed potent activity against cancer cell lines as colon 26-L5, B16-BL6 and HeLa, whereas formononetin, biochanin A and liquiritigenin showed cytotoxic activity against the colon 26-L5 line. Kanazawa et al. (2003) showed that isoliquiritigenin has a potent activity against prostate cancer.

3.2. Total polyphenol content and antioxidant activity

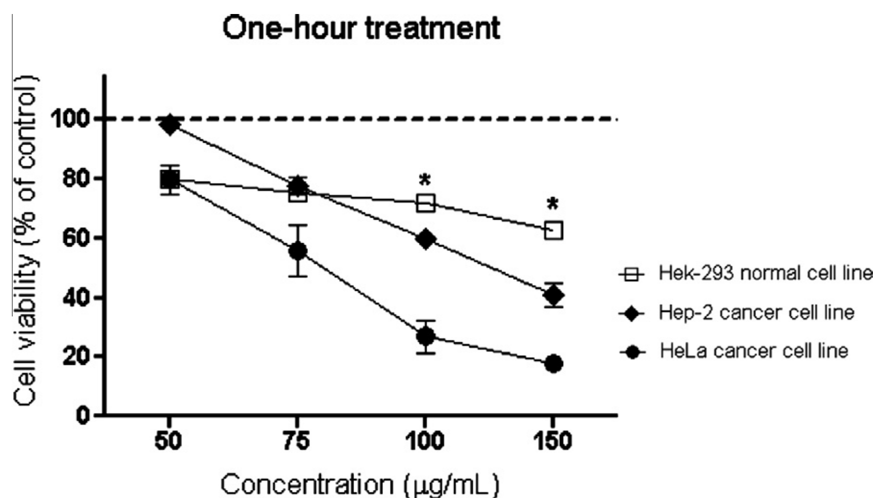
Hydroalcoholic extract obtained from red propolis showed high polyphenolic content (Table 2). Polyphenols are part of the chemical composition found in red propolis that varies according to the year and location of collection. Extracts taken from the northeast of Brazil revealed different amounts of polyphenols: 154 mg/g (Moraes, 2009), 232 mg/g (Alencar et al., 2007) and 257 mg/g (Cabral et al., 2009). This difference is possible due the different method of extraction, beside the geographic localization. Phenolic compounds present in propolis are known to possess antioxidant properties, and these properties may play a key role in the anticancer activity.

The hydroalcoholic extract of red propolis showed important DPPH· scavenging ability. DPPH· is a widely used method to evaluate antioxidant activity. This simple and highly sensitive assay measures the ability of the extract to donate electrons to the stable radical DPPH· (Moon and Shibamoto, 2009). Red propolis extract showed similar results (Table 2) to those obtained by Pinheiro

Table 2
Total phenolic content, DPPH. radical scavenging, superoxide dismutase-like, and catalase-like activities in red propolis extracts.

Sample	Total phenolic content mg/g dry extract[a]	DPPH. radical scavenging IC50[b]	Superoxide dismutase-like USOD-like	Catalase-like activity (mmol of H_2O_2 decomposed/min)
Red propolis	151.55 ± 1.95	270.13 ± 24.77	466.90 ± 12.40	13.13 ± 2.65

Results presented as mean ± SD.
[a] Gallic acid equivalents.
[b] Amount (μg/mL) of red propolis extract required to scavenge 50% of the DPPH. radical.



Values are presented as mean ± SD of three independent experiments performed in quadruplicate. *represent statistical significance between normal and tumor cells according to analysis of variance and Tukey's post hoc test (p ≤ 0.05).

Fig. 3. Inhibitory effects of red propolis on Hep-2 and HeLa cancer cell lines and on Hek-293 normal cell line.

(2009) with red propolis from the same geographic region, with IC50 of 294 μg/mL, confirming the potential DPPH· scavenging.

Superoxide dismutase is an important enzyme that catalyzes the dismutation of superoxide anion ($O2^{\cdot-}$) to oxygen and H_2O_2, while catalase converts H_2O_2 to water and molecular oxygen (Halliwell and Gutteridge, 2007). The extract prepared from red propolis showed important SOD-like and CAT-like activities (Table 2). It is known that antioxidant enzymes have an important role in maintaining physiological redox equilibrium, decreasing oxidative stress.

3.3. In vitro cytotoxic activity

3.3.1. MTT assay

In order to investigate cytotoxic effects of red propolis, this work analyzed the hydroalcoholic extract activity on tumor (Hep-2 and HeLa) and non-tumor (Hek-283) established cell lines. Fig. 3 shows the results from cell viability after 1 h extract incubation using a concentration range (10–150 μg/mL) of red propolis extract. Inhibitory effects of red propolis hydroalcoholic extract were more significant in higher concentrations on tumor cell lines when compared to the non-tumor ones, suggesting an increased sensitivity that may correlate with the higher proliferative index of the tumor vs. normal cells, once the same concentration of extract was able to inhibit cell proliferation more efficiently in the tumor lines here investigated. Results are even more prominent in 24 h propolis extract treatment (Table 3), where no IC50 was obtained for normal cell lines. Although longer red propolis extract incubation time has been reported in the literature for different cell lines (Alencar et al., 2007; Filardi, 2010; Franchi et al., 2012; Silva, 2007) previous results from our group (data not shown) generated spectra interference for long incubation periods, probably due to colorimetric responses caused by the inherit red pigmentation of the extract in higher doses.

A recent report using primary cultures of normal and malignant cells showed that methanol extracts of Portuguese propolis also presented *in vitro* selectivity (Valente et al., 2011). Results were also observed on primary prostate cancer cells compared to normal human prostate epithelial cells, which showed higher inhibitory efficiency in treated cells with propolis extracts (Moraes et al., 2010). It is known that selectivity is an important parameter in cancer prevention, as in therapy associated to the development of natural products, where new and effective drugs with reduced collateral effects are discovered.

Table 3
IC50 for 1 h and 24 h treatment with red propolis extracts in different cell lines.

Treatment (μg/mL)	IC50 (μg/mL)		
	Tumor cell lines		Normal cell line
	Hep-2	HeLa	Hek-293
1-h	128.12 ± 5.33	85.77 ± 1.00	>150
24-h	63.48 ± 3.30*	81.40 ± 6.40	>150

IC50 (μg/mL) presented as mean ± SD.
* Statistical significance between 1- and 24-h of treatment in Hep-2 cells. No statistical significance was found for HeLa and Hek-293 for 1- and 24-h of treatment, according to *t*-test ($p \leqslant 0.05$).

3.3.2. Clonogenic assay

To discard possible colorimetric interference, extracts tested in Hep-2 were also submitted to the clonogenic assays, which showed lower IC50 value (1.86 ± 0.01 μg/mL) compared to the absorbance-based (MTT) assay. The clonogenic test is a method that consists of a direct investigation with colony formation after 168 h, where colorimetry plays no interference. In the clonogenic assay, no colony formation was observed at 10 μg/mL of red propolis extract, showing the importance of evaluating effects of chronic exposure (168 h) compared to short-term (1 and 24 h) assays. Considering that MTT-based method is still one of the most widely used assays for measuring acute cytotoxic effects of compounds and that consists of an easy, sensitive, rapid and low cost assay, results here presented were based on this experimental protocol.

The inhibitory effect against cancer cell growth exhibited by different propolis samples may be related to an overall effect of the phenolic compounds present in each extract, the region and year where the material was collected. Our results confirm the *in vitro* cytotoxic effects of red propolis in different tumor cell lines, indicating a clear anticancer activity by inhibiting cell growth proliferation at different levels.

4. Concluding remarks

The data here presented indicate that red propolis is composed of complex molecules and exhibits important biological properties with capacity to scavenge free radicals and to inhibit tumor cell growth. Although the molecular mechanisms by which propolis interacts with cell metabolism remain unclear, further studies including isolation of major bioactive compounds with cytogenetic and molecular tests will better elucidate the antioxidant and anticancer activities here observed. Isolated compounds should also be investigated regarding its biological properties against different tumor and non-tumor cell lines, using both MTT-based and clonogenic assays. The biological effects of chronic exposure for clonogenic assays observed by natural products including propolis should be considered as a validation method and a critical tool for testing natural compounds.

Conflict of Interest

The authors have declared that there is no conflict of interest.

Acknowledgments

This work was supported by CAPES and FAPERGS.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Alencar, S.M., Oldoni, T.L., Castro, M.L., Cabral, I.S., Costa-Neto, C.M., Cury, J.A., Rosalen, P.L., Ikegaki, M., 2007. Chemical composition and biological activity of a new type of Brazilian propolis: red propolis. J. Ethnopharmacol. 113, 278–283.
Awale, S., Li, F., Onozuka, H., Esumi, H., Tezuka, Y., Kadota, S., 2008. Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition. Bioorg. Med. Chem. 16, 181–189.
Bannister, J.V., Calabrese, L., 1987. Assays for superoxide dismutase. Methods Biochem. Anal. 32, 279–312.
Barreto, A.L.S., 2008. Estudo histomorfológico do efeito de membranas de colágeno contendo própolis vermelha sobre o processo de reparo cicatricial por segunda intenção em ratos. Universidade Tiradentes, Aracaju.
Burdock, G.A., 1998. Review of the biological properties and toxicity of bee propolis (propolis). Food Chem. Toxicol. 36, 347–363.
Cabral, I.S.R., Oldoni, T.L.C., Prado, A., Bezerra, R.M.N., Alencar, S.M.d., 2009. Composição fenólica, atividade antibacteriana e antioxidante da própolis vermelha brasileira. Quim. Nova 32, 1523–1527.
Castaldo, S., Capasso, F., 2002. Propolis, an old remedy used in modern medicine. Fitoterapia 73 (Suppl. 1), S1–S6.
Da Costa, L.A., Badawi, A., El-Sohemy, A., 2012. Nutrigenetics and modulation of oxidative stress. Ann. Nutr. Metab. 60 (Suppl. 3), 27–36.
Daugsch, A., Moraes, C.S., Fort, P., Park, Y.K., 2008. Brazilian red propolis – chemical composition and botanical origin. Evid. Based Complement. Altern. Med. 5, 435–441.
Filardi, M.A., 2010. Potencial antitumoral de extratos da própolis Brasileira e de folhas de graviola (*Annona muricata*): efeito citotóxico sobre células hepatocarcinogênicas HEPG2. Universidade Federal de Viçosa, Viçosa.
Franchi Jr., G.C., Moraes, C.S., Toreti, V.C., Daugsch, A., Nowill, A.E., Park, Y.K., 2012. Comparison of effects of the ethanolic extracts of Brazilian propolis on human leukemic cells as assessed with the MTT assay. Evid. Based Complement. Altern. Med. 2012, 918956-1–918956-6. http://dx.doi.org/10.1155/2012/918956.
Gómez-Romero, M., Zurek, G., Schneider, B., Baessmann, C., Segura-Carretero, A., Fernández-Gutiérrez, A., 2011. Automated identification of phenolics in plant-derived foods using library search approach. Food Chem. 124, 379–386.
Halliwell, B., Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine, fourth ed. Clarendon Press, Oxford, UK.
Kanazawa, M., Satomi, Y., Mizutani, Y., Ukimura, O., Kawauchi, A., Sakai, T., Baba, M., Okuyama, T., Nishino, H., Miki, T., 2003. Isoliquiritigenin inhibits the growth of prostate cancer. Eur. Urol. 43, 580–586.
Lacorte, S., Fernandez-Alba, A.R., 2006. Time of flight mass spectrometry applied to the liquid chromatographic analysis of pesticides in water and food. Mass Spectrom. Rev. 25, 866–880.
Li, F., Awale, S., Tezuka, Y., Kadota, S., 2008. Cytotoxic constituents from Brazilian red propolis and their structure–activity relationship. Bioorg. Med. Chem. 16, 5434–5440.
Lu, Y., Wu, C., Yuan, Z., 2004. Determination of hesperetin, cinnamic acid and nicotinic acid in propolis with micellar electrokinetic capillary chromatography. Fitoterapia 75, 267–276.
Lustosa, S.R., Galindo, A.B., Nunes, L.C.C., Randau, K.P., Neto, P.J.R., 2008. Própolis: atualizações sobre a química e a farmacologia. Rev. Bras. Farmacogn. 18, 447–454.
Mirabelli, C.K., Johnson, R.K., Sung, C.M., Faucette, L., Muirhead, K., Crooke, S.T., 1985. Evaluation of the in vivo antitumor activity and in vitro cytotoxic properties of auranofin, a coordinated gold compound, in murine tumor models. Cancer Res. 45, 32–39.
Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., et al., 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J. Natl. Cancer Inst. 83, 757–766.
Moon, J.K., Shibamoto, T., 2009. Antioxidant assays for plant and food components. J. Agric. Food. Chem. 57, 1655–1666.
Moraes, C.S., 2009. Isolamento e identificação de formononetina da própolis de João Pessoa-PB, estudo de sua sazonalidade e avaliação de suas atividades biológicas. Universidade Estadual de Campinas, Campinas.
Moraes, C.S., Daugsch, A., Li, H., Rhim, J.S., Park, Y.K., 2010. Comparative antiproliferation of human prostate cancer cells by ethanolic extracts of two groups of Brazilian propolis. Ciênc. Tecnol. Aliment. 30, 539–543.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Park, Y.K., Ikegaki, M., Alencar, S.M., Moura, F.F., 2000. Evaluation of Brazilian propolis by both hysicochemical methods and biological activity. Honeybee Sci. 21, 85–90.
Piccinelli, A.L., Lotti, C., Campone, L., Cuesta-Rubio, O., Campo Fernandez, M., Rastrelli, L., 2011. Cuban and Brazilian red propolis: botanical origin and comparative analysis by high-performance liquid chromatography-photodiode array detection/electrospray ionization tandem mass spectrometry. J. Agric. Food Chem. 59, 6484–6491.
Pinheiro, M.S., 2009. Avaliação da atividade antimicrobiana e citoprotetora gástrica dos extratos de mangaba, caju e própolis vermelha. Universidade Tiradentes, Aracaju.
Righi, A.A., Alves, T.R., Negri, G., Marques, L.M., Breyer, H., Salatino, A., 2011. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. J. Sci. Food Agric. 91, 2363–2370.
Sforcin, J.M., 2007. Propolis and the immune system: a review. J. Ethnopharmacol. 113, 1–14.
Silva, B.B., Rosalen, P.L., Cury, J.A., Ikegaki, M., Souza, V.C., Esteves, A., Alencar, S.M., 2008. Chemical composition and botanical origin of red propolis, a new type of Brazilian propolis. Evid. Based Complement. Altern. Med. 5, 313–316.
Silva, M.S.e.C., 2007. Estudo do efeito da fração BRVD obtida a partir da própolis brasileira tipificada, na proliferação de células tumorais, São Paulo.
Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolic with phosphomolybdic–phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–158.
Siqueira, A.B., Gomes, B.S., Cambuim, I., Maia, R., Abreu, S., Souza-Motta, C.M., de Queiroz, L.A., Porto, A.L., 2009. Trichophyton species susceptibility to green and red propolis from Brazil. Lett. Appl. Microbiol. 48, 90–96.
Sulaiman, G.M., Al Sammarrae, K.W., Ad'hiah, A.H., Zucchetti, M., Frapolli, R., Bello, E., Erba, E., D'Incalci, M., Bagnati, R., 2011. Chemical characterization of Iraqi propolis samples and assessing their antioxidant potentials. Food Chem. Toxicol. 49, 2415–2421.
Valcic, S., Montenegro, G., Mujica, A.M., Avila, G., Franzblau, S., Singh, M.P., Maiese, W.M., Timmermann, B.N., 1999. Phytochemical, morphological, and biological investigations of propolis from Central Chile. Z. Naturforsch. 54c, 406–416.
Valente, M.J., Baltazar, A.F., Henrique, R., Estevinho, L., Carvalho, M., 2011. Biological activities of Portuguese propolis: protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth in vitro. Food Chem. Toxicol. 49, 86–92.
Vessecchi, R., Zocolo, G.J., Gouvea, D.R., Hubner, F., Cramer, B., de Marchi, M.R., Humpf, H.U., Lopes, N.P., 2011. Re-examination of the anion derivatives of isoflavones by radical fragmentation in negative electrospray ionization tandem mass spectrometry: experimental and computational studies. Rapid Commun. Mass Spectrom. 25, 2020–2026.
Yamaguchi, T., Takamura, H., Matoba, T., Terao, J., 1998. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. Biosci. Biotechnol. Biochem. 62, 1201–1204.
Yang, M., Wang, X., Guan, S., Xia, J., Sun, J., Guo, H., Guo, D.A., 2007. Analysis of triterpenoids in ganoderma lucidum using liquid chromatography coupled with electrospray ionization mass spectrometry. J. Am. Soc. Mass Spectrom. 18, 927–939.