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Caracterização química e avaliação da atividade biológica da própolis vermelha em células tumorais e não tumorais

Caroline Olivieri da Silva Frozza

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LISTA DE ABREVIATURAS

2D	Eletroforese bidimensional
Cat	Enzima catalase
DMEM	*Dulbecco's Modified Eagle Medium*
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
$DPPH^{\bullet}$	Radical 2,2-difenil 1-picrilhidrazil
ESI	Ionização por *electrospray*
EUA	Estados Unidos da América
GPx	Glutationa-peroxidase
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
MS	Espectrometria de massas
MTT	3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio
PBS	Tampão fosfato salina
SC	Santa Catarina
SDS-PAGE	Eletroforese em gel de poliacrilamida-dodecil sulfato de sódio
SFB	Soro fetal bovino
Sod	Enzima superóxido-dismutase

RESUMO

A própolis vermelha brasileira tem atraído interesses científicos e econômicos devido às suas variadas atividades biológicas. Este produto natural possui composição química diferente 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 possivelmente responsáveis por estas atividades. Dentre as atividades biológicas mais investigadas, destacam-se as atividades antioxidante e antitumoral. Neste trabalho, buscou-se caracterizar quimicamente o extrato hidroalcoólico da própolis vermelha brasileira, avaliar as atividades antioxidantes e antitumorais, além de investigar o padrão proteico de células tumorais de laringe tratadas e não tratadas com extratos da própolis vermelha através da análise proteômica comparativa.

A caracterização química realizada através de espectrometria de massas com ionização por *electrospray* mostrou que a própolis apresenta moléculas complexas, principalmente isoflavonoides, compostos com importantes atividades biológicas. Os extratos hidroalcoólicos obtidos a partir da própolis vermelha revelaram um significante conteúdo de polifenóis associados a uma habilidade de sequestrar radicais livres $DPPH^{\bullet}$. Os extratos também apresentaram atividades superóxido-dismutase-*like* e catalase-*like*, indicando que podem exercer um papel fundamental na manutenção fisiológica do equilíbrio redox quando em um organismo.

A atividade citotóxica dos extratos da própolis foi avaliada nas linhagens tumorais Hep-2, HeLa e não tumoral Hek-293, sendo que os valores de IC50 foram menores para a linhagens tumorais em relação a não tumoral. Desta forma, sugere-se uma seletividade da própolis vermelha quanto às linhagens tumorais.

A análise proteômica, usando eletroforese bidimensional associada à cromatografia

líquida de alta eficiência acoplada a espectrômetro de massa, permitiu a comparação dos mapas proteicos da linhagem Hep-2, na ausência ou presença de extratos da própolis vermelha, nas concentrações 6 μg/mL (não citotóxica) e 120 μg/mL (IC50). A excisão manual de 325 *spots* foi efetuada nos géis 2D- SDS-PAGE, em que 177 proteínas foram identificadas. Estas proteínas foram relacionadas com diversos processos metabólicos e estruturais como produção e conversão de energia, transporte e metabolismo de carboidratos, modificação pós-traducional, reciclagem de proteínas e chaperonas, proteínas do citoesqueleto, proteínas de reparo, entre outros. Das proteínas identificadas com expressão diferencial, cinco apresentaram expressão reduzida na presença do extrato da própolis, este em sua maior concentração (120 μg/mL). Apenas duas proteínas identificadas neste estudo mostraram expressão aumentada na concentração não citotóxica (6 μg/mL) do extrato da própolis vermelha.

Os resultados da proteômica comparativa 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 as atividades antioxidantes e antitumorais aqui observadas.

Palavras-chave: própolis vermelha, antitumoral, antioxidante, proteômica

ABSTRACT

Brazilian red propolis has attracted scientific and economic interests because of its varied biological activities. This natural product has different chemical compositions according to the region in which it is found, requiring a robust chemical characterization to elucidate the compounds responsible for the activities in each type of propolis investigated. So far antioxidant and antitumor properties are amongst the most studied biological activities. This study aimed to characterize chemically the hydroalcoholic extract of Brazilian propolis from the state of Sergipe, evaluate the antitumor and antioxidant activities, and to investigate the pattern of proteins in tumor cells of the larynx treated or not with extracts of propolis through comparative proteomic analysis.

The chemical characterization by mass spectrometry with electrospray ionization showed that propolis presents complex molecules, especially isoflavones, which has important biological and antioxidant capacity. The hydroalcoholic extracts obtained from propolis revealed a significant content of polyphenols associated with the ability to scavenge $DPPH^{\bullet}$ radicals. The extracts also showed significant activities for superoxide dismutase-like and catalase-like, indicating an important role in maintaining physiological redox equilibrium, decreasing oxidative stress.

Cytotoxic activity was assessed for tumor cell lines Hep-2, HeLa and non-tumor cell line Hek-293, showing IC50 values greater for Hek-293 compared to Hep-2 and HeLa cells, which suggests a selectivity of propolis for the tumor lines.

The proteomic analysis using two-dimensional electrophoresis associated with high performance liquid chromatography coupled with mass spectrometer allowed the comparison of the protein maps of Hep-2 cell line in the absence or presence of propolis extracts in concentrations of 6 µg/mL (not cytotoxic) and 120 µg/mL (IC50). 325 spots were

manually excised from the gels 2D SDS-PAGE and 177 proteins were identified. These proteins have been linked to various structural and metabolic processes, such as production and energy conversion, transport and carbohydrate metabolism, post-translational modification, protein turnover and chaperones, cytoskeletal proteins, repair proteins, among others. From the identified proteins that showed differential expression five were down-regulated in the presence of propolis extract in the highest concentration (120 µg/mL). Only two proteins identified in this study showed increased expression in the no cytotoxic concentration (6 µg/mL) of the red propolis extract.

The results of comparative proteomic showed that the propolis interacts with a series of intracellular events and hence becomes a promising candidate to inhibit cellular growth and contribute to the different steps related to the process of carcinogenesis. Although the molecular mechanisms by which propolis interacts with the metabolism of the cells remain unknown, additional studies will better elucidate the antitumor and antioxidant activities here observed.

Keywords: red propolis, antitumor, antioxidant, 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 caso de infecções, cânceres, imunodeficiências, entre outras. A avaliação do potencial terapêutico dos produtos naturais e de alguns de seus constituintes, tais como flavonóides, alcaloides, triterpenos, taninos, etc, tem sido objeto de incessantes estudos, onde já foram comprovadas várias ações farmacológicas. Desta forma, muitas substâncias que são descobertas, isoladas ou caracterizadas têm grandes possibilidades de futuramente virem a ser aproveitadas como agentes medicinais.

A própolis tem sido empregada popularmente como agente terapêutico na medicina alternativa por muitas décadas e vem se destacando entre uma série de produtos naturais em virtude de seu imenso potencial na prevenção e tratamento de diversas doenças. Ela é um produto resinoso elaborado pelas abelhas, as quais a utilizam para se protegerem contra a invasão de insetos e para assepsia da colmeia.

Dentre os diversos tipos de própolis brasileiras, a vermelha tem despertado interesses científico e econômico devido às atividades biológicas recentemente apresentadas. Ainda que a maioria dos estudos tenha seu foco na própolis verde, por ser bem caracterizada e conhecida em todo o mundo, a variedade vermelha, encontrada principalmente no Nordeste brasileiro, vem sendo estudada por apresentar características físico-químicas e biológicas diferenciais. Daugsch *et al.* (2008) classificaram a própolis vermelha como um novo tipo de própolis, diferente das 12 anteriormente classificadas por Park *et al.* (2000), e identificaram-na como sendo pertencente a um novo grupo, conhecido como 13º grupo.

Os diferentes tipos da própolis possuem composição química variada de acordo com a região em que são encontrados, desta forma, se faz necessária uma caracterização química precisa para que sejam identificados os compostos responsáveis por suas atividades biológicas. Dentre as ações biológicas mais estudadas em própolis, destacam-se a atividade antioxidante e a antitumoral.

Antioxidantes são conhecidos por protegerem contra o estresse oxidativo, o qual está envolvido no desenvolvimento de várias doenças como as neurodegenerativas, vasculares, cânceres, entre outras. Eles podem prevenir a formação de espécies reativas, neutralizar ou remover estas espécies, além de inibir reações em cadeia eliminando os radicais livres.

A atividade antitumoral da própolis, por sua vez, tem sido estudada como novo recurso terapêutico frente à alta incidência de diferentes tipos de tumores que acometem a população. O câncer é a segunda causa de morte registrada no mundo, precedida apenas de doenças de origem vascular. Desta forma, buscam-se um melhor entendimento quanto à etiopatogenia das neoplasias e o desenvolvimento de novas estratégias preventivas e terapêuticas no seu combate.

O estudo dos extratos totais da própolis e de seus compostos químicos, de forma isolada ou em associação, é empregado para determinar citotoxicidade e seletividade destas moléculas em linhagens tumorais e não tumorais. A determinação quanto à atividade antioxidante destes extratos traz uma informação adicional importante em relação à associação redox destes com as células estudadas.

A proteômica consiste de uma ferramenta de grande potencial que utiliza uma combinação de métodos analíticos, tais como eletroforese bidimensional, cromatografia líquida e espectrometria de massas, o que permite a identificação de alterações específicas e perfis proteicos associados com as neoplasias. Os estudos proteômicos também contribuem

para determinação de diagnósticos precoces, novas possibilidades terapêuticas e melhor entendimento dos mecanismos biológicos.

Em face ao exposto, este trabalho teve como objetivo caracterizar quimicamente o extrato da própolis vermelha brasileira, avaliar as atividades antioxidantes e antitumorais e analisar o padrão proteico de células tumorais de laringe tratadas com os extratos da própolis vermelha por análise proteômica comparativa. Assim, os objetivos específicos foram: 1) caracterizar quimicamente o extrato da própolis vermelha por espectrometria de massa com ionização por *electrospray*; 2) quantificar o teor de polifenóis, determinar a capacidade de sequestrar radicais livres e a atividade das enzimas antioxidantes superóxido dismutase e catalase no extrato da própolis vermelha; 3) determinar a citotoxicidade dos extratos da própolis em culturas de células tumorais de laringe (Hep-2), de câncer cervical (HeLa) e em células não tumorais de rim (Hek-293); e 4) avaliar o padrão proteico de células tumorais de laringe tratadas com os extratos da própolis vermelha através da análise proteômica comparativa.

2. REVISÃO BIBLIOGRÁFICA

2.1 Própolis

Própolis é um produto resinoso elaborado pelas abelhas do gênero *Apis melífera,*o qual tem origem de várias plantas, brotos ou exsudatos (Daugsch *et al.*, 2008; Silva *et al.*, 2008). A palavra própolis é derivada do grego pro-, em defesa, e polis-, comunidade, considerado assim, um produto utilizado pelas abelhas na defesa da colmeia (Marcucci, 1996). 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 (Marcucci, 1996; Marcucci *et al.*, 2001; Bankova, 2009).

A coloração da própolis pode variar de amarelo claro até marrom avermelhado dependendo de sua procedência (Marcucci 1996; Burdock, 1998; Salatino *et al.*, 2005). Apresenta-se com odor característico de acordo com a amostra analisada, ou mesmo pode ainda não possuir qualquer odor (Marcucci, 1996).

As abelhas fragmentam os ápices vegetativos das plantas com as mandíbulas, coletando, neste processo, as resinas liberadas. 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 (**Figura 1**) (Teixeira *et al.*, 2005).

O Brasil é o terceiro maior produtor mundial de própolis, com 150 toneladas produzidas a cada ano (Tribuna Hoje, 2012). O Japão é o principal mercado importador da própolis brasileira, absorvendo cerca de 80% da produção (EMBRAPA, 2012). Por apresentar alto valor agregado, a comercialização da própolis pode ser uma alternativa de

renda importante para o apicultor. Os preços variam de acordo com sua qualidade, origem botânica e mercado a que é destinada (EMBRAPA, 2012).

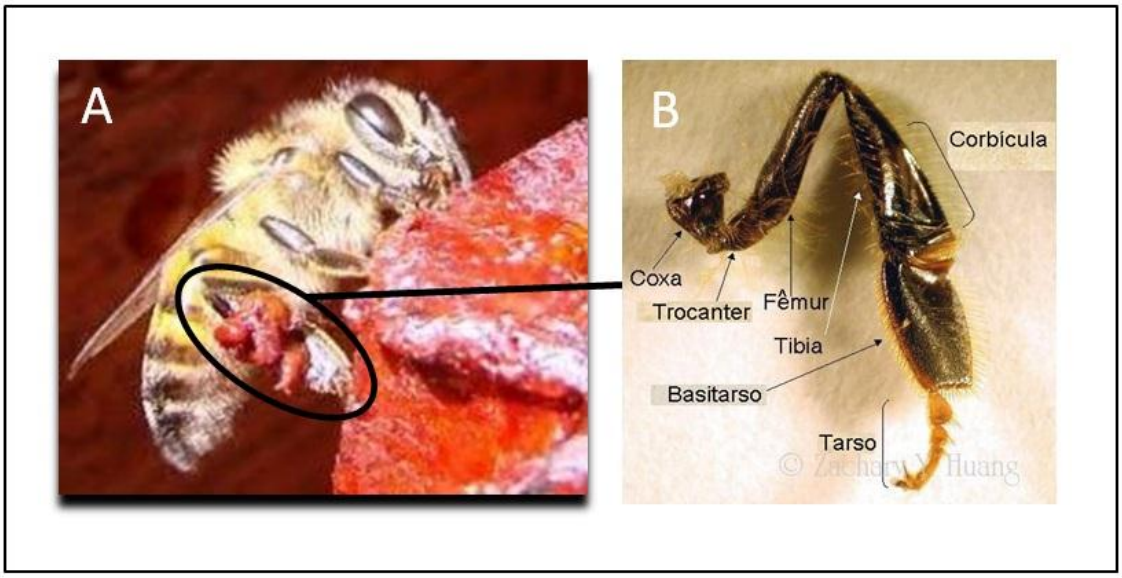

Figura 1. A- Abelha com própolis vermelha depositada na corbícula; B- estruturas da pata da abelha.

O Brasil, devido a sua biodiversidade, possui uma grande variedade de própolis. Em 2000, 12 tipos distintos de própolis brasileira foram quimicamente classificados (Park *et al.*, 2000). Alguns anos após, em 2008, Daugsch *et al.* (2008) classificaram uma 13ª própolis, a qual recebeu o nome de própolis vermelha.

2.1.1 Própolis Vermelha

A própolis vermelha, denominada assim devido a sua intensa coloração vermelha, é encontrada na região Nordeste brasileira, principalmente 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[1], 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 o único produtor dessa espécie de própolis no mundo com a denominação de origem (IG 201101) concedida em julho de 2012 (**Figura 2**), pelo Instituto Nacional de Propriedade Industrial (INPI, 2012). Pela sua singularidade, o quilograma deste produto bruto chega a custar 500 reais no mercado externo e se tornou a solução para o sustento das famílias de pescadores que enfrentavam dificuldades com a expressiva redução de peixes e caranguejos na região (Tribuna Hoje, 2012), além de ter mudado a vida dos criadores de abelha que produzem própolis vermelha em Alagoas.

Figura 2. Símbolo da denominação de origem da própolis vermelha de Alagoas.

2.2 Composição química da própolis

A própolis é considerada uma das misturas biológicas mais heterogêneas encontradas em fontes naturais. Mais de 300 componentes de própolis já foram identificados e/ou caracterizados em diferentes amostras (Salatino *et al.*, 2005; Araujo, 2009). A composição química é bastante complexa e variada. Ela está relacionada com a flora de cada região

[1] **Escandente** – espécie de planta que trepa, prendendo-se de alguma forma ao seu suporte.

visitada pelas abelhas (Park *et al.*, 2002; Bankova 2005) e com as características geográficas e climáticas do local (Silva *et al.*, 2008). Além disso, a variabilidade genética das abelhas também pode influenciar na composição química da própolis (Koo & Park, 1997).

As amostras de própolis de regiões tropicais, especialmente as brasileiras, têm mostrado diferenças significantes na sua composição química em relação à própolis da zona temperada, devido a sua rica biodiversidade. Por esta razão, a própolis brasileira tem se tornado objeto de grande interesse por parte dos cientistas (Bankova, 2005; Trusheva *et al.*, 2006). Silva *et al.* (2008) acreditam que esta grande variedade de compostos necessita ser investigada como uma fonte de novas substâncias bioativas.

A resina obtida das plantas é mastigada pelas abelhas, que adicionam enzimas salivares durante a coleta e o material, parcialmente digerido, é misturado com a cera de abelha e usado na colméia (Burdock, 1998). Os resultados desta modificação enzimática são hidrólises dos compostos fenólicos como, por exemplo, os flavonoides heterosídeos (Najafi *et al.*, 2007).

De modo geral, as própolis de diversas partes do mundo contêm 50% de resinas, 30% de ceras, 10% de óleos essenciais, 5% de grãos de pólen, 5% de várias outras substâncias como, por exemplo, elementos inorgânicos (alumínio, cálcio, ferro, cobre, manganês) e pequenas quantidades de vitaminas B1, B2, B6, C e E (Marcucci, 1996; Burdock, 1998; Menezes, 2005).

A composição química da própolis em geral inclui flavonoides (como a galangina, quercetina, pinocembrina e kaempferol), ácidos e ésteres aromáticos, terpenóides, fenilpropanoides (como os ácidos cafeico e clorogênico), esteroides, polissacarídeos e ácidos graxos (Lustosa *et al.*, 2008).

A própolis verde apresenta os seguintes componentes principais: flavonoides, compostos fenólicos, terpenoides (incluindo sesqui, di e triterpenoide) e fenilpropanoides

prenilados. Estes últimos consistem de ácidos fenólicos contendo um grupo prenila e são formados a partir do ácido cinâmico e seus derivados, como por exemplo, a artepilina C (ácido 3,5 diprenil-4-hidroxicinâmico), muito conhecida por sua atividade antitumoral (Bankova, 2005; Teixeira *et al.,* 2008).

A própolis vermelha difere quimicamente das demais por possuir compostos como os isoflavonoides (daidzeina, formononetina, biochanina A, medicarpina, vestitol), chalcona (isoliquiritigenina), flavanona (liquiritigenina), neoflavonoide (dalbergina) e isoflavanol (neovestitol) (Alencar *et al.,* 2007; Daugsch *et al.*, 2008; Oldoni *et al.*, 2011). Além disso, Nunes *et al.* (2009) identificaram 34 constituintes voláteis, sendo os majoritários *trans*-anetol, *α*-copaeno e metil *cis*-isoeugenol.

Os flavonoides são considerados os principais compostos responsáveis pelos efeitos benéficos da própolis, sendo a eles atribuídas as propriedades antibacteriana, antiviral e antioxidante (Barbosa *et al.,* 2009). Os flavonoides apresentam estrutura hidrocarbonada do tipo C6-C3-C6 nas quais as duas unidades C6 (anel A e anel B) possuem natureza fenólica (**Figura 3**) (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, como por exemplo, em *D. ecastophyllum* e possuem na sua estrutura o anel B ligado na posição C3 do anel C (**Figura 3**) (Tsao, 2010). De modo geral, os flavonoides apresentam-se hidroxilados, metoxilados e/ou glicosilados, sendo que o açúcar ligado frequentemente é uma glicose ou ramnose (Singh *et al.,* 2008).

A identificação e caracterização dos diversos compostos químicos podem ser efetuadas por espectrometria de massas com ESI, considerada uma técnica analítica rápida e confiável para a análise direta de extratos hidroalcoólicos de própolis (Buriol *et al.,* 2009).

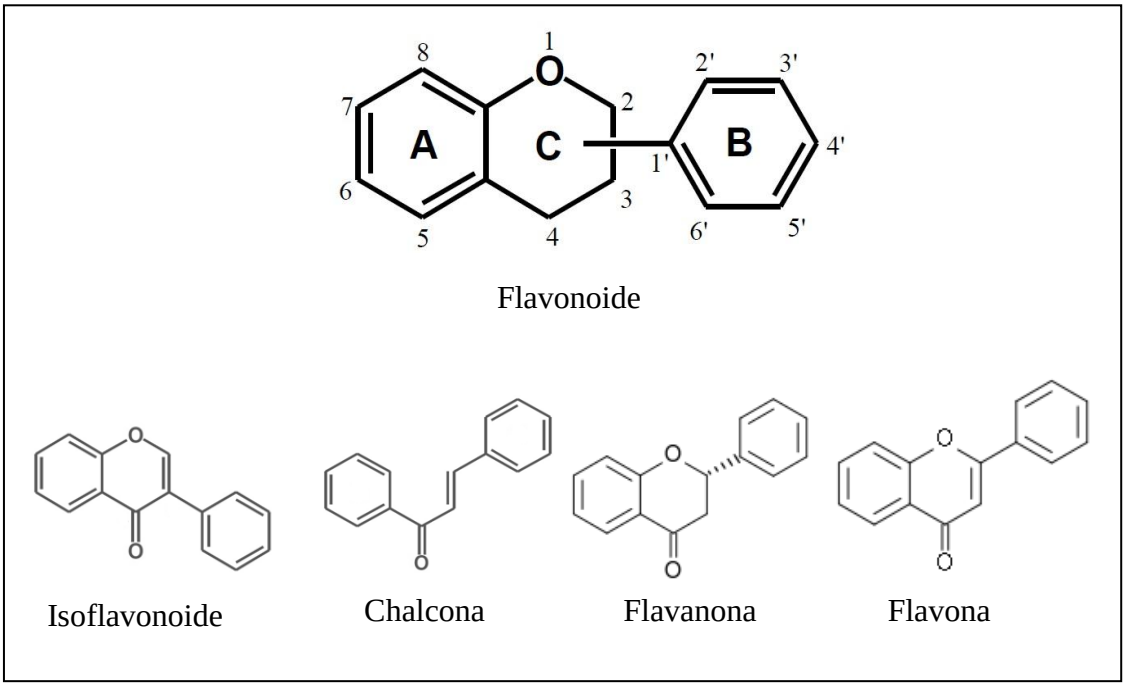

Figura 3. Estrutura básica dos flavonoides e subclasses (Fonte: Tsao, 2010).

2.3 Atividades biológicas da própolis vermelha

A própolis é um dos muitos produtos naturais que vem sendo utilizado desde a antiguidade pela humanidade (Castaldo & Capasso, 2002). Os egípcios conheciam as propriedades antiputrefativas da própolis e a empregavam para embalsamar cadáveres Além disso, existem relatos da utilização da própolis como antisséptico e cicatrizante no tratamento de feridas na Idade Média.

A própolis vermelha é conhecida pelos seus benefícios à saúde humana e entre as atividades biológicas mais estudadas, encontram-se a antibacteriana (Cabral *et al*., 2009; Righi *et al.*, 2011), antifúngica (Siqueira *et al.*, 2009), anti-inflamatória (Barreto, 2008), antiulcerativa (Pinheiro, 2009), antioxidante (Cabral *et al.*, 2009; Righi *et al.*, 2011) e antitumoral (Alencar *et al.*, 2007; Li *et al.*, 2008).

2.3.1 Atividade antioxidante

O termo antioxidante é utilizado para denominar a função de proteção celular contra os efeitos danosos dos radicais livres. Radicais livres são átomos, moléculas ou íons com elétrons desemparelhados que são altamente instáveis e reativos (Carocho & Ferreira, 2013). Eles podem ser derivados de três elementos químicos: oxigênio, nitrogênio e enxofre. Entre as espécies reativas oriundas do oxigênio, podem ser incluídos o ânion superóxido (O_2^-), o radical hidroxil ($OH^{\cdot}$), o peróxido de hidrogênio (H_2O_2), entre outros (Carocho & Ferreira, 2013). No organismo humano, existem dois sistemas de defesas antioxidantes que agem contra os danos causados pelas espécies reativas, as defesas enzimáticas e as não enzimáticas. Contudo, quando ocorre um desequilíbrio entre estas defesas e a formação de espécies reativas, ocorre uma condição fisiológica designada como estresse oxidativo (Halliwell & Gutteridge, 2007).

Quanto às defesas antioxidantes enzimáticas, podem ser citadas as enzimas superóxido-dismutase (Sod), catalase (Cat), glutationa-peroxidase (GPx), entre outras (Da Costa *et al.,* 2012). A enzima Sod dismuta o ânion superóxido ($O_2^{\bullet -}$) formando H_2O_2 (Equação 1) o qual é menos reativo, e pode ser degradado por outras enzimas, como a Cat ou a GPx, formando água e oxigênio molecular (Equação 2) (Fukai & Ushio-Fukai, 2011).

(1) $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$ Ação da enzima superóxido dismutase

(2) $2H_2O_2 \rightarrow 2H_2O + O_2$ Ação da enzima catalase

O sistema de defesa não enzimático, por sua vez, é formado pelos compostos provenientes da dieta, tais como vitaminas, carotenóides e polifenóis. Dentre estes, os polifenóis são os antioxidantes exógenos mais estudados (Singh *et al.*, 2008; Tsao, 2010). Também conhecidos como compostos fenólicos, os mesmos estão presentes nos vegetais,

como substâncias antioxidantes mais abundantes da dieta humana e podem ser classificados em flavonóides, citados anteriormente, e não flavonoides (Singh *et al.,* 2008). Os compostos não flavonoides podem ser divididos principalmente em dois grandes grupos: os derivados do ácido benzoico (C6-C1), como os ácidos vanílico e gálico, e os derivados do ácido cinâmico (C6-C3), como os ácidos p-cumárico e cafeico (Tsao, 2010).

Diferentes metodologias têm sido desenvolvidas para avaliar o conteúdo polifenólico total de um extrato/produto e sua capacidade antioxidante. O método mais utilizado para a determinação de polifenóis é a técnica colorimétrica Folin-Ciocalteu (Singleton *et al.,* 1999). Já para a determinação da atividade antioxidante, utiliza-se a medida da capacidade da inibição do radical livre 2,2-difenil 1-picrilhidrazil ($DPPH^{\bullet}$). Esta técnica vem sendo empregada para avaliar o potencial antioxidante de diferentes compostos, de forma sensível e rápida (Brand-Williams *et al*., 1995; Cheng *et al*., 2006). O radical livre em questão caracteriza-se por ser um cromóforo extremamente estável que apresenta uma faixa de absorção no comprimento de onda de 515 nm e conforme vai sendo reduzido por um antioxidante tem o desaparecimento da sua absortividade (Brand-Williams *et al*., 1995). Adicionalmente, os ensaios de Sod e Cat-*like*, que avaliam a capacidade das amostras em mimetizar a ação das enzimas Sod e Cat, também são utilizados com a mesma finalidade de avaliar a capacidade antioxidante (Dani *et al*., 2007; Spada *et al*., 2008).

A atividade antioxidante da própolis foi verificada em amostras de diversas localidades através de várias metodologias. Extratos da própolis vermelha de Alagoas e Sergipe apresentaram significativa atividade de varredora de radicais livres $DPPH^{\bullet}$ e grande teor de polifenóis totais (Cabral *et al.,* 2009; Araujo, 2009; Righi *et al.,* 2011). Contudo, não há relatos da medida de Sod e Cat-*like* nestes extratos.

2.3.2 Atividade antitumoral da própolis

Segundo o Instituto Nacional do Câncer (INCA) o termo câncer é empregado para designar mais de uma centena de diferentes doenças. No Brasil, as estimativas para o ano de 2013 apontam a ocorrência de mais de 500 mil novos casos de câncer, incluindo os casos de pele não melanoma, reforçando a magnitude do problema do câncer no país (INCA 2012). Os tipos mais incidentes são os cânceres de pele do tipo não melanoma, próstata, pulmão, cólon, reto e estômago para o sexo masculino; e os cânceres de pele não melanoma, mama, colo do útero, cólon e reto e glândula tireoide para o sexo feminino (INCA 2012).

Atualmente, o desenvolvimento de estratégias para otimização da terapêutica complementar e adjuvante e consequente diminuição do número de vítimas desta doença tem sido um foco na terapia antitumoral (Choi *et al.,* 2012). 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. Existe hoje uma grande tendência para o aproveitamento de recursos naturais na terapêutica de tumores (Shan *et al.,* 2011; Choi *et al.,* 2012), por apresentarem vantagens econômicas, serem eficientes e mostrarem poucos efeitos nocivos à saúde.

Extratos brutos e substâncias isoladas da própolis vêm sendo estudados quanto a sua atividade antitumoral com linhagens sensíveis a sua ação citotóxica. Os testes com culturas celulares (método *in vitro*) vêm, neste contexto, sendo utilizados com sucesso por serem reprodutíveis, rápidos, sensíveis e financeiramente viáveis. Watanabe *et al.* (2011) relatam que amostras de própolis de vários países foram testadas em diferentes células tumorais e apresentaram capacidade antitumoral dose e linhagem dependentes. 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 tratadas com própolis vermelha.

Linhagem celular humana		**Origem da própolis vermelha**	**Referência (Método utilizado)**
HepG-2	Carcinoma hepatocelular	Pharma Nectar Ltda.	Filardi, 2010 (vermelho neutro)
HeLa	Cervical adenocarcinoma	Alagoas	Alencar *et al.*, 2007 (MTT)
PANC-1	Câncer pancreático	Paraíba	Awale *et al.*, 2008 (WST-8 kit)
RPMI-8226	Mieloma múltiplo	Alagoas	Silva, 2007 (Azul tripan)
HL60	Leucemia promielocítica		
K562	Leucemia mielóide crônica		
FP	Fibroblastos de prepúcio		
MRC-5	Fibroblastos de pulmão		
RC-58T/h/AS	Câncer de próstata	Nordeste brasileiro	Moraes *et al.*, 2010 (Celltiter kit)
PrEC	Primárias Epiteliais de próstata		
K562	Leucemia crônica mielogena	Nordeste brasileiro	Franchi Jr. *et al.*, 2012 (MTT)
HL60	Leucemia promielocítica		
NB4	Leucemia promielocítica		
Raji HBL	Linfoma de Burkitt		
Ramos HBL	Linfoma de Burkitt		
B15	Precursora de leucemia		
REH	Precursora de leucemia		
Nalm16	Precursora de leucemia		
Nalm6	Precursora de leucemia		
RS4	Precursora de leucemia		

As linhagens celulares utilizadas neste trabalho foram a linhagem Hep-2, que consiste de células epiteliais de carcinoma de laringe, muito utilizada em modelos de carcinogênese e mutagênese (Lima *et al.*, 2005), a qual não possui relato de estudos envolvendo tratamento de própolis vermelha; a linhagem HeLa, caracterizada por células de câncer cervical, sendo uma das linhagens celulares humanas mais antigas e mais comumente usada em pesquisas; e a linhagem Hek-293, que se refere a células epiteliais embrionárias de

rim humano, representando um tipo de célula não tumoral que possui a mesma morfologia epitelial das células tumorais investigadas.

Dentre os métodos que avaliam a citotoxicidade em linhagens celulares, o que vem se destacando por ser um método colorimétrico rápido e preciso é o ensaio em que ocorre a redução do sal 3-(4,5-dimetiltiazol 2-il)-2,5difenil brometo de tetrazolina (MTT) (Mosmann, 1983). O MTT, quando incubado com células metabolicamente ativas, é reduzido por enzimas mitocondriais, transformando-se de um composto amarelo para um composto azul escuro pela formação dos cristais de formazan (Mosmann, 1983). O ensaio clonogênico vem como alternativa e permite verificar a capacidade de uma célula em proliferar-se indefinidamente, mantendo assim a sua capacidade reprodutiva e a habilidade de formar colônias após o tratamento com os compostos em estudo (Mirabelli *et al.*, 1985). Através destes métodos, pode-se avaliar a citotoxicidade do extrato da própolis vermelha em diferentes linhagens celulares e obter a concentração do extrato necessária para inibir em 50% o crescimento celular (IC50).

2.4 Avaliação do perfil proteico de células tumorais

A proteômica é uma ferramenta sensível empregada na identificação de alterações específicas e perfis proteicos em amostras biológicas, oferecendo diagnósticos precoces e possibilidades terapêuticas quando associada a estudos de neoplasias (Plebani, 2005). Esta utiliza uma combinação de métodos, incluindo eletroforese bidimensional, cromatografia líquida e espectrometria de massas (MS), permitindo a caracterização diferencial de moléculas proteicas (Canelle *et al.,* 2005; Chuthapisith *et al.,* 2007; Bianchi *et al.,* 2011).

Atualmente, verifica-se que a comunidade científica tem aumentado o seu interesse na quantificação global das proteínas de amostras biológicas (tecidos, órgãos ou

organismos). Juntamente com o avanço das técnicas envolvendo espectrometria de massas, surgem variações de métodos de quantificação, como por exemplo, a técnica utilizando a afinidade de marcadores isotópicos (ICAT, do inglês *isotope-coded affinity tags*) (Molloy *et al.*, 2005; Ramus *et al.*, 2006), marcações metabólicas com ^{15}N ou ^{13}C (Snijders *et al.*, 2005a e 2005b), marcadores isobáricos para quantificação relativa ou absoluta (iTRAQ, do inglês *isobaric tags for relative and absolute quantitation*) (Unwin *et al.*, 2005; Zhang *et al.*, 2005) e marcadores isotópicos estáveis através de aminoácidos em cultura celular (SILAC) (Berard *et al.*, 2012).

A MS é uma técnica que determina a relação entre massa e carga (*m/z*) de espécies ionizadas. O espectrômetro de massas possui uma fonte de ionização, um analisador de massas, um detector e um sistema de aquisição de dados (Cantú *et al.*, 2008). As fontes de ionização utilizadas na análise das proteínas caracterizam-se por adicionar cargas negativas ou positivas às moléculas, evitando a ruptura da estrutura polipeptídica e por transferir para a fase gasosa as espécies que serão analisadas (Cantú *et al.*, 2008). As razões para que a identificação de proteínas seja feita a partir de peptídeos inclui a sensibilidade do espectrômetro de massas, menor para as proteínas quando comparada aos peptídeos, e propriedades físico-químicas em relação à molécula inteira e/ou hidrolisada (Steen & Mann, 2004).

Segundo os estudos de Klose (2009), a separação de proteínas por sistema bidimensional é uma alternativa para o estudo de variabilidade do organismo a nível proteico, pois permite a detecção de variantes causadas por substituições de aminoácidos, isoformas de proteínas, mudanças quantitativas ou variações devido à presença/ausência de moléculas no organismo. As variantes podem ser detectadas entre centenas de proteínas e avaliadas lado a lado no mesmo sistema (Klose, 2009). Um desequilíbrio do proteoma celular pode representar um primeiro passo para o desenvolvimento de uma doença, como

no caso do câncer. À medida que uma proteína esteja superexpressa na célula, outras proteínas devem estar expressas em menor intensidade para manter um arranjo quantitativo em equilíbrio no sistema metabólico.

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 prognóstico (Bianchi *et al.*, 2011; Kim *et al*., 2010). Contudo, ainda não há relatos da utilização da plataforma 2D acoplada a análise de massas com amostra da própolis vermelha. Assim, a aquisição de perfis proteicos de células Hep-2 tratadas com extratos da própolis vermelha pode fornecer informações importantes sobre alterações moleculares observadas em células tumorais antes e após a ação destes extratos, revelando também mapas de proteínas diferencialmente expressas que possam atuar em uma série de funções biológicas celulares.

3. RESULTADOS E DISCUSSÃO

Os resultados deste trabalho estão apresentados na forma de dois artigos científicos. O primeiro (em anexo), intitulado ***Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis***, DOI 10.1016/j.fct.2012.11.013, o qual foi aceito pela revista *Food and Chemical Toxicology* em novembro de 2012 e publicado no volume 52 (2013) nas páginas 137–142. O segundo, intitulado ***Proteomic analysis identifies differentially expressed proteins after red propolis extract treatment in Hep-2 cells,*** o qual será submetido a uma revista internacional.

3.1 CAPÍTULO 1

Chemical Characterization, Antioxidant and Cytotoxic Activities of Brazilian Red Propolis

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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 agents.

Keywords: propolis, chemical characterization, antioxidants, cell line.

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 et al., 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, a 13th type, named red propolis, has been an important source of investigation since 2007 by local and international research groups. Besides differences in chemical composition, Franchi Jr. 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 Jr 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) and antitumoral (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, besides inhibiting oxidative chain reactions, chelating reactive metals, and repairing damage to biological molecules (Da Costa et al., 2012). The antitumoral 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 extracts from red propolis of Brazilian northeast, Sergipe. Moreover, this study also evaluated the antioxidant activity and the cytotoxic effect of red propolis extracts against well-known classic tumor cancer cell lines (Hep-2, HeLa), which was compared with 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 ($DPPH^{\bullet}$), 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, 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.

2.3 Preparation of red propolis extract

The twelve samples of red propolis was ground to a fine powder forming a pool and 1 g (dry weight) from it 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 fine red 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) LTQ Orbitrap XL 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$^{\bullet}$ radical scavenging Assay

DPPH$^{\bullet}$ 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$^{\bullet}$ 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$^{\bullet}$).

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/mLin EtOH - H_2O 50% (v/v). 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 Sod-

like units.

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 cultures

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 ºC, 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 ºC 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.

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 compounds 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 Jr 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 Figure 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 Jr 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) (Figure 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 molecules investigated, isoliquiritigenin and medicarpin showed potent activity against cancer cell lines as murine colon carcinoma (26-L5), murine melanoma (B16-BL6) and cervical human cancer (HeLa), whereas formononetin, biochanin A and liquiritigenin showed cytotoxic activity against the murine 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 $DPPH^{\bullet}$ scavenging ability. $DPPH^{\bullet}$ 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 $DPPH^{\bullet}$ (Moon and Shibamoto, 2009). Red propolis extract showed similar results (Table 2) to those obtained by Pinheiro (2009) with red propolis from the same geographic region, with IC50 of 294 μg/mL, confirming the potential $DPPH^{\bullet}$ scavenging.

Superoxide dismutase is an important enzyme that catalyzes the dismutation of

superoxide anion ($O2^{\bullet-}$) 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-293) established cell lines. Figure 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 Jr 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.

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 remains 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 (indirect) and clonogenic assays (direct). 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 statement

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

Acknowledgments

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References

Aebi, H., 1984. Catalase in vitro. Meth. 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, Brasil

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, 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 Alternat 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. Brasil

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. 2012, article ID 918956, DOI: 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, 4th ed. Clarendon Press, Oxford, UK.

Ishihara, M., Naoi, K., Hashita, M., Itoh, Y., Suzui, M., 2009. Growth inhibitory activity of ethanol extracts of Chinese and Brazilian propolis in four human colon carcinoma cell lines. Oncol. Rep. 22, 349-354.

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 physicochemical 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. Alternat. 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. C 54, 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.

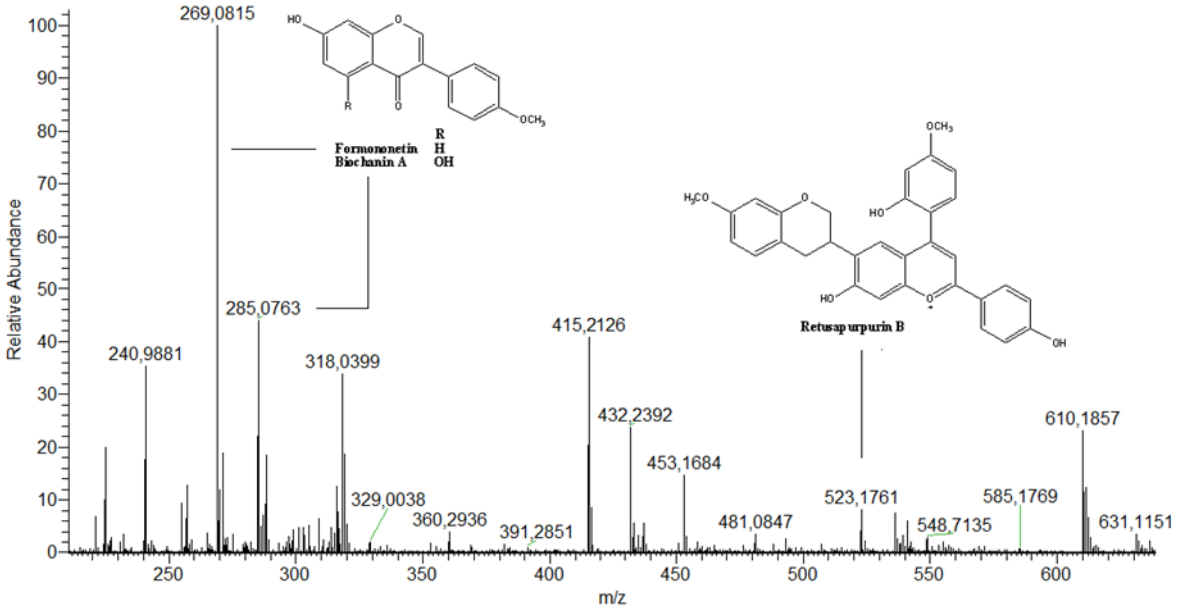


Figure 1. ESI(+)-MS Fingerprint for red propolis from Sergipe.

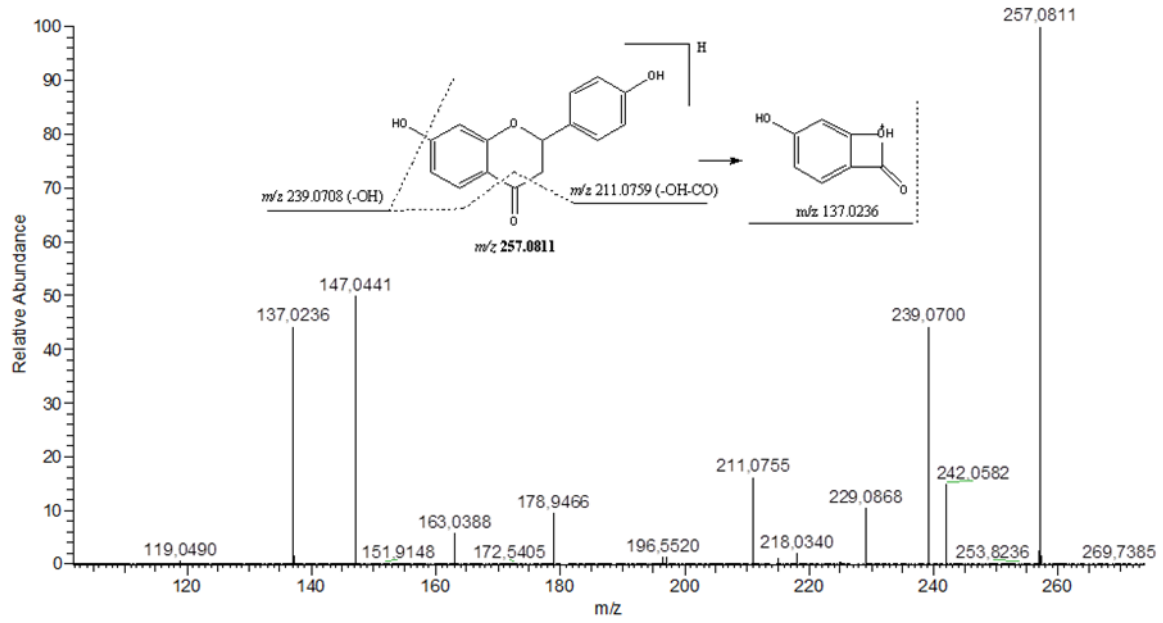
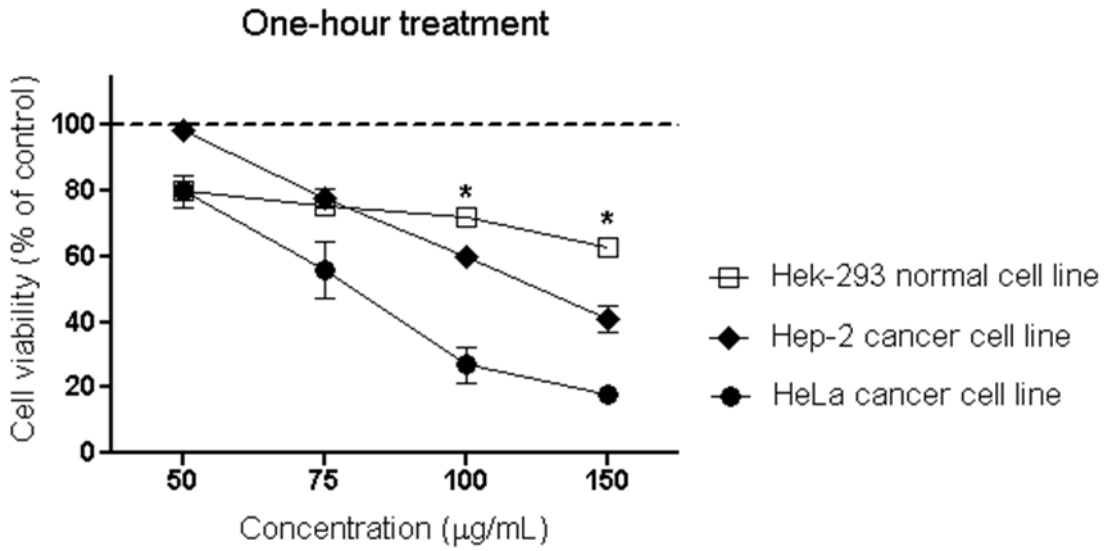


Figure 2. ESI(+)-MS/MS of liquiritigenin (*m/z* 257.0811).



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 ($p \leq 0.05$).

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

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)];	399.1220 [M-Ph-OH-OCH3]; 387.1244 [M-Ph-	(Piccinelli et al.,

					387.1244(100) [371.0919(100); 355.0979(30); 279.0645(30); 196.5436(5)]; 386.1164(30); 371.0919(15).	OCH_3-OCH_2]; 386.1164 $[C_{24}H_{18}O_5]^+$; 371.0919 $[C_{23}H_{15}O_5]^+$	2011)
8	611.1965 (10)	Hesperetin 7-rhamnoglucoside	$C_{28}H_{35}O_{15}$	1.792			(Sulaiman et al., 2011)

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.

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

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

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

3.2 CAPÍTULO 2

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

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Abstract

Here we investigated alterations in the protein profile of Hep-2 treated with red propolis using two-dimensional electrophoresis associated to mass spectrometry (MS). A total of 325 spots were manually excised from the two-gel electrophoresis and 177 proteins were identified using LC-TOF-MS. Among all proteins identified that presented differential expression (n°7), most were down-regulated, wich one were expressed in presence of red propolis extract at a concentration of 120 μg/mL (IC50), namely: GRP78, PRDX2, LDHB, VIM and TUBA1A. Only two up-regulated proteins we identified in this study, however in the non-cytotoxic (6 μg/mL) red propolis treated group: RPLP0 and RAD23B. Hep-2 cells treated with red propolis revealed 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 tumour 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.

Keywords: red propolis, Hep-2, proteome

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). The propolis's composition varies according to its botanical origin; generally contains resin, wax, pollens, 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)* that characterizes the propolis red color (Daugsch et al., 2008; Franchi Jr et al., 2012; Piccinelli et al., 2011; Silva, 2007). Red propolis has shown 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), and antitumor (Alencar et al., 2007; Li et al., 2008). The propolis antitumor activity has been widely studied (Valente et al., 2011). The resistance mechanisms to cancer drugs promote the development of new medicines with potential source of novel bioactive molecules (Umthong, 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 non tumour cell lines (Frozza et al., 2013). In the present study, we used 2-DE associated to mass spectrometry to reveal the comprehensive protein profiles of Hep-2 cells under a comparative investigation between the cells treated with and without red propolis extracts.

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% (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 analized 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 ºC, 5% CO_2 and 95% air. The study was performed when cells reached 70–80% confluence.

Cells (26×10^5) 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 lyses buffer (7 M (w/v) ureia, 2 M (w/v) thiourea, 40 mM (w/v) Tris base pH 8.5, 20 mM (w/v) DTT, 4% (w/v) CHAPS and 1% (w/v) protease inhibitor). After 30 min at room temperature, shaking at 90 rpm, the lysed were scrapped, put into microtubes and centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was collected and frozen at -20 °C. Procedure was repeated at least three times.

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 (w/v) urea, 2 M (w/v) tiourea, 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 14,000 rpm 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 ºC 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 (w/v) urea, 30% (v/v) glycerol, and 1% (w/v) DTT and for 15 min in the same buffer but with DTT replaced by 4% 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 using an ImageScanner III (GE Healthcare, USA), digitalized with LabScan^{TM} v. 6.0 software and analyzed with the ImageMaster^{TM} 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 an Eppendorf tube, and washed twice with 50% (v/v) acetonitrile and 25 mM (w/v) 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 (w/v) 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) (Ultima API Mass Spectrometer, Waters Micromass, Milford, USA) coupled to a capillary liquid chromatography system (CapLC, 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.

3. Results

Cells to each group were harvested and lysed 3 times to obtein a pool of protein extractation and at least triplicates of gels were performed for each group of cell extract. Samples were initially loaded in pH 3-10 gels and 2-DE maps confirmed more concentrated pattern of spots at pH 4-7. Proteins spots were resolved in each of the 9 coomassie brilhant blue (CBB) stained 2-DE SDS-PAGE gels with pH 4-7 IPG strips and molecular weights ranging from 10 – 250 kDa. Image analysis of the 2-DE maps taken from LabScan^{TM} v. 6.0 and ImageMaster^{TM} 2D Platinum v. 7.0 softwares (GE Healthcare, USA) indicated good resolution and reproducibility. The software indicated different patterns of proteins in the groups treated with red propolis extract compared to the control group.

Spots were manually excised from 3 groups of 2-DE map gels (**Figure 1**) that received different concentration of red propolis extract. Altogether, 325 spots were excised and submitted to identification. All spots from group A (control) were excised (n= 251). Only differentially expressed spots from the treated groups (B, C) were submitted to identification according to differences in protein profiles observed in the ImageMaster^{TM} 2D Platinum software. Therefore, 65 spots were excised from group B and only 9 spots from group C. The identified proteins are listed in **Table 1** and **Table 2.**

Altogether, MS results identified 177 proteins out of 325 spots. The molecular mass (MW) and the isoeletric 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 **Table 1**. Many of the gel-estimated MW/pI corresponded well to the theoretical values but when experimentally determined MW and/or pI values were significantly distinct from the predicted ones; this could be explained by a relatively high level of post-translational

modification. 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 (**Table 1**). The proteins from control are presented in **Figure 2** 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). 8% were not 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 2**.

Five proteins were identified as down-regulated in the group treated with 120 μg/mL of hydroalcoholic extract of red propolis (Figure 1-C) compared to the control group (Figure 1-A): 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 (Figure 1-B) using non-cytotoxic concentrations of extract (6 μg/mL) compared to the control group (Figure 1-A): 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 **Table 1** and **Table 2** and differential expressed proteins are seen in **Figure 1** and **Figure 3,** at a maximized perspective.

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 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. So far, however, there are no reports using proteomics with Brazilian red propolis in tumor cell lines.

Altogether 325 spots from both control and treated groups were excised, in-gel digested with trypsin, and 177 proteins were identified. All spots 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^{TM} 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 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 2**).

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. Both HSp27 and Hsp70 expressions were reduced in human breast adenocarcinoma adriamycin-resistant and paclitaxel-resistant cell lines in comparison to the parental cell ones (Chuthapisith et al., 2007). HSp27 showed down-regulation 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). Microenviroment of tumor cells may be similar to a physiological stress condition and cells manage to acquire a survival function (Koumenis, 2006), PRDX2 encodes a member of the peroxiredoxin family of antioxidant enzymes (PRDX 1–6), which play important roles in maintaining the intracellular redox homeostasis (Shen et al., 2012a; Shen et al., 2012b). Peroxiredoxins have been linked with regulation of proliferation, differentiation, and apoptosis (Park et al., 2006). Peroxiredoxin 2 has been attributed to have both proliferative and antiapoptotic functions, inducing carcinogenic changes upon treatment (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. Glioma cells in which PRDX2 was decreased by RNA silencing exhibit increased hyperoxidation, suggesting that the redox environment is more oxidizing, 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 upregulated 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 protective role against radiation induced oxidative stress. Stresing et al (2012) have demonstrated that overexpression of PRDX2 in lung metastatic cells effectively removes the intracellular ROS, suggesting that this phenotype is helpful 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 Reactive Oxygen Species (ROS) in cancer cells is important for developing successful therapy, in which most of the anticancer agents induce ROS generation in order to kill cancer cells by apoptosis through common molecular pathways (Wang et al., 2005). 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. Human cancer cells cultured under hypoxic conditions convert glucose to lactate and extrude it, whereas aerobic cancer cells take up lactate via monocarboxylate transporter 1 (MCT1) and utilize it for oxidative phosphorylation by an oxygen-dependent expression of LDHB (Semenza, 2008). Lactate dehydrogenase (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 advanced melanomas compared with nevi (Ho et al., 2012). LDH is also expressed in breast tumors (Hussien and Brooks, 2011), gastric tumors (Liu et al., 2009), and melanoma (Ho et al., 2012).

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 a selective diversion of pyruvate metabolism from the tricarboxylic acid cycle to lactic acid (Liu et al., 2009). Aerobic lactate production of which the final step is executed by lactate dehydrogenase is one of the typical phenotypes in invasive tumor development, and suppression of LDHB expression plays critical 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 stomal cells, regulation of its expression levels have been recently associated to many epithelial transformed tumor cells. VIM is frequently overexpressed in tumours undergoing epithelial to mesenchymal transition (EMT), a condition correlated with invasiveness and poor prognosis (Bozzuto et al., 2010). EMT is a key process during embryonic development, also associated to an invasive cancer phenotype (Gavert and Ben-Ze'ev, 2008), characterized by a change in the

morphology with loss of polarity and cell contacts by the epithelial cells with increased vimentin expression and concomitant decrease of E-cadherin (Chen et al., 2010).

Vimentin expression in epithelial tumours is considered a marker of de-differentiation and aggressiveness, which has been recently indicated as a novel therapeutic target (Lahat et al., 2010). At functional levels, however, the down-regulation of vimentin here observed seems to correlate 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 virinostat 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: mitotic progression, proper positioning of the nucleus, cell motility, actin filament growth, organelle biogenesis, mitochondrial tabulation, among others. The α and β-tubulins are the building blocks of the cytoskeletal microtubules (Sahab et al., 2012). Identification of differential modifications in tubulin is especially important in the research area of cancer therapeutics. These proteins are crucial in cell division, which make them attractive targets for anticancer drug design, Drugs based on natural products that target the tubulin and the microtubule system, also referred to as anti-mitotics, remain an important component in combination chemotherapy for the treatment of many malignancies (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. Together with other recognition factors like XPA, RPA and the TFIIH complex, RAD23B is part of the pre-incision (or initial recognition) process of DNA repair. The XPC complex recognizes a wide spectrum of damaged DNA characterized by distortions of the DNA helix. RAD23B dimer preferentially binds to platinum based chemotherapeutic agents, like cysplatin and UV-damaged double-stranded DNA (Janicijevic et al., 2003; Sugasawa et al., 2009). A recent model in which XPC–RAD23B is the actual damage sensor has been proposed. After recognition, RAD23B exposes the XPC damage–binding sites by dissociating from it, allowing XPC (without RAD23B) to stably bind the DNA lesions (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 60 S acidic ribosomal protein P0 (RPLP0) on the 2-DE pattern of non-apoptotic cell group. The

pathways for programmed cell death, or apoptosis, are presumably activated when other cellular response pathways will not be sufficiently efficacious (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 contribute significantly to the malignant phenotype, including deregulation of tumour-cell growth, apoptosis and invasiveness (Ji et al., 2007). Phosphor 60S acidic ribosomal protein P0 was suppressed by the introduction of oxythiamine (OT) into pancreatic cells, OT has been demonstrated in many studies to inhibit cancer cell growth through suppression 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 60 S ribosomal compartment (L-14A) and up-regulation of RPLP0.

Tumour suppressor genes and proto-oncogenes have been found either to affect the formation of the mature ribosome or to regulate the activity of proteins known as translation factors (Ruggero et al., 2003; Ruggero and Pandolfi, 2003). It seems that the disruption in one or more of the steps that control protein biosynthesis has been associated with alterations in the cell cycle and regulation of cell growth, thus certain tumour suppressors and proto-oncogenes might regulate to malignant progression by altering the protein synthesis machinery.

In summary, upon red propolis treatment in Hep-2 cells, we were able to identify candidate proteins that participate of the regulatory metabolism of laryngeal cell lines. 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 tumour 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. It is clear that red propolis interacts with a whole set of intracellular events and turns to be a promising and suitable 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 in the larynx. Cell-based proteomic assays are particularly valuable when searching for therapeutic agents once they can reveal activity against a particular molecular target and 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 trough immunofluorescence and western blot analysis will contribute to elucidate the antioxidant and anticancer activities reported.

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.

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.

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.

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.

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.

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

Frozza, C.O.S., Garcia, C.S.C., Gambato, G., Souza, M.D.O.S., Salvador, M., Moura, S., Padilha, F.F., Seixas, F.K., Collares, T., Borsuk, S., Dellagostin, O.A., Henriques, J.A.P., 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. The transcriptional repressor Delta EF1 controls the EMT phenotype and resistance to the EGFR inhibitor erlotinib in human head & 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.

Kavallaris, M., 2010. Microtubules and resistance to tubulin-binding agents. Nat Rev Cancer 10, 194-204.

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.

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.

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, 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, J., Lee, A.S., 2006. Stress induction of GRP78/BiP and its role in cancer. Curr Mol Med 6, 45-54.

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.

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. 1(2): 1-5.

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.

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.

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.

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.

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.

Ruggero, D., Grisendi, S., Piazza, F., Rego, E., Mari, F., Rao, P.H., Cordon-Cardo, C., Pandolfi, P.P., 2003. Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. Science 299, 259-262.

Ruggero, D., Pandolfi, P.P., 2003. Does the ribosome translate cancer? Nat Rev Cancer 3, 179-192.

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.

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.

Shen, K., Cui, D., Sun, L., Lu, Y., Han, M., Liu, J., 2012a. Inhibition of IGF-IR increases chemosensitivity in human colorectal cancer cells through MRP-2 promoter suppression. J Cell Biochem 113, 2086-2097.

Shen, K., Han, X., Lu, X., 2012b. Cationic Pd(II)-catalyzed highly enantioselective arylative cyclization of alkyne-tethered enals or enones initiated by carbopalladation of alkynes with arylboronic acids. Org Lett 14, 1756-1759.

Silva, M.S.C., 2007. Estudo do efeito da fração BRVD obtida a partir da própolis brasileira tipificada, na proliferação de células tumorais, 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.

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.P., Preecha; Puthong, Songchan and Chanchao, Chanpen 2011. In vitro antiproliferative activity of partially purified Trigona laeviceps propolis from Thailand on human cancer cell lines. Complementary and Alternative Medicine 11.

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.

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.

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.

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, 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.

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.

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.

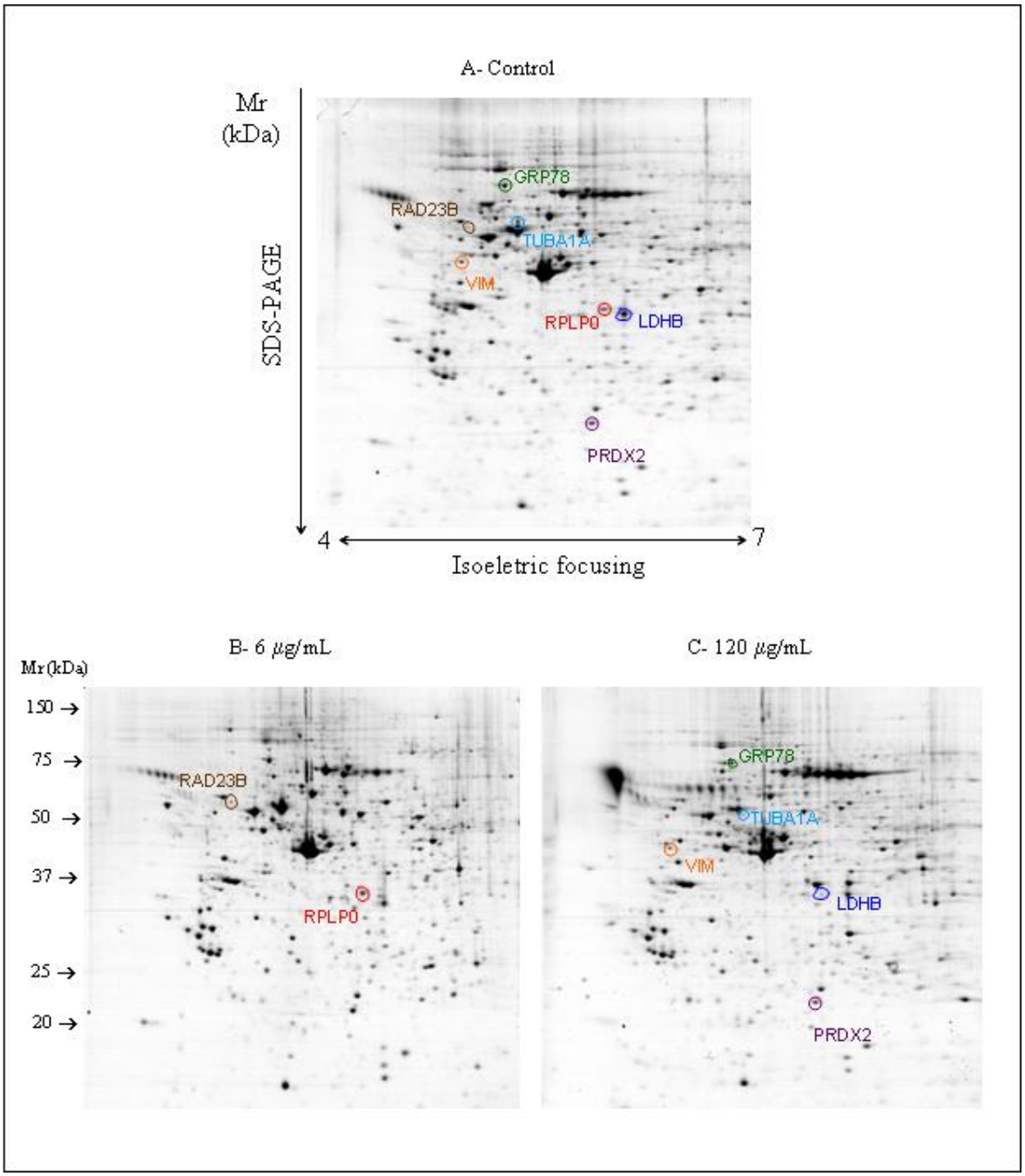
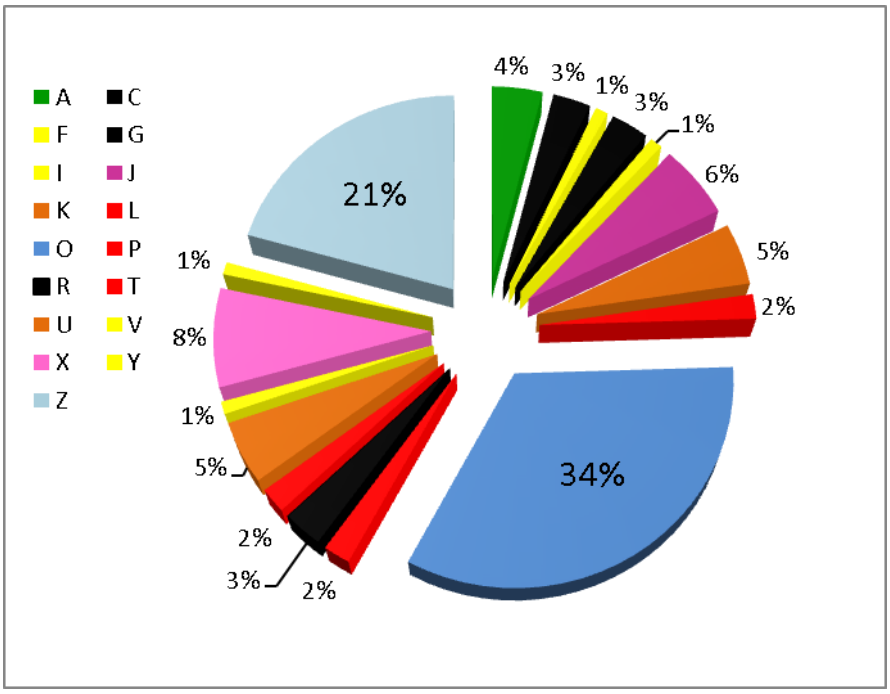


Figure 1: 2-DE profiles of the control (A) and red propolis treated group (B, 6 µg/mL; C, 120 µg/mL). Total protein extracts were separated on pH 4-7, 17 cm IPG strips, following by 12% SDS-PAGE and staining with CBB R-250.



***A** (RNA processing and modification); **C** (Energy production and conversion); **F** (Nucleotide transport and metabolism); **G** (Carbohydrate transport and metabolism**); I** (Lipid transport and metabolism); **J** (Translation, ribosomal structure and biogenesis); **K** (Transcription); **L** (Replication, recombination and repair); **O** (Posttranslational modification, protein turnover, chaperones); **P** (Inorganic ion transport and metabolism); **R** (General function prediction only**); T** (Signal transduction mechanisms); **U** (Intracellular trafficking, secretion, and vesicular transport); **V** (Defense mechanisms); **X** (Null); **Y** (Nuclear structure); **Z** (Cytoskeleton).

Figure 2: Functional distribution of the identified proteins by 2-DE SDS-PAGE found in control group of Hep-2 cell line (KOG- EuKaryotic Orthologous Group).

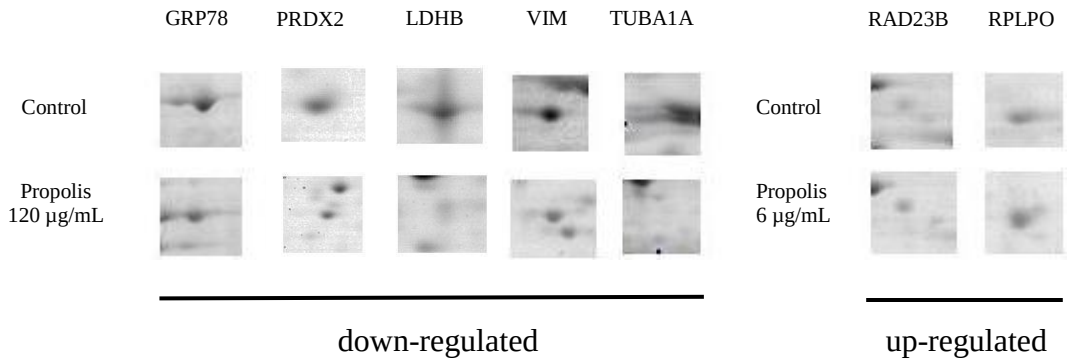


Figure 3: Magnified view expression profile of the 7 significant altered proteins: 5 down-regulated in the C panel from Figure 1 and 2 up-regulated in the B panel form Figure 1.

Table 1: Identified proteins from Hep-2 cells by MS.

Spot Number	Name Description Protein ID	Acession Number	Mascot Score	Theor. MW (Da)	Theor. pI	(%)	Matched Pept.	KOG
7	calreticulin precursor	gi|4757900	105	48283	4.29	9	4	O
11	calumenin	gi|2809324	414	37164	4.47	29	8	TU
13	nascent-polypeptide-associated complex alpha polypeptide	gi|62897731	335	23344	4.52	25	5	K
16	hCG1983058	gi|119619436	159	25031	4.59	28	4	X
17	proliferating cell nuclear antigen	gi|4505641	256	29092	4.57	24	4	L
18	tropomyosin alpha-1 chain isoform 5	gi|27597085	506	32846	4.69	35	13	Z
19	tropomyosin alpha-4 chain isoform 2	gi|4507651	625	28619	4.67	42	13	Z
20	tropomyosin alpha-3 chain isoform 2	gi|24119203	354	29243	4.75	29	7	Z
21	14-3-3 protein epsilon	gi|5803225	451	29326	4.63	37	9	O
22	14-3-3 protein zeta/delta	gi|4507953	452	27899	4.73	30	5	O
23	14-3-3 protein theta	gi|5803227	410	28032	4.68	29	5	O
24	14-3-3 protein zeta/delta	gi|4507953	524	27899	4.73	37	8	O
25	14-3-3 protein zeta/delta	gi|4507953	344	27899	4.73	30	5	O
26	14-3-3 protein beta/alpha	gi|4507949	154	28179	4.76	26	4	O
27	tyrosine 3-monooxygenase tryptophan 5 - monooxygenase activation protein beta polypeptide	gi|377656701	399	28076	4.76	34	7	O
28	eukaryotic translation initiation factor 6 isoform a	gi|4504771	254	27095	4.56	26	4	J
33	RNA polymerase II subunit	gi|1017823	131	17203	4.50	24	2	K
34	smooth muscle myosin alkali light chain	gi|467828	66	17772	4.57	14	2	Z
35	smooth muscle myosin alkali light chain	gi|467828	193	17772	4.57	23	4	Z

36	tumor rejection antigen (gp96) 1 variant	gi|62088648	95	66140	5.08	12	5	O
37	nucleolin	gi|189306	90	76355	4.59	4	2	K
38	tumor rejection antigen (gp96) 1 variant	gi|62088648	107	66140	5.08	10	4	O
39	heat shock protein gp96 precursor	gi|15010550	463	90309	4.73	19	11	O
40	nucleolin	gi|189306	203	76355	4.59	13	6	K
41	nucleolin	gi|189306	182	76355	4.59	12	6	K
42	90kDa heat shock protein	gi|306891	187	83584	4.97	5	3	O
44	tropomyosin alpha-4 chain isoform 2	gi|4507651	139	28619	4.67	15	4	Z
45	90kDa heat shock protein	gi|306891	226	83584	4.97	7	4	O
46	90kDa heat shock protein	gi|306891	141	83584	4.97	11	6	O
47	90kDa heat shock protein	gi|306891	327	83584	4.97	12	7	O
49	90kDa heat shock protein	gi|306891	341	83584	4.97	12	7	O
50	90kDa heat shock protein	gi|306891	507	83584	4.97	18	11	O
51	heat shock protein HSP 90-beta	gi|20149594	940	83554	4.97	26	18	O
52	90kDa heat shock protein	gi|306891	491	83584	4.97	23	15	O
53	thyroid hormone binding protein precursor	gi|339647	156	57468	4.82	16	5	O
54	78 kDa glucose-regulated protein precursor	gi|16507237	374	72402	5.07	18	8	O
58	tubulin, beta	gi|18088719	522	50096	4.75	32	11	Z
59	tubulin, beta	gi|18088719	891	50096	4.75	35	13	Z
60	90kDa heat shock protein	gi|306891	248	83584	4.97	8	5	O
61	alpha-tubulin	gi|37492	103	50810	5.02	11	3	Z
62	vimentin	gi|340219	441	53738	5.03	26	11	Z
64	vimentin	gi|340219	370	53738	5.03	25	9	Z
67	UV excision repair protein RAD23 homolog B isoform 1	gi|4506387	112	43202	4.79	16	4	L
69	laminin-binding protein	gi|34234	257	31888	4.84	20	5	J
70	ribonuclease inhibitor	gi|21361547	114	51766	4.71	6	2	A
71	B23 nucleophosmin	gi|825671	53	31090	4.71	20	2	A
72	vimentin	gi|340219	183	53738	5.03	12	4	Z

73	tubulin, beta	gi|18088719	1002	50096	4.75	45	17	Z
74	alpha-tubulin	gi|340021	469	50804	494	21	6	Z
75	vimentin	gi|340219	965	53738	5.03	38	14	Z
76	ATP synthase subunit beta, mitochondrial precursor	gi|32189394	927	56525	5.26	42	14	C
77	protein disulfide isomerase-related protein 5	gi|1710248	463	46512	4.95	26	7	O
78	vimentin	gi|340219	243	53738	5.03	22	8	Z
79	vimentin	gi|340219	274	53738	5.03	26	10	Z
80	vimentin	gi|340219	567	53738	5.03	29	11	Z
83	NSFL1 cofactor p47 isoform a	gi|20149635	123	40548	4.99	22	5	O
84	chain C, Human Pcna	gi|2914385	147	29072	4.53	16	2	L
85	laminin-binding protein	gi|34234	322	31888	4.84	32	6	J
88	TPMsk3	gi|19072649	158	28906	4.72	19	4	Z
89	C protein	gi|306875	81	32004	5.10	9	2	U
90	14-3-3 protein epsilon	gi|5803225	368	29326	4.63	25	7	O
91	tropomyosin beta chain isoform 2	gi|47519616	145	33027	4.63	19	4	Z
92	14-3-3 protein theta	gi|5803227	342	28032	4.68	25	4	O
93	14-3-3 protein zeta/delta	gi|4507953	290	27899	4.73	30	5	O
94	14-3-3 protein zeta/delta	gi|4507953	551	27899	4.73	34	6	O
95	proteasome subunit alpha type-5	gi|7106387	129	26565	4.74	19	3	O
96	14-3-3 protein beta/alpha	gi|4507949	285	28179	4.76	26	4	O
97	nucleophosmin isoform 1	gi|10835063	185	32726	4.64	18	3	A
98	B23 nucleophosmin (280 AA)	gi|825671	227	31090	4.71	22	4	A
100	HSPC263	gi|6841176	113	31951	4.90	21	4	O
101	human elongation factor-1-delta	gi|38522	213	31316	4.95	17	3	K
102	thioredoxin-like protein 1	gi|4759274	61	32630	4.84	13	2	O
103	chain A, human annexin V with incorporated methionine analogue azidohomoalanine	gi|342350777	609	35811	4.89	45	12	U

104	WD repeat-containing protein 61	gi|13376840	118	33731	5.16	12	2	R
106	rho GDP-dissociation inhibitor 1 isoform a	gi|4757768	174	23250	5.02	14	3	T
107	C protein	gi|306875	126	32004	5.10	16	4	U
108	C protein	gi|306875	114	32004	5.10	8	2	U
111	transcription intermediary factor 1-beta	gi|5032179	112	90261	5.52	5	3	K
112	alpha actinin 4	gi|2804273	136	102661	5.27	5	3	Z
113	90kDa heat shock protein	gi|306891	231	83584	4.97	13	7	O
114	90kDa heat shock protein	gi|306891	196	83584	4.97	9	5	O
115	90kDa heat shock protein	gi|306891	178	83584	4.97	7	4	O
116	eukaryotic translation initiation factor 5A-1 isoform B	gi|4503545	40	17049	5.08	30	3	J
118	human Cu, Zn superoxide dismutase	gi|349905	84	15861	5.70	18	2	P
119	stathmin isoform a	gi|5031851	252	17292	5.76	50	10	X
120	nucleoside diphosphate kinase A isoform b	gi|4557797	141	17309	5.83	34	4	F
121	chain A, human dutp pyrophosphatase complex with dudp	gi|34810576	173	16322	6.77	42	4	F
122	thiol-specific antioxidant protein	gi|438069	48	22014	6.84	19	3	O
123	glutathione S-transferase	gi|2204207	223	23595	5.43	21	3	O
128	cofilin-1	gi|5031635	110	18719	8.22	18	2	Z
130	protein DJ-1	gi|31543380	101	20050	6.33	30	4	RV
131	chain A, horf6 a novel human peroxidase enzyme	gi|3318841	94	25011	6.00	22	4	X
137	alpha-enolase isoform 1	gi|4503571	503	47481	7.01	28	9	G
138	L-lactate dehydrogenase B chain	gi|4557032	314	36900	5.71	19	5	C
139	proteasome activator complex subunit 1 isoform 1	gi|5453990	100	28876	5.78	15	3	O
141	proteasome (prosome, macropain) activator subunit 2 (PA28 beta), isoform CRA_b	gi|119586505	159	22023	5.40	13	2	O
142	prohibitin	gi|4505773	302	29843	5.57	34	7	O

143	60S acidic ribosomal protein P0	gi|4506667	219	34423	5.71	26	6	J
145	BTB/POZ domain-containing protein KCTD12	gi|19923973	46	35964	5.51	11	2	P
146	regulation of nuclear pre-mRNA domain-containing protein 1B	gi|11034845	60	36991	5.73	9	2	K
147	L-lactate dehydrogenase B chain	gi|4557032	56	36900	5.71	4	1	C
149	F-actin-capping protein subunit alpha-1	gi|5453597	90	33073	5.45	19	4	Z
151	actin, cytoplasmic 1	gi|4501885	710	42052	5.29	34	38	Z
152	actin, cytoplasmic 1	gi|4501885	676	42052	5.29	34	36	Z
153	actin, cytoplasmic 1	gi|4501885	429	42052	5.29	32	11	Z
154	actin, cytoplasmic 1	gi|4501885	387	42052	5.29		10	Z
155	eukaryotic initiation factor 4A-I isoform 1	gi|4503529	370	46353	5.32	23	7	J
157	actin, cytoplasmic 1	gi|4501885	404	42052	5.29	32	11	Z
162	alpha-tubulin	gi|340021	243	50804	4.94	18	5	Z
163	peptidyl-prolyl cis-trans isomerase FKBP4	gi|4503729	233	52057	5.35	18	6	O
164	transformation upregulated nuclear protein	gi|460789	393	51325	5.13	24	9	A
165	T-complex protein 1 subunit epsilon	gi|24307939	217	60089	5.45	12	6	X
166	transformation upregulated nuclear protein	gi|460789	684	51325	5.13	24	8	A
167	chaperonin (HSP60)	gi|306890	213	61157	5.70	22	8	O
168	importin subunit alpha-2	gi|4504897	135	58168	5.25	12	4	U
169	alpha-tubulin	gi|340021	366	50804	4.94	25	8	Z
170	lamin-B1 isoform 1	gi|5031877	274	66653	5.11	21	12	Y
171	heat shock cognate 71 kDa protein isoform1	gi|5729877	677	71082	5.37	26	14	O
172	heat shock cognate 71 kDa protein isoform1	gi|5729877	1181	71082	5.37	34	18	O
173	heat shock cognate 71 kDa protein isoform1	gi|5729877	454	71082	5.37	21	11	O
174	HSP70-2	gi|4529892	644	70267	5.48	24	13	OT
175	heat shock 70kDa protein 9 (mortalin)	gi|12653415	694	73967	6.03	23	11	O
176	chaperonin (HSP60)	gi|306890	1060	61157	5.70	36	19	O
177	PRO2044	gi|6650826	125	30084	6.97	5	2	X

178	PRO2044	gi|6650826	124	30084	6.97	5	2	X
179	albumin, isoform CRA_t	gi|119626083	132	60211	6.66	4	3	X
180	albumin, isoform CRA_t	gi|119626083	139	60211	6.66	4	3	X
181	chain A, tapasinERP57 heterodimer	gi|220702506	350	54541	5.61	15	7	X
182	t-complex polypeptide 1	gi|36796	228	60869	6.03	13	4	X
183	t-complex protein 1 subunit beta isoform 1	gi|5453603	584	57794	6.01	34	12	X
184	heterogeneous nuclear ribonucleoprotein H	gi|5031753	266	49484	5.89	13	5	A
186	translation initiation factor eIF3 p44 subunit	gi|3264859	161	35959	6.10	16	3	J
188	human rab GDI	gi|285975	145	51088	5.94	15	4	O
190	2-phosphopyruvate-hydratase alpha-enolase	gi|693933	320	47421	7.01	18	5	G
191	alpha-enolase isoform 1	gi|4503571	345	47481	7.01	23	7	G
192	actin-related protein	gi|381964	39	42659	6.35	8	2	Z
193	succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial precursor	gi|4557817	72	56578	7.14	9	2	X
195	glucose-6-phosphate dehydrogenase	gi|26224790	67	55188	6.91	6	2	G
220	F-actin-capping protein subunit beta isoform 1	gi|4826659	142	30952	5.69	17	5	Z
221	annexin A3	gi|4826643	466	36524	5.63	31	10	U
223	proteasome activator complex subunit 3 isoform 2	gi|30410796	232	31038	5.79	15	5	O
224	dnaJ homolog subfamily C member 9	gi|27597059	101	30062	5.58	24	4	O
226	exosome complex component MTR3	gi|17402904	108	28503	6.06	11	2	J
228	glutathione S-transferase omega-1 isoform 1	gi|4758484	107	27833	6.23	18	4	O
229	endoplasmic reticulum resident protein 29 isoform 1 precursor	gi|5803013	122	29032	6.77	16	3	R
230	ran-specific GTPase-activating protein	gi|4506407	50	23467	5.19	5	2	U
231	proteasome subunit alpha type-3 isoform 1	gi|4506183	114	28643	5.19	18	4	O
233	26S protease regulatory subunit 7 isoform 1	gi|4506209	321	49002	5.71	18	6	O
235	alpha-enolase isoform 1	gi|4503571	157	47481	7.01	14	4	G

237	leukocyte elastase inhibitor	gi|13489087	53	42829	5.90	13	4	P
240	CKB	gi|49457530	323	42933	5.34	20	3	C
244	actin, cytoplasmic 1	gi|4501885	349	42052	5.29	20	7	Z
245	eukaryotic translation initiation factor 3 subunit F	gi|4503519	191	37654	5.24	15	5	J
249	chain A, 3.10 a crystal structure of maspin, space group P 4 21 2	gi|56554671	150	43690	5.99	14	4	R
250	acyl-CoA thioesterase 8	gi|13929451	38	10080	5.37	60	4	I
251	eukaryotic translation initiation factor 2 subunit 1	gi|4758256	172	36374	5.02	18	5	J
252	trafficking protein particle complex 2, isoform CRA_c	gi|119619239	37	16373	5.84	60	6	U
258	5Sa/antisecretory factor protein, partial	gi|2351562	109	33206	4.75	18	3	X
261	thyroid hormone binding protein precursor	gi|339647	259	57468	4.82	25	9	O
268	78 kDa glucose-regulated protein precursor	gi|16507237	556	72402	5.07	29	15	O
276	transformation upregulated nuclear protein	gi|460789	63	51325	5.13	7	2	A
285	triosephosphate isomerase isoform 1	gi|4507645	296	26938	6.45	32	6	G
289	proteasome activator complex subunit 3 isoform 2	gi|30410796	193	31038	5.79	11	2	O
292	L-lactate dehydrogenase B chain	gi|4557032	210	36900	5.71	12	3	C
293	L-lactate dehydrogenase B chain	gi|4557032	217	36900	5.71	16	4	C
297	eukaryotic initiation factor 4A-I isoform 1	gi|4503529	352	46353	5.32	22	6	J
298	alpha-tubulin	gi|340021	151	50804	4.94	19	5	Z
306	vimentin	gi|340219	294	53738	5.03	20	9	Z
307	vimentin	gi|340219	63	53738	5.03	9	3	Z
309	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide, isoform CRA_b	gi|119596301	81	24717	7.66	14	2	O
310	14-3-3 protein epsilon	gi|5803225	181	29326	4.63	21	4	O

311	14-3-3 protein theta	gi|5803227	216	28032	4.68	20	3	O
314	thiol-specific antioxidant protein	gi|438069	125	22014	6.84	19	4	O
321	UV excision repair protein RAD23 homolog B isoform 1	gi|4506387	139	43202	4.79	7	2	L
323	60S acidic ribosomal protein P0	gi|4506667	248	34423	5.71	22	4	J
324	chain A, crystal structure of human Eif3k	gi|55669683	138	26273	5.21	18	3	J
325	chain A, crystal structure of protein phosphatase 2a (Pp2a) holoenzyme with the catalytic subunit carboxyl terminus truncated	gi|122921194	125	65173	5.07	9	3	X

Table 2: 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	0.400	0.188	2.12	O	78 kDa glucose-regulated protein precursor
PRDX2	0.264	0.206	1.28	O	thiol-specific antioxidant protein
LDHB	0.532	0.091	5.84	C	L-lactate dehydrogenase B chain
VIM	0.162	0.112	1.45	Z	vimentin
TUBA1A	0.198	0.028	7.07	Z	alfa-tubulin
UP-REGULATED					
RAD23B	0.069	0.087	0.79	L	UV excision repair protein RAD23 homolog B isoforms 1
RPLP0	0.247	0.320	0.77	J	60 acidic ribosomal protein P0

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 hydroalcoolic solution instead of propolis.
e)Propolis treated groups: 120 μg/mL (IC50) and 6 μg/mL (non cytotoxic).

3.3 Discussão Geral

Este é o primeiro trabalho que investiga os principais compostos da própolis vermelha de Sergipe pela técnica de espectrometria de massas de alta resolução e que confirma efeitos antioxidantes do extrato e citotóxicos contra células cancerosas. A identificação química aqui apresentada reforça a complexidade de compostos encontrados no extrato da própolis vermelha, por exemplo, flavonóides como a formononetina, liquiritigenina, medicarpina e biochanina A. Os flavonoides são considerados os principais compostos responsáveis pelos efeitos benéficos observados pela ação da própolis, sendo a eles atribuídas as propriedades antibacteriana, antiviral e antioxidante (Barbosa, 2009). Li *et al.* (2008) investigaram as atividades citotóxicas *in vitro* de vários compostos isolados de própolis vermelha brasileira. Entre as moléculas investigadas, isoliquiritigenina e medicarpina mostraram uma potente atividade contra as linhagens celulares de câncer como 26-L5, B16-BL6 e HeLa, enquanto que a formononetina, biochanina A e liquiritigenina demonstraram atividade citotóxica contra a célula tumoral de cólon murino 26-L5.

As concentrações de própolis vermelha mostraram-se mais citotóxicas nas células tumorais, Hep-2 e HeLa, respectivamente 128,12 ± 5,33 e 85,77 ± 1,00 μg/mL, quando comparadas a célula não tumoral Hek-293 (> 150 μg/mL), indicando uma possível seletividade para as células tumorais, a qual ficou mais evidente no tratamento de 24 h. O trabalho de Valente *et al.* (2011) corrobora com estes resultados, uma vez que ele relata que extratos metanóicos de própolis portuguesa também apresentaram seletividade para células tumorais em células de cultura primária. Moraes *et al.* (2010) também constataram que extratos da própolis apresentaram maior inibição do crescimento das células primárias de câncer da próstata quando comparadas a células normais da próstata.

O ensaio clonogênico se mostrou como mais um método de comprovação da

atividade inibitória da própolis vermelha, a qual apresentou citotoxicidade de 100% em células Hep-2 a partir da concentração de 10 µg/mL. Ou seja, o tratamento de 24 h com esta concentração de própolis vermelha impediu o crescimento de qualquer colônia. A citotoxicidade e a seletividade são parâmetros importantes na prevenção e tratamento do câncer, visto a necessidade do desenvolvimento de novos produtos a partir de fontes naturais que sejam efetivos, porém que não apresentem efeitos indesejáveis ao corpo humano.

O efeito inibitório no crescimento celular de células cancerosas pode estar relacionado à presença de compostos fenólicos encontrados em cada extrato, que varia de acordo com a região, clima e ano em que o material foi coletado. Foi comprovado através do ensaio Folin-Ciocalteu que o extrato hidroalcoólico da própolis vermelha de Sergipe possui na sua constituição um significante conteúdo de polifenóis totais (151,55 ± 1,95 mg/g de extrato seco). Através dos ensaios colorimétricos, também se pode observar que a própolis possui capacidade antioxidante de varrer o radical livre $DPPH^{\bullet}$ e apresenta atividade enzimática Sod (466,90 ± 12,40 USod-*like*) e Cat-*like* (13,13 ± 2,65 mmol de H_2O_2 decomposto/min). A enzima superóxido dismutase catalisa a dismutação do ânion superóxido em oxigênio e peróxido de hidrogênio, enquanto que a catalase converte o peróxido de hidrogênio em água e oxigênio molecular (Halliwell & Gutteridge, 2007). As enzimas antioxidantes desempenham importante papel no equilíbrio redox e na diminuição do estresse oxidativo, o qual está envolvido no desenvolvimento de várias doenças como as neurodegenerativas, vasculares, cânceres, entre outras.

A análise proteômica foi realizada utilizando as concentrações 6 µg/mL (não citotóxica) e 120 µg/mL (IC50) de extrato hidroalcoólico de própolis vermelha em células tumorais Hep-2. Os resultados indicam padrões proteicos diferentes quando comparado ao controle que recebeu a mesma quantidade de solução hidroalcoólica. Até o momento, este estudo é mostra-se pioneiro ao utilizar a própolis vermelha do nordeste brasileiro associada à

separação de células tumorais por gel de poliacrilamida bidimensional e espectrometria de massas. Por meio da análise de 9 géis utilizando o programa ImageMaster^{TM} 2D Platinum 7.0, foram identificados os *spots* de proteínas dos quais 325 foram cortados manualmente e 177 proteínas identificadas. As proteínas identificadas foram relacionadas com diversos processos metabólicos, como produção e conversão de energia, transporte e metabolismo de carboidratos, proteínas do citoesqueleto, proteínas de reparo, entre outros. Contudo, a maior porcentagem ficou classificada dentro do grupo relacionado à modificação pós-traducional, reciclagem de proteínas e chaperonas (letra O na classificação feita pelo KOG). Das proteínas identificadas com expressão diferencial, cinco apresentarm sua expressão reduzida na presença do extrato da própolis (120 μg/mL): GRP78, PRDX2, LDHB, VIM, TUBA1A. A proteína GRP78 modula as funções do retículo endoplasmático apresentando propriedades pró-sobrevivência e antiapoptótica (Wang *et al*., 2009; Chiu *et al.*, 2008). Acredita-se que ela possa interagir na ativação dos componentes da via apoptótica em uma série de moléculas. A proteína PRDX2 possui as funções proliferativa e antiapoptótica, induzindo mudanças carcinogênicas sob tratamento (Noh *et al.*, 2001). LDHB é uma enzima chave que converte o lactato a piruvato e sua expressão reduzida sugere a inibição na atividade proliferativa das células tumorais. A proteína VIM indica diferenciação e agressão celular, tendo sido recentemente indicada como um novo alvo terapêutico (Lahat *et al*., 2010). A expressão reduzida desta proteína parece estar relacionada com a inibição da proliferação celular tumoral (Nodale *et al.*, 2012). TUBA1A é a principal componente dos microtúbulos, sendo a sua identificação muito importante na pesquisa do câncer. Esta proteína é crucial na divisão celular, que a torna interessante para o *design* de novas drogas anticancerígenas (Kavallaris, 2010).

Apenas duas proteínas identificadas neste estudo, RPLP0 e RAD23B, mostraram expressão aumentada na concentração não citotóxica. A RPLP0 é uma proteína envolvida na

tradução do RNAm. Recentes relatos indicam um aumento da sua expressão em condições de estresse induzido pelo fator de necrose tumoral próapoptótico (TRAIL) em células de câncer de mama (Wilmet *et al.,* 2011) e em quimioterápicos a base de platina para câncer de pulmão (Kageyama *et al.,* 2011). A proteína RAD23B está envolvida no reparo por excisão de nucleotídeos (NER), o seu dímero se liga preferencialmente a agentes quimioterápicos com platina, como a cisplatina, e em dupla fita de DNA com dano causado por ultravioleta (Sugasawa *et al*., 2009, Janicijevic *et al.,* 2003).

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 de algumas das proteínas aqui identificadas sugerem outras aplicações como alvos moleculares para intervenção terapêutica em vários tipos tumorais. 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 o isolamento de compostos bioativos importantes com testes citogenéticos e moleculares servirão para melhor elucidar a atividade antioxidante e antitumoral aqui observada. Compostos isolados também devem ser investigados quanto as suas propriedades biológicas em linhagens tumorais e não tumorais.

4. CONCLUSÕES

Os dados obtidos neste estudo permitem concluir que:

- O extrato hidroalcoólico de própolis vermelha possui em sua constituição principalmente flavonoides que conferem importantes propriedades biológicas;
- O extrato hidroalcoólico de própolis vermelha possui grande quantidade de polifenóis totais e capacidade de varredura do radical livre $DPPH^{\bullet}$, bem como ação enzimática Sod e Cat-*like* comprovando sua ação antioxidante;
- As concentrações de extrato hidroalcoólico de própolis vermelha utilizadas foram capazes de inibir o crescimento de células tumorais;
- A linhagem Hep-2 apresentou perfil proteico distinto na presença ou ausência de extratos da própolis vermelha;
- Das proteínas identificadas com expressão diferencial, a maioria apresentou sua expressão reduzida na presença do extrato da própolis relativo à concentração inibitória de 50% quando comparada ao controle.

5. PERSPECTIVAS FUTURAS

Como continuidade deste trabalho seria importante:

- Isolar e quantificar os compostos da própolis e analisar a sua atividade biológica (antioxidante e antitumoral);
- Avaliar e validar a expressão de proteínas diferencialmente expressas através de imunofluorescência indireta e *Western blot;*
- Realizar um *screening* da atividade biológica de extratos da própolis em bateria de linhagens tumorais e não tumorais;
- Avaliar dano de DNA utilizando ensaio cometa;
- Fazer estudo comparativo entre a própolis vermelha do Nordeste brasileiro e a verde encontrada nas regiões Sul e Sudeste do Brasil.

6. REFERÊNCIAS BIBLIOGRÁFICAS

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.

Araujo, Y.L.F.M. (2009). Estudo da atividade antimicrobiana de variedade de própolis da região da foz do Rio São Francisco-Brasil. **Dissertação de mestrado.** Universidade de Tiradentes. Aracaju, Brasil.

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.

Bankova, V. (2005). Recent trends and important developments in propolis research. **Evid Based Complement Alternat Med**. 2: 29-32.

Bankova, V. (2009). Chemical diversity of propolis makes it a valuable source of new biologically active compounds. **J of ApiProduct and ApiMedical Sci.**. 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.

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. **Dissertação de mestrado**. Universidade Tiradentes. Aracaju, Brasil.

Berard, A.R.; Cortens, J.P.; Krokhin, O.; Wilkins, J.A.; Severini, A.; Coombs, K.M. (2012). Quantification of the host response proteome after mammalian reovirus T1L infection. **PLoS One.** 7(12): e51939.

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.

Brand-Williams, W.; Cuvelier, M. E.; Berset, C. (1995). Use of free radical method to evaluate antioxidant activity. **Lebensm. Wiss. Technol**. 28: 25-30.

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

Buriol, L.; Finger, D.; Schmidt, E.M.; Santos, J.M.T.; Rosa, M.R.; Quinaia, S.P.; Torres, Y.R. (2009). Composição química e atividade biológica de extrato oleoso de própolis: uma alternativa ao extrato etanólico. **Quim. Nova**. 32: 296-302.

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.

Canelle, L.; Bousquet, J.; Pionneau, C.; Deneux, L.; Imam-Sghiouar, N.; Caron, M.; Joubert-Caron, R. (2005). An efficient proteomics-based approach for the screening of autoantibodies. **J Immunol Methods**. 299: 77-89.

Cantu, M.D.; Carrilho, E.; Wulff, N.A.; Palma, M.S. (2008). Sequenciamento de peptídeos usando espectrometria de massa: um guia prático. **Quim. Nova**. 31: 669-675.

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

Castaldo, S.; Capasso, F. (2002). Propolis, an old remedy used in modern medicine. **Fitoterapia**. 73: S1-S6.

Cheng, Z.; Moore, J.; Yu, L. (2006). High-throughput relative DPPH radical scavenging capacity assay. **J. Agric. Food. Chem.** 54: 7429-7436.

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.

Choi, W.; Jung, H.; Kim, K.; Lee, S.; Yoon, S.; Park, J.; Kim, S.; Cheon, S.; Eo, W. (2012). Rhus verniciflua stokes against advanced cancer: a perspective from the Korean Integrative Cancer Center. **J Biomed Biotechnol**. 2012: 874276.

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.

Da Costa, L.A.; Badawi, A.; El-Sohemy, A. (2012). Nutrigenetics and modulation of oxidative stress. **Ann. Nutr. Metab.** 60: 27-36.

Dani, C.; Oliboni, L.S.; Vanderlinde, R.; Bonatto, D.; Salvador, M. (2007). Phenolic content and antioxidant activities of White and purple juices manufactured with organically or conventionally-produced grapes. **Food Chem. Toxicol**. 45(12): 2574-2580.

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.

EMBRAPA. Empresa Brasileira de Pesquisa Agropecuária (2012). Própolis: uma alternativa para diversificar a produção apícola (17/06/2009). Disponível (*on line*): http://www.embrapa.br/imprensa/artigos/2008/propolis-uma-alternativa-para-diversificar-a-producao-apicola/ (25 de outubro).

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

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.**

Fukai, T.; Ushio-Fukai, M. (2011). Superoxide dismutases: role in redox signaling, vascular function, and diseases. **Antioxid Redox Signal**. 15: 1583-1606.

Halliwell, B.; Gutteridge, J.M.C. (2007). **Free Radicals in Biology and Medicine.** 4 ed. Oxford, New York.

INCA. Instituto Nacional de Câncer (2012). Disponível (*on line*): http://www2.inca.gov.br/wps/wcm/connect/cancer/site/oquee (20 de outubro).

INPI. Instituto Nacional de Propriedade Industrial (2012). Disponível (*on line*): http://www.inpi.gov.br/images/stories/downloads/indicacao_geografica/pdf/LISTA_COM_AS_INDICAES_GEOGRFICAS_RECONHECIDAS_-_31-07-2.pdf (25 de outubro).

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.

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.

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.

Klose, J. (2009). From 2-D electrophoresis to proteomics. **Electrophoresis**. 30 Suppl 1: S142-149.

Koo, M.H.; Park, Y.K. (1997). Investigation of flavonoid aglycones in propolis collected by two different varieties of bees in the same region. **Biosci. Biotechnol. Biochem.** 61: 367-369.

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, 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.

Lima, E.M.; Rissino, J.D.; Guimaraes, A.C.; Overal, D.J.; Khayat, A.S.; Souza, P.C.; Christofolini, D.M.; Smith, M.A.C.; Burbano, R.R. (2005). Drifter technique: a new method to obtain metaphases in Hep-2 cell line cultures. **Braz. Arch. of Biol. and Techn**. 48: 537-540.

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.

Marcucci, M.C. (1996). Propriedades biológicas e terapêuticas dos constituintes químicos da própolis. **Quim. Nova**. 19: 529-536.

Marcucci, M.C.; Ferreres, F.; Garcia-Viguera, C.; Bankova, V.S.; De Castro, S.L.; Dantas, A.P.; Valente, P.H.; Paulino, N. (2001). Phenolic compounds from Brazilian propolis with pharmacological activities. **J Ethnopharmacol**. 74: 105-112.

Menezes, H. (2005). Própolis: uma revisão dos recentes estudos de suas propriedades farmacológicas. **Arq. Inst. Biol.** 72: 405-411.

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.

Molloy, M.P.; Donohoe, S.; Brzezinski, E.E.; Kilby, G.W.; Stevenson, T.I.; Baker, J.D.; Goodlett, D.R.; Gage, D.A. (2005). Large-scale evaluation of quantitative reproducibility and proteome coverage using acid cleavable isotope coded affinity tag mass spectrometry for proteomic profiling. **Proteomics**. 5: 1204-1208.

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.

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.

Nunes, L.C.C.; Galindo, A.B.; de Deus, A.S.O.; Rufino, D.A.; Randau, K.P.; Xavier, H.S.; Cito, A.M.G.L.; Neto, P.J.R. (2009). Variabilidade sazonal dos contituintes da própolis vermelha e bioatividade em *Artemia salina.* **Rev. Bras. Farmacogn.** 19: 524-529.

Oldoni, T.L.; Cabral, I.S.; d´Arce, M.A.B.; Rosalen, P.L.; Ikegaki, M.; Nascimento, A.M.; Alencar, S.M. (2011). Isolation and analysis of bioactive isoflavonoids and chalcone from a new type of Brazilian propolis. **Separation and Purification Technology**. 77: 208-213.

Park, Y.K.; Alencar, S.M.; Aguiar, C.L. (2002). Botanical origin and chemical composition of Brazilian propolis. **J Agric Food Chem**. 50: 2502-2506.

Park, Y.K.; Ikegaki, M.; Alencar, S.M.; Moura, F.F. (2000). Evaluation of Brazilian propolis by both physicochemical methods and biological activity. **Honeybee Sci.** 21: 85-90.

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.

Plebani, M. (2005). Proteomics: the next revolution in laboratory medicine? **Clin Chim Acta**. 357: 113-122.

Ramus, C.; Gonzalez de Peredo, A.; Dahout, C.; Gallagher, M.; Garin, J. (2006). An optimized strategy for ICAT quantification of membrane proteins. **Mol Cell Proteomics**. 5: 68-78.

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.

Salatino, A.; Teixeira, E.W.; Negri, G.; Message, D. (2005). Origin and Chemical Variation of Brazilian Propolis. **Evid Based Complement Alternat Med**. 2: 33-38.

Shan, T.; Ma, Q.; Guo, K.; Liu, J.; Li, W.; Wang, F.; Wu, E. (2011). Xanthones from mangosteen extracts as natural chemopreventive agents: potential anticancer drugs. **Curr Mol Med**. 11: 666-677.

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.

Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. **Meth. Enzymol.** 299: 152-178.

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.

Snijders, A.P.; de Vos, M.G.; de Koning, B.; Wright, P.C. (2005). A fast method for quantitative proteomics based on a combination between two-dimensional electrophoresis and 15N-metabolic labelling. **Electrophoresis**. 26: 3191-3199.

Snijders, A.P.; de Vos, M.G.; Wright, P.C. (2005). Novel approach for peptide quantitation and sequencing based on 15N and 13C metabolic labeling. **J Proteome Res**. 4: 578-585.

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

Spada, P.D.S.; de Souza, G.G.N.; Bortolini, G.V.; Henriques, J.A.P.; Salvador, M. (2008) Antioxidant, Mutagenic and Antimutagenic activity of frozen fruits. **J. Med. Food.** 11(1):144-151.

Steen, H.; Mann, M. (2004). The ABC's (and XYZ's) of peptide sequencing. **Nat Rev Mol Cell Biol**. 5: 699-711.

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.

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.

Teixeira, E.W.; Message, D.; Negri, G.; Salatino, A.; Stringheta, P.C. (2008). Seasonal variation, chemical composition and antioxidant activity of Brazilian propolis samples. **Evid Based Complement Alternat Med**. 7: 307-315.

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.

TribunaHoje. Própolis vermelha é reconhecida como produto exclusivo do Brasil (18/10/2012). Disponível (*on line*): http://www.tribunahoje.com/noticia/43356/economia/2012/10/18/propolis-vermelha-e-reconhecida-como-produto-exclusivo-do-brasil.html (26 de outubro).

Unwin, R.D.; Pierce, A.; Watson, R.B.; Sternberg, D.W.; Whetton, A.D. (2005). Quantitative proteomic analysis using isobaric protein tags enables rapid comparison of changes in transcript and protein levels in transformed cells. **Mol Cell Proteomics**. 4: 924-935.

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.

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.

Xiang, Y.; Sekine, T.; Nakamura, H.; Imajoh-Ohmi, S.; Fukuda, H.; Nishioka, K.; Kato, T. (2004). Proteomic surveillance of autoimmunity in osteoarthritis: identification of triosephosphate isomerase as an autoantigen in patients with osteoarthritis. **Arthritis Rheum**. 50: 1511-1521.

Zhang, Y.; Wolf-Yadlin, A.; Ross, P.L.; Pappin, D.J.; Rush, J.; Lauffenburger, D.A.; White, F.M. (2005). Time-resolved mass spectrometry of tyrosine phosphorylation sites in the epidermal growth factor receptor signaling network reveals dynamic modules. **Mol Cell Proteomics**. 4: 1240-1250.

ANEXO

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Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis

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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.

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,

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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-

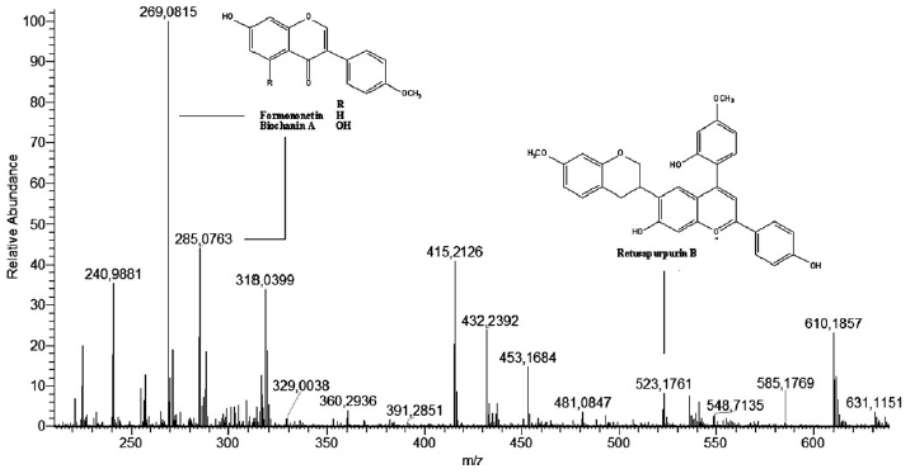


Fig. 1. ESI(+)-MS fingerprint for red propolis from Sergipe.

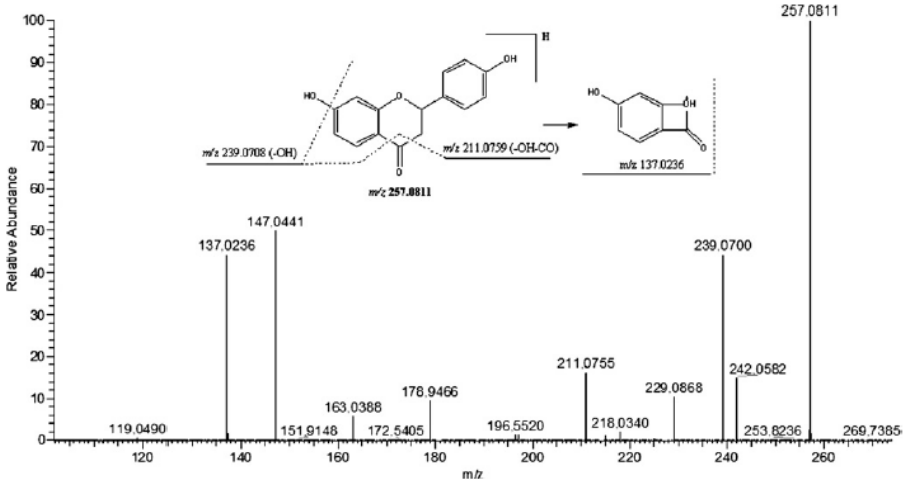


Fig. 2. ESI(+)-MS/MS of liquiritigenin (*m/z* 257.0811).

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

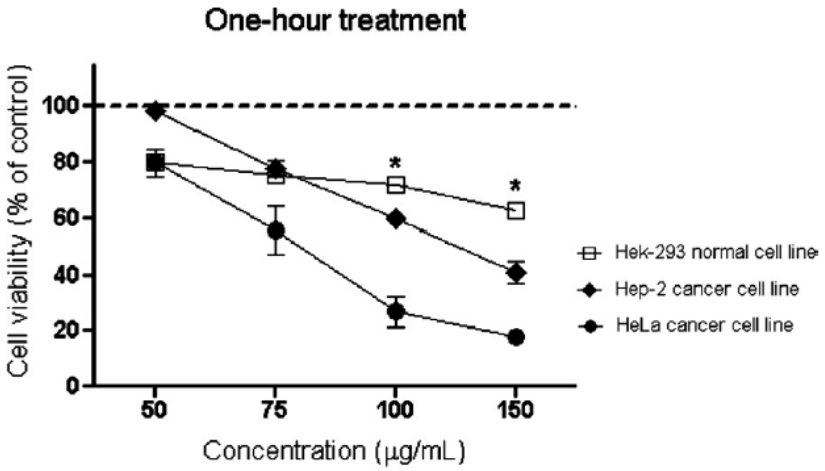
Hydroalcoholic extract obtained from red propolis showed high polyphenolic content (Table 2). Polyphenols are part of the chemical composition found in red propolis that varies according to the year and location of collection. Extracts taken from the northeast of Brazil revealed different amounts of 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 ($p \leq 0.05$).

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 $DPPH^{\cdot}$ 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.

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 ($p \leqslant 0.05$).

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.

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

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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.