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AVALIAÇÃO DA ATIVIDADE BIOLÓGICA DE
FRAÇÕES OBTIDAS DA PRÓPOLIS VERMELHA EM
CULTIVO CELULAR

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Dissertação apresentada ao Programa de Pós-Graduação em
Biotecnologia da Universidade de Caxias do Sul, visando a
obtenção de grau de Mestre em Biotecnologia

Orientação: Mariana Roesch Ely

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RESUMO

A própolis vermelha é uma resina natural produzida por abelhas do gênero *Apis* a partir de exsudados vegetais e tem atraído a atenção dos pesquisadores de todo o mundo devido à sua potente atividade em diversos modelos biológicos. Essa resina natural é rica em polifenóis e compostos voláteis, sendo que mais 300 compostos químicos já foram identificados em amostras de diferentes regiões. Técnicas de identificação química de alta precisão são imprescindíveis para a correta elucidação dos compostos químicos presentes nas amostras e para o entendimento dos efeitos biológicos. Neste estudo, buscou-se utilizar técnicas de fracionamento como uma metodologia capaz de ser explorada em estudos envolvendo amostras de própolis vermelha, buscando-se a identificação e caracterização de frações ativas, bem como o uso de modelos celulares de câncer de cólon para o *screening* de moléculas bioativas. As frações ativas foram identificadas por meio HPLC-MS e ESI-MS/MS, evidenciando uma composição química complexa baseada em compostos fenólicos e flavonoides. Entre os resultados obtidos, destacam-se as frações 05 e 06, as quais foram capazes de produzir inibição significativa sobre as linhagens tumorais HT-29 e HCT-116, respectivamente, com menores efeitos sobre as células não-tumorais. As frações obtidas no estudo apresentaram menor diversidade química do que a encontrada no extrato total da própolis, favorecendo a sua bioatividade *in vitro*, e, como resultado, esta técnica pode favorecer a descoberta e o desenvolvimento de novos fármacos utilizando a própolis como fonte de potencial de moléculas contra o câncer.

Palavras-chave: Própolis, fracionamento, citotoxicidade, câncer colorretal.

ABSTRACT

Red propolis is a natural resin produced by bees of the genus *Apis* from plant exudates and has attracted the attention of researchers worldwide due to its potent activity in several biological models. This natural resin is rich in polyphenols and volatile compounds and more than 300 chemical compounds have been identified in samples from different regions. High precision chemical identification techniques are essential for the correct elucidation of the chemical compounds present in the samples and for the understanding of the biological effects. In this study, we sought to use fractionation techniques as a methodology able to be explored in studies involving samples of red propolis, seeking the identification and characterization of active fractions, as well as the use of cellular models of colon cancer for screening of bioactive molecules. The active fractions were identified by techniques of HPLC-MS and ESI-MS/MS, evidencing a complex chemical composition based on phenolic compounds and flavonoids. Among the obtained results, we highlight the fractions 05 and 06, which are able to produce significant inhibition on the HT-29 and HCT-116 tumor lines. The fractions obtained in this study showed lower chemical diversity than that found in the total extract of propolis, favoring its bioactivity, and as a result, this technique may favor the discovery and development of new drugs using propolis as a potential source of molecules against cancer.

Keywords: Propolis, fractionation, cytotoxicity, colorectal cancer.

1. INTRODUÇÃO

Os produtos naturais possuem uma diversidade química e estrutural mais abundante do que a encontrada nas bibliotecas de compostos sintéticos atualmente disponíveis. Assim, a grande diversidade química observada na Natureza continua a inspirar novas descobertas nos campos da química, biologia e medicina (Shen, 2015).

Compostos naturais têm sido empregados para propósitos terapêuticos desde a Antiguidade. Registros históricos indicam o envolvimento entre as sociedades e os fármacos naturais como a utilização da casca do salgueiro por gregos e egípcios para o alívio de diversos tipos de dores. No século XIX, químicos da empresa Bayer conseguiram o isolamento do ácido salicílico, dando origem à aspirina, disponível comercialmente até os tempos atuais. No século XX a descoberta da penicilina também revolucionou o campo dos antibióticos (Awan et al., 2016).

A fonte principal de compostos químicos está no reino vegetal. As plantas produzem compostos que contribuem para a sua defesa e sobrevivência e que também podem contribuir para a saúde humana. Estima-se que um terço dos fármacos atualmente disponíveis no mercado foi obtido a partir de derivados do metabolismo vegetal (Chae et al., 2014). Estudos com produtos naturais demandam um profundo conhecimento das técnicas de química analítica, biológica e orgânica, assim como das técnicas de separação e fracionamento, com o intuito de obtenção de isolados químicos para testes como potenciais novos fármacos (Pauli et al., 2012).

Extratos obtidos a partir de materiais botânicos podem ser considerados de inestimável valor como fonte de novos metabólitos ativos. Tem sido demonstrado que o *screening* de produtos naturais, pode ser uma abordagem de sucesso para a descoberta de novas drogas. Porém, todo esse trabalho é laborioso, de alto custo, e envolve um

grande consumo de tempo (Ciésła & Moaddel, 2016).

Apesar das dificuldades intrínsecas desta ciência, compostos com potenciais terapêuticos têm surgido com as pesquisas realizadas em todo o mundo. No campo da saúde humana, as drogas derivadas de produtos naturais constituem a principal fonte de agentes quimioterápicos contra o câncer, atuando nas etapas de divisão e proliferação celular (Klautke & Müller, 2016).

1.1 Produtos naturais na medicina tradicional

As civilizações têm utilizado produtos originados de fontes naturais por muitos séculos para prevenção e tratamento de um amplo espectro de enfermidades (Cragg & Newman, 2013). Esse conhecimento empírico chama-se etnobotânica e trata das propriedades medicinais de produtos obtidos da natureza. Este termo foi cunhado em 1895 por Harshberger para definir o estudo e a avaliação de todas as relações existentes entre humanos e plantas, bem como os usos e os efeitos proporcionados pela flora nas populações humanas (Sharma & Kumar, 2013).

Esse acervo cultural tem uma importância global tanto para fins de documentação dos usos populares dos produtos naturais, como para o direcionamento de investigações científicas que objetivam a busca de novos fármacos (Popovic et al., 2016). As vantagens do uso da terapêutica tradicional inclui a grande disponibilidade dos recursos naturais, os aspectos relacionados às culturas locais e também o aumento da demanda por produtos orgânicos e naturais (Carmona & Pereira, 2013).

Durante todas as etapas do desenvolvimento das sociedades humanas, o uso de produtos naturais esteve inserido tanto em contextos relacionados às atividades curativas quanto para finalidades mágico-religiosas, sendo que diferentes pontos de

vista sobre os conceitos de saúde e doença existiram dentro de cada cultura. A Revolução Industrial e o desenvolvimento da química orgânica trouxeram um maior uso de produtos sintéticos para os tratamentos farmacológicos. As razões envolvidas nesse processo estão relacionadas à maior facilidade de obtenção de compostos puros com estruturas e isômeros definidos, bem como as modificações estruturais que produziram compostos mais ativos e seguros (Rates, 2001).

O uso de compostos derivados de síntese quebrou a conexão entre as plantas e a saúde humana, fazendo com que a medicina moderna se tornasse dependente dessas moléculas, preferencialmente com mecanismos de ação definidos (Carmona & Pereira, 2013). Essa nova abordagem dos compostos farmacológicos começou a predominar no pensamento das sociedades modernas ocidentais, sendo que o uso de drogas naturais para a cura de enfermidades passou a ser discriminado e considerado uma opção somente das pessoas com baixo nível de escolaridade, ou ainda simplesmente uma prática supersticiosa, sem considerar as suas potenciais atividades curativas (Rates, 2001).

Entretanto, dados recentes têm comprovado o potencial farmacológico dos produtos naturais. Um levantamento das drogas aprovadas pelo FDA entre os anos de 1981 a 2010 revelou que 34% dos fármacos foram baseados ou derivados de pequenas moléculas de origem natural, sendo que, dentre esses compostos, estão drogas anticâncer e imunossuppressores (Harvey et al., 2015).

Neste contexto, há um grande potencial a ser explorado, pois 95% de toda a biodiversidade terrestre ainda não foi estudada para a avaliação de seus efeitos biológicos, sendo estimada uma biodiversidade biológica mundial de cerca de 2 milhões de espécies de plantas, animais, fungos e micro-organismos. Além disso, produtos derivados de fontes naturais podem mitigar os efeitos adversos provocados por drogas

sintéticas, como os que ocorrem em decorrência de sessões de quimioterapia e radioterapia (David et al., 2014). De qualquer modo, é preciso avaliar a extensão e os limites de ação dos compostos de origem natural, não somente através dos conhecimentos etnobotânicos, mas também através de investigações científicas que confirmem os efeitos observados (Carmona & Pereira, 2013).

1.2 Metabolismo secundário das plantas

O metabolismo é definido como a soma de todas as reações bioquímicas efetuadas por um organismo. As vias do metabolismo primário convergem para produção de uma pequena gama de moléculas, que atuam principalmente nos aspectos metabólitos essenciais para a vida. Os metabólitos primários, como fitoesteróis, nucleotídeos e ácidos orgânicos são encontrados em todos os vegetais.

Enquanto isso, o metabolismo secundário é responsável pela produção de um grande número de moléculas que atuam em resposta ao ambiente e para a defesa do vegetal, sendo o fator chave na adaptação das plantas às condições estressantes de origem biótica ou abiótica (Hussain et al., 2012).

Entre os compostos produzidos pelo metabolismo secundário estão os compostos fenólicos. O termo “compostos fenólicos” se refere a um grande número de compostos (mais de 8000) de ampla ocorrência no reino vegetal e que são caracterizados pela presença de pelo menos um anel aromático ligado a um ou mais grupos hidroxila. Esses compostos são produzidos através da via do ácido chiquímico, onde a enzima principal, fenilalanina amônia-liase, catalisa a biossíntese dos fenólicos a partir do aminoácido fenilalanina (Ferrer-Galego et al., 2014).

Os compostos fenólicos são classificados em compostos não-flavonóides (ácidos fenólicos, hidroxicinamatos, stilbenos, entre outros) e flavonoides (flavonóis, flavonas, flavan-3-ols, antocianidinas, flavanonas, isoflavonas entre outros). Os flavonoides surgiram há cerca de 450 milhões de anos atrás quando as plantas pioneiras iniciaram seus processos de ocupação de novos ambientes, afastando-se de condições ambientais ricas em umidade (Ferreira et al., 2012). Esse grupo de compostos apresentam diversas funções biológicas nas plantas, como proteção contra fitopatógenos e bloqueio de radiação UV, atuação na sinalização durante a nodulação, transporte de auxinas, bem como são responsáveis pela coloração das flores para atração de polinizadores (Ferreira et al., 2012).

Os flavonoides são sintetizados através da via dos fenilpropanóides, transformando fenilalanina e 4-coumaroyl-CoA, que finalmente entra na via de biossíntese dos flavonoides. A primeira enzima específica dessa via, chalcona sintase, produz derivados de chalconas a partir dos quais todos os demais flavonoides são derivados (Ferreira et al., 2012).

A própolis vermelha brasileira é composta principalmente por flavonoides e compostos fenólicos (Alencar et al., 2007) que são derivados do metabolismo vegetal, no caso da própolis vermelha, a sua origem botânica é a espécie *Dalbergia ecastophyllum* (L.) Taub. (Silva et al., 2008).

1.3 Própolis

A palavra própolis é originária dos termos gregos *pro* (em frente a, na entrada de) e *polis* (comunidade) e identifica um produto com a finalidade original de atuar

como material selante e também antimicrobiano, formando uma barreira física e biológica para a proteção das colmeias (Salatino et al., 2005). As abelhas da espécie *Apis mellifera* (L.) fabricam a própolis para isolar a colmeia da entrada de insetos e micro-organismos patogênicos, para o preparo de locais assépticos, para a postura de ovos da abelha rainha e para a mumificação de insetos invasores (Sforcin & Bankova, 2011). As abelhas operárias produzem a própolis através da coleta de exsudatos vegetais de origem diversificada e posterior enriquecimento com secreções enzimáticas e salivares (Valença et al., 2013). Esse enriquecimento proporciona a hidrolização dos compostos fenólicos, que, por sua vez, apresentam atividade farmacológica aumentada (Najafi et al., 2007).

A própolis apresenta consistência resinosa e tem sido utilizada por séculos na medicina tradicional em todas as partes do mundo (Daugusch et al., 2008). Os antigos médicos gregos e romanos utilizavam a própolis como agente antisséptico e cicatrizante. As civilizações incas também empregavam a própolis nas práticas curativas, principalmente para fins anti-piréticos (Sforcin & Bankova, 2011), já os egípcios utilizavam a própolis nas práticas de mumificação de corpos. Durante a Segunda Guerra Mundial, a própolis teve aplicação importante na fabricação de pomadas cicatrizantes para o atendimento de feridos (Marcucci, 1995). Atualmente, continuam sendo atribuídas atividades curativas à própolis como: atividades antissépticas, antifúngicas, anti-inflamatórias, anestésicas e propriedades antioxidantes, que fazem com que o seu uso continue tanto na medicina tradicional como em produtos industrializados que utilizam própolis em sua formulação (Kasote et al., 2014).

A própolis é uma das misturas mais heterogêneas encontradas na natureza, sendo que já foram identificados mais de 300 compostos a partir de amostras de regiões geográficas distintas (Salatino et al., 2005; Anexo I). Esta variação química está

fortemente relacionada à diversidade da flora local (Marcucci, 1995; Bankova et al., 2000), bem como à diversidade genética das abelhas (Koo & Park, 1997). De modo geral, as própolis de diferentes regiões têm em sua composição 50% de resinas e bálsamos vegetais, 30% de ceras, 10% de óleos essenciais, 5% de pólen e 5% de outras substâncias, incluindo elementos inorgânicos (Burdock, 1998).

Existem diversos tipos de própolis, as quais variam de acordo com a sua região de origem, fonte botânica e composição química. Os principais tipos que ocorrem no mundo são a própolis verde, vermelha, de álamo, de bétula, do Pacífico e das Ilhas Canárias. A própolis de álamo ocorre principalmente na Europa, América do Norte e regiões não-tropicais da Ásia e apresenta como compostos principais flavonas, flavanonas e ácidos cinâmicos; sua origem botânica é associada ao gênero *Populus*, mais frequentemente à espécie *Populus nigra* L. (Bankova et al., 2000; Bankova, 2005).

Já a própolis de bétula ocorre nas regiões da Rússia e sua origem botânica é associada ao gênero *Betula*, sua composição química inclui flavonas e flavonóis (diferentes dos que ocorrem na própolis de álamo) (Isidorov et al., 2014). As própolis do Pacífico e das Ilhas Canárias possuem em sua composição química C-prenilflavonas e lignanas, respectivamente, e suas origens botânicas ainda não foram identificadas (Bankova, 2005).

A própolis verde apresenta os seguintes compostos principais: flavonoides, compostos fenólicos, terpenóides (incluindo sesqui, di e triterpenoides) e fenilpropanoides prenilados. Esses últimos são constituídos por ácidos fenólicos contendo o grupo funcional prenila e são formados a partir do ácido cinâmico e seus derivados, como por exemplo, a artepilina C, muito conhecida por sua atividade antitumoral (Bankova, 2005; Teixeira et al., 2010).

Devido à sua biodiversidade, o Brasil possui uma grande variedade de própolis

que pode ser encontradas em diferentes regiões. No ano 2000, 12 tipos de distintos de própolis foram quimicamente classificadas para o país (Park *et al.*, 2000). Em 2008, Dausch e colaboradores (2008), classificaram uma 13ª própolis, a qual recebeu o nome de própolis vermelha.

A existência da própolis vermelha tem sido reportada em muitos países como na costa nordeste do Brasil (estados de Alagoas, Sergipe, Bahia, Paraíba e Pernambuco), Cuba, México e China. A ampla ocorrência geográfica desse tipo de própolis indica que sua origem botânica é diferente nos diversos países, bem como as características da flora, fauna e clima locais (López *et al.*, 2014).

Para a própolis vermelha do nordeste brasileiro, as pesquisas de Silva e colaboradores, (2008) indicam que a sua origem botânica é a espécie *Dalbergia ecastophyllum* (L.) Taub. devido à correspondência química existente entre a própolis e os exsudados resinosos produzidos por esta planta.

A própolis vermelha difere quimicamente das demais por possuir compostos classificados como isoflavonoides (daidzeina, formononetina, biochanina A, medicarpina, vestitol), chalcona (isoliquiritigenina), flavanona (liquiritigenina), neoflavonoide (dalbergina) e isoflavonol (neovestitol) (Alencar *et al.*, 2007; Dausch *et al.*, 2008; Oldoni *et al.*, 2011; Anexo I).

De todos os grupos químicos, os flavonoides e os compostos fenólicos são os que mais têm chamado a atenção dos pesquisadores (Volpi & Bergonzini, 2006). Segundo Barbosa e colaboradores (2009), os flavonoides são considerados os principais compostos bioativos da própolis, com vários estudos comprovando as suas propriedades antibacterianas, antivirais e antioxidantes. Esses compostos apresentam estrutura hidrocarbonada do tipo C6-C3-C6 nas quais as duas unidades C6 (anéis A e B) possuem natureza fenólica (Tsao, 2010). Essas estruturas químicas podem ser divididas em

subclasses de acordo com o grau de oxidação do anel heterocíclico como: flavanol, flavona, flavonol, flavanona, antocianinas e isoflavonas. De modo geral os flavonoides apresentam-se hidroxilados, metoxilados e/ou glicosilados, sendo que o açúcar ligado geralmente é uma glicose ou ramnose (Singh et al., 2008).

Estudos envolvendo polifenóis (flavonóides e ácidos fenólicos) derivados da própolis também têm sido realizados devido aos grandes benefícios que esses compostos podem trazer à saúde humana como efeitos antimutagênicos (Volpi & Bergonzini, 2006), antioxidantes (Kurek-Górecka et al., 2014) e antiaterogênicos (Daleprane et al., 2012). Nas diferentes amostras de própolis também ocorrem elementos inorgânicos como o cobre, manganês, ferro, cálcio, alumínio, vanádio e silício. Em amostras de própolis francesas também foi identificada a presença das vitaminas B1, B2, B6, C e E (Marcucci, 1995).

Li e colaboradores (2008) isolaram e identificaram 42 compostos químicos presentes na própolis vermelha de Alagoas, a maioria deles da classe dos flavonoides (flavanoides, flavonol, isoflavonas, isoflavanonas, isoflavans, chalconas, auronol, pterocarpanas, 2-arylbenzofuran e neoflavonoides), bem como lignanas. Esse tipo de própolis é rica em isoflavonóides, existindo diversos estudos na literatura que associam esses compostos à diversas atividades biológicas como ação antimicrobiana (Nedji & Loucif-Ayad, 2014), antioxidante, citotóxica (Campos et al., 2014; Anexo I), anti-inflamatória e anti-alérgica (Orsolich et al., 2014).

1.4 Métodos empregados para extração de compostos naturais

A descoberta de drogas a partir de produtos naturais tem envolvido diversas áreas do saber. Esse processo se inicia tipicamente com um botânico ou um

etnobotânico, que coleta e identifica plantas de interesse medicinal (Baker et al., 1995). Em seguida, métodos de extração são utilizados para a obtenção dos extratos, sendo que estes então passam por ensaios para a avaliação de seu potencial biológico. Diversos processos de isolamento e de caracterização das frações são realizados com a finalidade de elucidar as estruturas químicas envolvidas nas atividades observadas (Balunas & Kinghorn, 2005).

De modo geral, antes de se iniciar uma metodologia de extração é necessário verificar a estabilidade dos compostos químicos já relatados para a amostra a ser analisada em relação a cada método. Processos de degradação podem ser originados a partir de diversos fatores como presença de luz, exposição ao ar, tempo de extração e temperatura. Enzimas presentes nas amostras também podem ser liberadas para o solvente de extração e levar à reações de degradação. Radicais livres podem, por exemplo, ser formados e contribuir para a modificação estrutural dos compostos de interesse (Biesaga, 2011).

Um dos grandes desafios nos processos de extração de moléculas a partir de matrizes complexas (como o caso dos materiais vegetais) se deve ao fato de que os compostos de interesse estão impregnados dentro das matrizes. Dessa forma, é importante a utilização de técnicas seguras que permitam a extração adequada desses compostos bioativos (Tiwari, 2015). A maioria dessas técnicas é baseada no poder de extração de diferentes solventes e também na aplicação de aquecimento ou mistura e são compostas pelo uso de sohxlet, maceração e hidrodestilação, sendo que outras técnicas também estão atualmente sendo utilizadas como digestão enzimática, extrusão, uso de micro-ondas, fluidos supercríticos e solventes acelerados (Azmir et al., 2013).

Cada técnica apresenta vantagens e desvantagens intrínsecas, porém a escolha da metodologia de extração deve levar em conta a capacidade de solubilização dos

compostos de interesse, evitando-se a ocorrência de modificações químicas. A taxa de eficiência de cada método de extração também é influenciada por diversos fatores como o tipo e concentração dos solventes utilizados, razão solvente-sólido, tempo de extração, temperatura empregada, pH, entre outros (Ćujic et al., 2016).

A seleção do método para a extração de componentes ativos com o máximo de rendimento e altos níveis de pureza depende principalmente da natureza e da estabilidade térmica dos compostos químicos, bem como da natureza do material vegetal a ser processado (Shirsath et al., 2012).

O método de extração para compostos fenólicos simples (ácidos benzoicos, aldeídos benzoicos, ácidos cinâmicos e catequinas) a partir de materiais sólidos e semi-sólidos se baseia principalmente em técnicas de maceração utilizando solventes orgânicos (Canals et al., 2005). Nessa técnica, a amostra é colocada em contato com solventes, com ou sem agitação, por um longo período de tempo, à temperatura ambiente ou sob aquecimento, até a completa solubilização dos compostos químicos no solvente. Porém devido ao longo tempo envolvido nesse processo de extração, os compostos podem sofrer modificações químicas (Garcia-Salas et al., 2010). Em relação a compostos termossensíveis, técnicas de extração conduzidas em altas temperaturas podem prejudicar a qualidade dos extratos, bem como provocar a destruição dos compostos ativos (Shirsath et al., 2012).

Diferentemente da maceração, as técnicas de ultrassom são bem conhecidas por apresentarem alta eficiência e redução dos tempos necessários para a extração de compostos biológicos, bem como menor utilização de solventes nos processos além de sua alta reprodutibilidade (Khan et al., 2010). Muitas classes de compostos têm sido extraídas por técnicas de ultrassom como aromas, pigmentos, antioxidantes e outros

compostos orgânicos e minerais a partir de diferentes tipos de matrizes como tecidos animais, microalgas, leveduras, alimentos e vegetais (Chemat et al., 2017).

Segundo Yeo e colaboradores (2015), o uso de diferentes técnicas de sonicação reduz significativamente o tempo de extração para a própolis, produzindo extratos com maior atividade antibacteriana, com redução no consumo de solventes, assim como esta técnica poderia ser utilizada comercialmente por ser mais econômica e sustentável, sem o comprometimento da qualidade dos extratos. Esse método de extração emprega o uso de ondas de ultrassom, que provocam colisões de alta velocidade entre as partículas do material usado na extração e o solvente, promovendo uma maior dissolução dos compostos químicos. As taxas de rendimento obtidas nesse tipo de extração estão diretamente ligadas à estrutura bem como à reologia e à composição do material vegetal, que pode apresentar diferentes graus de resistência às ondas de ultrassom (Vilkhu et al., 2008).

As técnicas envolvendo extração por micro-ondas são conhecidas pela eficiência da extração de metabólitos a partir de materiais biológicos diversos, entretanto os fatores limitantes dessa técnica estão relacionados com a potência e a frequência utilizadas no equipamento, temperatura, pressão aplicada e escolha do tempo de exposição da amostra à radiação de micro-ondas. Outro fator limitante é a escolha do solvente adequado que deve permitir a absorção da energia liberada pelo equipamento (Zhang et al., 2011). Por outro lado, os benefícios apresentados por essa técnica estão relacionado ao menor tempo de extração e menor consumo de solventes em relação aos métodos convencionais.

Os estudos de Trusheva e colaboradores (2007) indicam que o uso das técnicas de maceração utilizando própolis produz baixos rendimentos, sendo que o emprego de micro-ondas propicia a geração de extratos com alta concentração em ceras e na

degradação dos compostos ativos. Entretanto, o emprego de ultrassom pode resultar em altos rendimentos, alta seletividade de compostos de interesse, bem como em redução do tempo de extração. Os estudos de Khacha-Ananda e colaboradores (2013), também comprovaram que as técnicas de ultrassom resultaram em extratos de própolis com maior concentração de flavonoides e fenólicos totais, bem como maior atividade antioxidante em relação aos extratos obtidos por maceração. A **Figura 1** mostra um quadro comparativo entre os métodos de extração por maceração, ultrassom e micro-ondas.

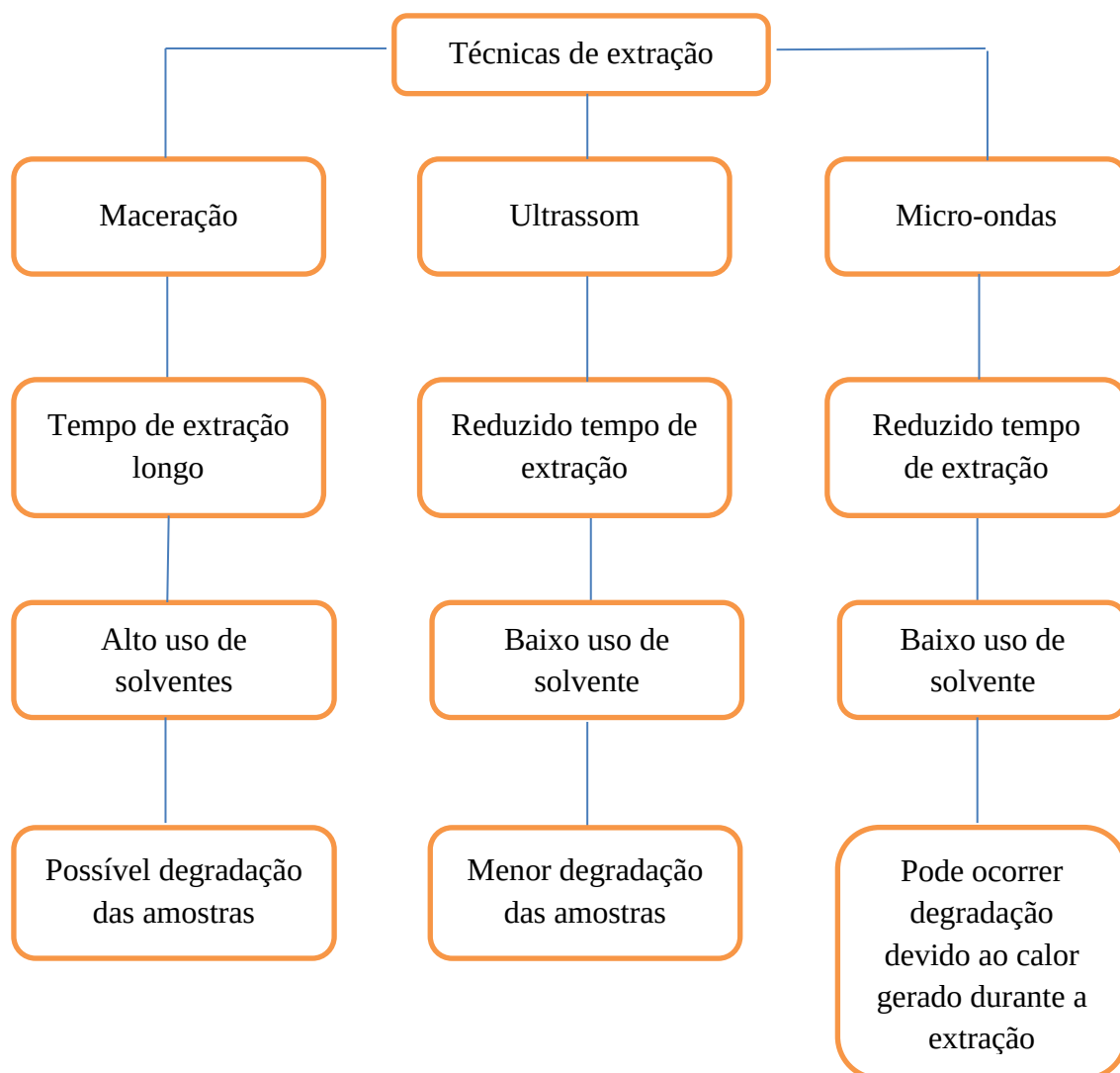


Figura 1 – Esquema comparativo entre as técnicas de maceração, ultrassom e micro-ondas, mostrando vantagens e desvantagens para cada método. O presente estudo utilizou a técnica de ultrassom.

1.5 Atividades biológicas da própolis

A própolis apresenta um amplo espectro de atividades biológicas, tendo um grande potencial para a descoberta de novos fármacos a partir de sua composição química complexa. Até o presente, mais de 300 compostos químicos diferentes já foram reportados para amostras de própolis provenientes de diversas regiões (Huang et al., 2014; Anexo I). Nesse contexto, a produção de extratos brutos tem como consequência direta a geração de extratos com uma grande mistura de compostos químicos diferentes. Técnicas simples de extração, entretanto, não são capazes de produzir extratos com compostos isolados ou frações com menor diversidade química (Sarker & Nahar, 2012).

A mistura de compostos presentes dentro dos extratos brutos pode produzir efeitos biológicos sobre determinados alvos, porém, alguns compostos ativos podem estar em concentrações extremamente pequenas, produzindo, dessa forma, efeitos que não podem ser mensurados adequadamente (Harvey et al., 2015).

Estudos envolvendo a própolis vermelha têm comprovado que o uso de técnicas de fracionamento pode resultar em frações com melhores resultados químicos e biológicos. Mendonça e colaboradores (2015), observaram maior concentração de compostos fenólicos e de atividade antioxidante em frações em comparação a extratos brutos. Resultado semelhante também foi encontrado por Alencar e colaboradores (2007), em que frações hexânicas apresentaram maiores concentrações de flavonoides e maior atividade antioxidante comparado ao extrato bruto testado.

Em relação a efeitos citotóxicos, frações enriquecidas com os compostos xanthochymol e formononetina provocaram inibição de crescimento e indução de processo apoptótico via ativação de caspases em células tumorais de melanoma (Novak et al., 2014). Dados da literatura também apontam que através do isolamento do vestitol,

por técnicas de fracionamento, foi verificada a inibição de migração de neutrófilos em processos inflamatórios (Franchin et al., 2016), podendo existir uma possível aplicação de modulação do sistema imunológico com o uso dessa substância.

Diversos métodos de fracionamento são reportados na literatura para a própolis vermelha brasileira. A **Figura 2** mostra um comparativo entre alguns métodos de fracionamento reportados na literatura.

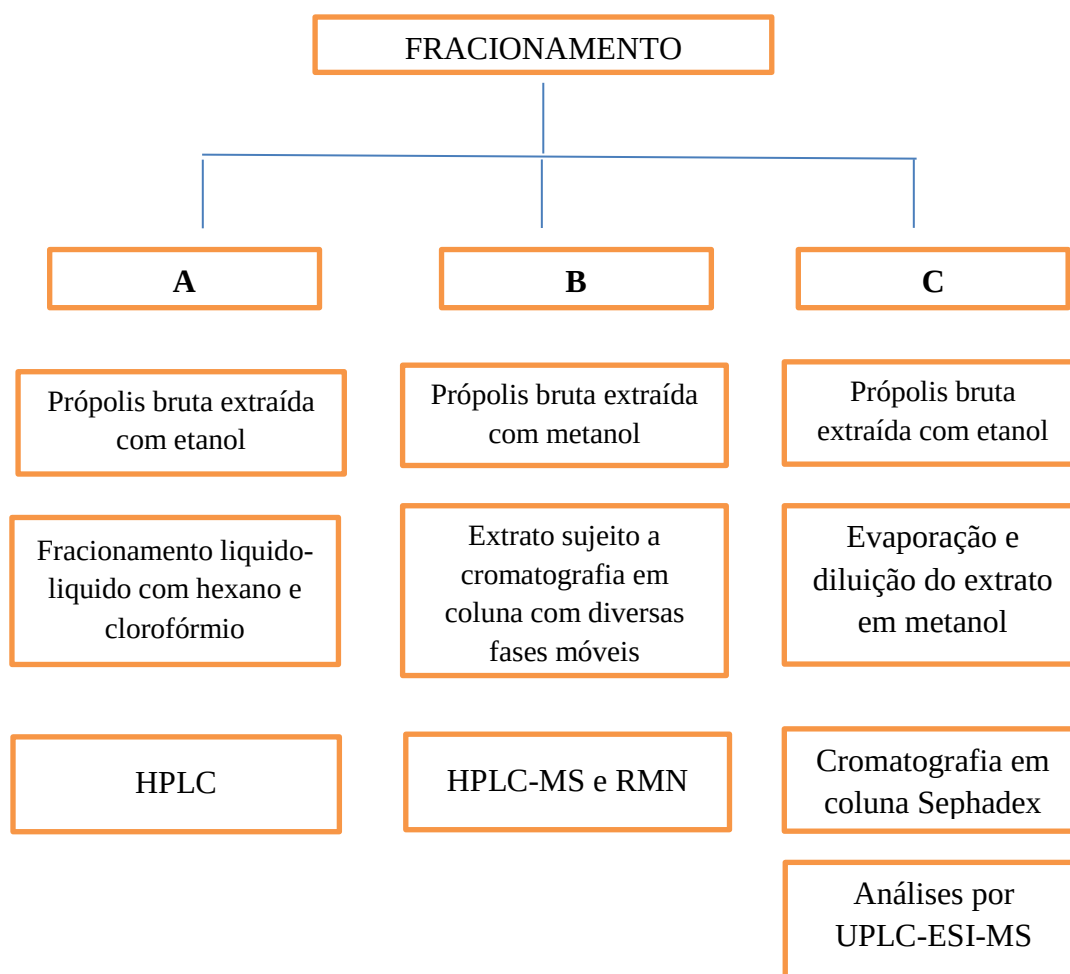


Figura 2- Esquema representativo de métodos de fracionamentos utilizados com a própolis vermelha brasileira. **A:** Alencar et al., (2007), **B:** Awale et al., 2008, **C:** Novak et al., 2014.

Apesar do grande potencial apresentado pela própolis vermelha brasileira, poucos estudos têm avaliado os possíveis efeitos biológicos que frações e substâncias isoladas poderiam apresentar em células em cultivo.

Estudos utilizando a própolis vermelha, juntamente com metodologias de fracionamento têm comprovado que frações são mais efetivas do que extratos brutos. Através do isolamento da molécula 3,8-dihydroxy-9-methoxypterocarpan, Awale e colaboradores (2008) verificaram 100% de morte celular contra a linhagem PANC-1 (célula tumoral de pâncreas) na concentração de 12,5µM.

Frações ricas em 7-hydroxy-6-methoxyflavanone apresentaram citotoxicidade nas linhagens B16-BL6 (IC50: 6,66µM), LLC (IC50: 9,29µM), A549 (IC50: 8,63µM) e HT-1080 (IC50: 7,94µM). A molécula mucronulatol, isolada de amostras da própolis vermelha, também apresentou efeitos inibitórios nas células LLC (IC50: 8,38 µM) e A549 (IC50: 9,9 µM), (Li et al., 2008; Anexo I).

Frações enriquecidas com formonetina e xanthochymol também foram eficientes em inibir o crescimento das linhagens tumorais B16F10 (IC50: 25,7±1,2 µg/mL), RPMI-8226 (32,6±2,6 µg/mL), HL-60 (20,5 ± 2,4 µg/mL) e K562 (30,3 ± 5,6), (Novak et al., 2014).

Além dos efeitos em células tumorais, Franchin e colaboradores (2016) também verificaram que a molécula vestitol isolada da própolis vermelha apresentou atividade anti-inflamatória, reduzindo a migração de neutrófilos em modelo *in vivo*.

1.6 Estudos da viabilidade de extratos naturais para aplicação terapêutica

Substâncias químicas derivadas de animais, plantas ou micro-organismos têm sido utilizadas desde os primórdios das civilizações para inúmeras finalidades curativas. Na indústria farmacêutica ocidental, pesquisas direcionadas para a investigação de novos fármacos a partir de compostos naturais atingiu o seu auge no período entre 1970-1980, o que resultou em um portfólio de fármacos fortemente influenciado por moléculas não-sintéticas. Entre as 877 moléculas registradas entre 1981 e 2002, 49% eram produtos naturais, compostos naturais semi-sintéticos ou análogos baseados em compostos naturais (Koehn & Carter, 2005).

Um grande número de extratos ou compostos derivados de fontes vegetais tem sido descritos como potenciais agentes terapêuticos contra o câncer. Os compostos fitoquímicos podem ser um caminho para a prevenção ou tratamento para neoplasias malignas, assim como uma janela para novas estratégias que podem limitar a capacidade metastática das células tumorais (AlQathama & Prieto, 2015).

Atualmente, uma porcentagem significativa de cânceres pode ser tratada através de cirurgia, radioterapia ou quimioterapia, especialmente se eles forem detectados precocemente (WHO, 2013). Porém, o tratamento continua sendo um grande desafio apesar dos avanços científicos nas últimas décadas. O tratamento clínico geralmente utiliza agentes antiproliferativos sistêmicos que destroem preferencialmente células em divisão (Bracke et al., 2008).

O câncer é uma das maiores causas de mortalidade no mundo. Para o ano de 2012 foram estimados aproximadamente 14 milhões de novos casos em todo o mundo e cerca de 8 milhões de mortes (WHO, 2014). Para o Brasil, os dados estimam a ocorrência de 420.310 novos casos de câncer, excluídos os tumores de pele não

melanoma para o ano de 2016. Em relação ao sexo masculino, os tumores com maior ocorrência são próstata (28,6%), traqueia, brônquio e pulmão (8,1%), cólon e reto (7,8%) e estômago (6,0%). Para o sexo feminino a distribuição dos tipos de câncer é a seguinte: mama (28,1%), cólon e reto (8,6%), colo do útero (7,9%) e traqueia, brônquio e pulmão (5,3%). Estima-se, para o Brasil, 16.660 casos novos de câncer de cólon e reto em homens e de 17.620 em mulheres, o que corresponde em um risco de 16,84 casos novos a cada 100 mil homens e de 17,10 para cada 100 mil mulheres, respectivamente (INCA, 2016).

Contudo, os fármacos antitumorais ainda apresentam baixa seletividade para células cancerosas e sua eficácia terapêutica fica limitada devido aos danos que podem ocorrer às células e aos tecidos normais (Duarte, 2010). Os efeitos colaterais apresentados pelos agentes quimioterápicos, atualmente disponíveis, também são fatores impeditivos para a adesão adequada aos tratamentos (Venditto & Szoka, 2013).

Neste contexto, a própolis pode servir como uma nova fonte de compostos para ser utilizada na área oncológica, com menores efeitos adversos do que as drogas convencionais. Estudos com extratos de própolis vermelha brasileira enriquecidos com polifenóis indicam efeitos anti-angiogênicos, uma aplicação que limitaria a formação de novos vasos sanguíneos na região do tumor (Daleprane et al., 2012).

Efeitos celulares também são relatados após a exposição a extratos de própolis vermelha como indução de apoptose e diminuição do potencial de migração em células de câncer de bexiga (Begnini et al., 2014; Anexo I). Em células tumorais de câncer de mama, a própolis vermelha induz processo apoptótico, sem afetar células normais de fibroblastos, confirmando que extratos de própolis tem grande potencial para aplicações antitumorais (Kamiya et al., 2012). Estudos do nosso grupo de pesquisa, por exemplo, apresentaram indução de processo apoptótico em células tumorais de laringe (Hep-2) e

de adenocarcinoma cervical (HeLa), sem afetar as células normais da linhagem Hek-293 (Frozza et al., 2013).

Porém, extratos brutos da própolis apresentam uma enorme diversidade de compostos químicos e diversos efeitos biológicos podem ser evidenciados. Nesse contexto, métodos de fracionamento são adequados para diminuir a diversidade química existente em cada amostra a ser testada, assim como produzir frações com compostos isolados ou em altas concentrações, permitindo uma melhor compreensão dos efeitos biológicos observados (Awale et al., 2008; Li et al., 2008).

Estudos comparativos de Novak e colaboradores, (2014) utilizando extrato etanólico de própolis em relação a frações obtidas por cromatografia em coluna, indicaram melhor atividade das frações em relação ao extrato inicial, sendo a fração IV, descrita no estudo, a mais ativa. Os resultados de IC₅₀ do extrato inicial sobre as células tumorais B16F10, RPMI-8226, HL-60 e K562 variaram entre 32,8±3,8 a 42,1±8,7 µg/mL, já a fração IV (composta majoritariamente por formononetina e xanthochymol) apresentou IC₅₀ variando entre 25,7±1,2 a 30,3±5,6µg/mL, mostrando uma maior atividade antitumoral em relação às demais frações.

Outro estudo utilizando técnicas de fracionamento utilizando amostras de própolis vermelha permitiram o isolamento de 43 compostos químicos, os quais mostraram diferentes níveis de citotoxicidade sobre células de tumorais de pâncreas, sendo que a molécula 3,8-dihydroxy-9-methoxypterocarpan foi a mais ativa (Awale et al., 2008).

As moléculas denominadas como mucronulatol e 7-hydroxy-6-methoxyflavanone, obtidas através de fracionamento da própolis vermelha, indicaram atividade citotóxica *in vitro* em relação às linhas de carcinoma de pulmão e de câncer de cólon (Li et al., 2008).

A partir desses resultados já reportados na literatura confirma-se o potencial terapêutico das moléculas orgânicas presentes na própolis vermelha, assim como indica novas perspectiva de aplicação para os compostos presentes nesta resina natural.

2. OBJETIVO GERAL

Caracterizar a composição química de compostos majoritários presentes em frações obtidas a partir de extratos da própolis vermelha e avaliar a citotoxicidade de extratos brutos e frações em células tumorais e não tumorais.

2.1 Objetivos Específicos

- Obter extratos da própolis vermelha e realizar fracionamento em coluna cromatográfica;
- Caracterizar as frações obtidas por HPLC/MS e ESI-MS/MS;
- Quantificar compostos fenólicos totais por metodologia de Folin-Ciocalteu;
- Avaliar a citotoxicidade em células tumorais de câncer de cólon (HT-29 e HCT-116) e células normais (Vero)
- Analisar alterações morfológicas de células após exposição a frações e extrato bruto da própolis vermelha.

3. RESULTADOS

Os resultados deste trabalho estão apresentados na forma de artigo científico.

Antitumor activity of Brazilian Red Propolis fractions against colon cancer cells

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ABSTRACT

Red propolis, a natural resin produced by bees with plant exudates, shows a great potential as source of new compounds against cancer. In this study, the potential activity of a total extract and fractions against colon cancer cell lines were investigated. At first, 255 fractions obtained by column chromatography was analyzed by thin layer chromatography and then arranged in 11 groups through profile similarity. Chemical characterization with HPLC/MS and ESI-MS/MS was able to identify 14 different compounds in the samples. High amounts of phenolic compounds were found at extracts and fractions (ranging from 110.38 ± 5.68 to 250.48 ± 13.83) and MTT viability assay showed different toxicity after total extract and fractions exposition at cell lines. Fractions 05 and 06 presented higher selectivity for tumor lines with lower biological effects in normal cells. May-Grouwald/Giemsa revealed cellular morphological changes after exposition to higher concentrations of total extract and fractions. The results indicate that fractionation techniques can contribute to reduce chemical diversity verified in propolis, generating fractions with improved biological activity and contributing to development of new strategies for discovery of natural compounds against cancer.

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Keywords: Propolis, fractionation, cell cytotoxicity, cancer

1. Introduction

Propolis is a complex mixture of compounds produced by honey bees with resins collected from leaves, buds and bark of certain tree species (CATCHPOLE et al., 2015) which are mixed with salivary enzymes (GRAIKOU et al., 2016). The chemical composition of propolis are linked to the geographical location, plant sources and bee species (HUANG et al., 2014) and it is composed of 50% resins, 30% waxes, 10% essential oils, 5% pollen and 5% other substances such as minerals and organic compounds (phenolic acids, esters, flavonoids, terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes and β -steroids) (SILVA-CARVALHO et al., 2015). More than 300 compounds have been identified in different samples and new ones are still being recognized by analytical methods. Chemical profile can be also modified by seasonality and climatic conditions, which makes even more complex the variety of molecules found in this product (WAGH, 2013).

Red propolis has been reported in several countries such as Cuba, Mexico, China and Brazil (states of Alagoas, Bahia, Paraiba, Sergipe and Pernambuco) (LÓPEZ et al., 2014). For the Brazilian red propolis the botanical origin has been attributed to specie *Dalbergia ecastophyllum* (L.) Taub due the chemical correspondence between propolis and resins produced by this plant (DAUGSCH et al., 2008). Compounds founded in propolis have shown important pharmacological effects such as antimicrobial, antioxidant, cytotoxic, anti-inflammatory and anti-allergic activities. Brazilian red propolis has great biological potential against cancer cells, decreasing cellullar migration and inducing cell death events (KAMIYA et al., 2012; BEGNINI et al., 2014).

Colon cancer is the third most prevalent neoplasia diagnosed in men and second in women in Brazil, and this illness has been associated with hereditary and lifestyle factors such as alimentary habits enriched with red meat and processed foods, a small consumption of fruits and sedentarism (INCA, 2016).

Given its complex chemical composition, recent studies have focused their investigation fractionating different extracts of red propolis. However, until present, few studies have evaluated the effects of these enriched fractions against tumor cell lines and no reports from propolis fractions against colon cancer cells are available. In this context, the aim of this work was to investigate the activity of different fractions obtained from Brazilian red propolis, identifying and elucidating possible active compounds against colorectal cancer cells.

2 Materials and Methods

2.1 Red propolis sample

Red propolis sample was collected in 2013 in state of Alagoas (Northeast region of Brazil), stored at room temperature and ground to a fine powder with liquid nitrogen. The extractions were conducted using ultrasound equipment (Vibracell, VCS 500) with a 60% of amplitude and under a thermostatic ice bath at 0 °C. At first, to remove waxes and resins, 50 g of propolis was extracted with 150 mL of hexane (30 min × 3 times). After each extraction, the hexane solution was removed by filtration and the residues were used for the next extraction procedure with solvent renovation. Then, a new extraction scheme were performed using residues not solubilized in the previous step with ethyl acetate as solvent (150 mL × 3 times), at same extraction and filtration

conditions. The solutions obtained with ethyl acetate were grouped and the solvent was removed using a rotary evaporator (40°C) followed by lyophilization. The ethyl acetate total extract (EAE) was stored at – 20 °C until utilization.

2.2 Fractionation

Fractionation was conducted using a column chromatography (4 × 45 cm) with 147 g of silica gel and 4 g of EAE. Hexane (100 – 60%) and ethyl acetate (0 – 40 %) was used as mobile phase. 255 fractions were collected and analyzed by thin layer chromatography (TLC) using mobile phase constituted by hexane:ethyl acetate (7:3). The TLC plates were visualized under UV light at 254 and 365 nm and stained with vanillin-sulphuric acid spray, followed by heating. The fractions with similar chromatographic profiles were grouped and the solvent was removed by rotary evaporator (40 °C) and lyophilization process. From this process, 11 different fractions were obtained and were kept at – 20 ° C.

2.3 Chemical characterization

EAE and the fractions were diluted in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid. The solutions were infused directly or with HPLC (Shymadzu) assistance into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate 10 $\mu\text{L}.\text{min}^{-1}$. ESI(+)-MS were acquired using a hybrid high-resolution and high accuracy microTOF-QII mass spectrometer (Bruker® Daltonics) under the following conditions: capillary and cone voltages were set to + 3500V and + 40V, respectively, with a de-

solvation temperature of 100 °C. Diagnostic ions were identified by the comparison of exact m/z with compounds determined in previous studies (**Table 1**). For data acquisition and processing, Hystar software (Bruker® Daltonics) was used. The data were collected in the m/z range of 70–800 at the speed of two scans per second. No important ions were observed below m/z 100 or above m/z 800.

2.4 Cell culture and cytotoxicity assay

HT-29 and HCT-116 cells were grown in RPMI 1640 medium. Vero cells were cultivated with DMEM (Dulbecco's Modified Eagle Medium). Cells lines were kept at 37° C in a humidified atmosphere enriched with 5% of CO₂. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin. EAE and the fractions were submitted to sequential dilutions with DMSO and culture media. A control solution was prepared with DMSO at 0.5%.

Initially, cells (1×10^4 cells/well) were seeded in 96-well plates with 100 μ L of supplemented culture medium for 24 hours for attachment. The cells were incubated with EAE and fractions in a range of concentrations (from 0 to 150 μ g/mL) for 24 hours. Then, the treatment was removed and a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (1 mg.mL^{-1}) was added and incubated for two hours. Formazan crystals were dissolved with DMSO for 30 minutes. Absorbance was measured using a microplate reader (Spectra Max 190, Molecular Devices) at 570 nm. The percent of growth inhibition was calculated in relation to control cells (100% viability) and the IC₅₀ (concentration that inhibit 50% of cell viability) value was calculated. Each experiment was performed in triplicate.

2.5 Total polyphenol content by Folin-Ciocalteu

EAE and fractions were individually diluted in a hydroethanolic solution (70 %) at a concentration of 0.5 mg/mL. Each sample (0.5 mL) was diluted in a Folin-Ciocalteu solution 0.2 N (2.5 mL) for 5 min. After, 2 mL of sodium carbonate solution (7.5%) was added and the samples were incubated at 50 °C for 5 min. The absorbance was measured at 760 nm at spectrophotometer. Galic acid was used as a standard compound in a range of 20-220 µg/mL to construct a standard curve.

2.6 Morphological assay

Vero, HT-29 and HCT-116 cells were seeded in 24 well plates (2×10^4 /well) with 10% serum supplemented media. After 24 hours, the culture medium was removed and the cells were treated with EAE and fractions at concentrations of 35 µg/mL and 70 µg/mL, for 24 h. Vehicle (solution of DMSO 0.5% in culture medium) was used as control. The cells were stained with May-Grounwald/Giemsa for 3 minutes in the same plate. After washing, cells were observed in microscope with 20x magnification.

2.7 Statistical Analysis

MTT results and phenolic quantification were expressed as mean $\pm$ standard deviation obtained from three independent experiments.

3. RESULTS

3.1 Chemical composition

From 255 fractions collected and analyzed by thin layer chromatography, 11 were obtained by profile similarity. However, only fractions that reached IC₅₀ at cell treatments were selected for chemical composition evaluation. Therefore, fractions 01 to 03 were excluded from analysis. The chemical composition of EAE and fractions (04 to 11) was determined with HPLC/MS and ESI-MS/MS (ESI+) in a Q-TOF (Q-TOF-II Bruker Daltonics) with UFLC. **Figure 1** shows the chemical analysis of total ethyl acetate extract. The compounds were identified with information provided by HMRS as exact *m/z* and fragmentation pathway in accordance with the literature. The acceptable threshold to confirm chemical compounds was established as 5 ppm, which provides highly secure identification of the chemical compounds.

In this work, it was possible to identified 14 compounds, which are described in the **Table 1**. By fragmentation pathways (tandem MS/MS), some compounds in relation to its mass isomers were distinguished. Liquiritigenin was differentiated of isoliquiritigenin by formation of ion 137.0271. In similar way, the differentiation of formononetin and dalbergin was possible by formation of ion 137.0277, recognized only from formononetin compound.

3.2 Viability assay

Cytotoxic activity of the EAE and fractions were investigated. The treatments with EAE have not shown significant statistical differences (**Table 2**). Fractions 04, 05, 08 and 10 presented more cytotoxicity in HT-29 than HCT-116 and normal cells; however no differences of cytotoxicity were verified between Vero and HCT-116 cells. In the other hand, HCT-116 was susceptible to fraction 06 in relation to HT-29 and Vero cells. The fractions 05 and 06 presented less cytotoxicity activity in normal cells in relation to colon cancer cells. Fraction 09 had less activity at HT-29 than HCT-116 and Vero cells. The results founded suggest existence of selectivity linked between chemical composition of each fraction that can produce different levels of cytotoxicity in relation to normal and cancer cells (**Table 1, Table 2**).

3.3 Total polyphenol quantification (Folin-Ciocalteu)

The results obtained from polyphenol quantification showed high level of phenolic compounds in samples. Fractions 04, 05, 06 and 11 presented less polyphenols than EAE. An increase of phenolic compounds was observed in fractions 08 and 09 and the fractions 10 and 11 presented slight decrease in the total polyphenol amounts in relation to EAE. However no direct interactions were observed between total amount of polyphenols and biological activity in the cell lines (**Table 3**).

3.4 Morphological assays

The same concentrations of EAE and fractions 05 and 06 (35 and 70 µg/mL) were tested to evidence possible morphological alterations in cells. These fractions showed activity against HT-29 and HCT-116, respectively, with fewer effects in Vero cells. Evidences of apoptotic alterations could be observed such as cell shrinkage, detachment and cytoplasm retraction. Higher concentrations of fractions (70 µg/mL) resulted in increased on morphological alterations in relation to lower levels of exposition (35 µg/mL). The data also confirm the results founded in MTT assay (**Table 3, Figure 3**).

3. DISCUSSION

Propolis has been used since ancient times and has gained attention of scientific community as a potential new source of drugs. The mixture of compounds found in this natural resin is largely dependent of the geographical origin and botanical sources (BANKOVA et al., 2014), presenting a very complex composition constituted mainly of waxes, essential oils and phenolic compounds (AWALE et al., 2008).

Several studies have applied crude extracts in cytotoxic investigations, in special, for anticancer screening (FREIRES et al., 2016). However, the large chemical diversity present in the total samples may mask the real pharmacological effects. Also, some of the active compounds may be in concentrations very low to provide relevant biological activity and still difficulty to proper identification by analytical methods (HARVEY et al., 2015). Identification of chemical profile of natural products requires high precision methods such as HPLC and ESI-MS to determine the chemical diversity

present in extracts and fractions originated from natural sources (MENDONÇA ET AL., 2015; NUNES & GUERREIRO, 2012).

Formononetin, identified in the fractions, is one of chemical markers of red propolis, and is founded in Brazilian and Cuban samples (CUESTA-RUBIO et al., 2007; MENDONÇA et al., 2015) and can promote decrease in tumor development in mice with prostate cancer (LI et al., 2014). Another study, using red propolis fractions obtained from ethanol extracts, has identified formononetin and xanthochymol as main compounds, showing growth inhibition on the development of cancer cells through caspase-3-dependent pathway apoptosis (NOVAK et al., 2014).

The flavonoids liquiritigenin, biochanin A and medicarpin identified in this work were cited in previous studies of our group using total extract (FROZZA et al., 2013). Liquiritigenin was shown *in vivo* cancer inhibition mediated by cellular effects and angiogenesis reduction (LIU et al., 2012). Vestitol, also founded in the fractions, can act as an anti-inflammatory agent showing inhibitory activity in neutrophil migration, suggesting modulation of the immune system (FRANCHIN et al., 2016).

Phenolic compounds derived from plant or synthetic sources have shown interesting anti-proliferative effects in breast, cervical, colon, leukemia, lung, prostate and skin tumor cell lines (ROLEIRA et al., 2015). Moreover, polyphenolic content is directly linked to antioxidant activities (MOUHOUBI-TAFININE et al., 2015) and to several health benefits such as prevention of major diseases (BOUDET, 2007). The fractions produced in this study have shown high levels of polyphenols by Folin-Ciocalteau technique, with values ranging from 110.38 to 250.48. These values are similar to those found by MACHADO et al., (2016), using extracts of propolis from different regions of Brazil.

Different levels of cytotoxicity were identified after exposition of total extract and fractions to cell lines. Selectivity mediated by chemical composition could be observed. In general, Vero cells were differentially affected by EAE and fractions, but less sensibility was verified to fractions 05 and 06. Fraction 06 presents a compound identified as Guttiferone E or Xanthochymol, absent in the fraction 05. Guttiferone E and Xanthochymol have been considered as an inseparable mixture and has been found in lipophilic extracts of Brazilian red propolis (FASOLO et al., 2016). Guttiferone E has shown induction of endoplasmic reticulum response that produce growth inhibition on colon cancer cell lines such as HCT-116, HT-29 and SW480 (PROTIVA et al., 2008). This compound can induces apoptosis via activation of caspases 8 and 9, effector caspase 3/7 and loss of mitochondrial membrane potential (KUETE et al., 2013). A study using Xanthochymol also has revealed the induction of cell cycle arrest and apoptosis in cell models (NOVAK et al., 2014).

Biochanin A founded in fraction 05, also presented anti-proliferative effects in HT-29 cells in other studies, showing apoptotic induction and enhancing effects of radiotherapy, opening new perspectives to combined treatments to colon cancer (PUTHLI et al., 2013). The flavanone liquiritigenin founded in fraction 08, demonstrated several antiproliferative effects in other studies, inhibiting migration and invasion processes in cells B16F10, as well as *in vivo* models. This compound is also associated to apoptosis induction on HeLa cells by release of cytochrome c from mitochondria (LIU et al., 2011). Formononetin founded from fraction 07, exhibit inhibitory effects in cancer cells with cell cycle arrest at G0/G1 phase (LI et al., 2014). In colorectal cancer, formononetin is also efficient to induce apoptosis using *in vivo* and *in vitro* models (HUANG et al., 2015) with great perspectives for clinical applications (WU et al., 2015). Another interesting compound, 7-O-Methylvestitol, founded in

fraction 05, apparently more activity in cancer cells, also shown potent inhibitory effects in HT-1080 and Colon 26-LS cells of colon adenocarcinoma (LI et al., 2008).

Morphological cell changes varied according to propolis concentration exposure (**Figure 3**), with more alterations in cellular morphology at higher concentrations. Data from scientific literature also observed cell alterations using ethanolic extracts of propolis in cancer cell lines, showing detachment, apoptotic blebbing, reduction in size and density at higher exposures (CHOUDHARI et al., 2013). Biochanin A founded in the fractions also produce morphological alterations in colon cancer cells, contributing to apoptosis and cell death (PUTHLI et al., 2013).

The results here presented indicate that the chemical compounds founded in fractions and EAE are able to produce *in vitro* cytotoxic effects. The results confirm that compounds of propolis can able to produce different effects at tumor cells and in consequence to chemical composition of the samples.

CONCLUSION

Researches involving natural products can bring new target compounds and strategies for treatment of human diseases as colon cancer. Our study has indicated that Brazilian red propolis, in specially enriched fractions, can produce several cytotoxic activities against cancer cells. Bioguided fractionation can be an efficient methodology to improve the development of new drugs from propolis samples, evidencing the activity of each group of compounds, individually or in combination with others.

The methodology used was efficient to produce fractions with different chemical composition that could be applied to discover new lead compounds from propolis and can aid in the understanding of the isolated activity of each compound,

improving the knowledge and favoring the development of target therapeutics against colorectal cancer.

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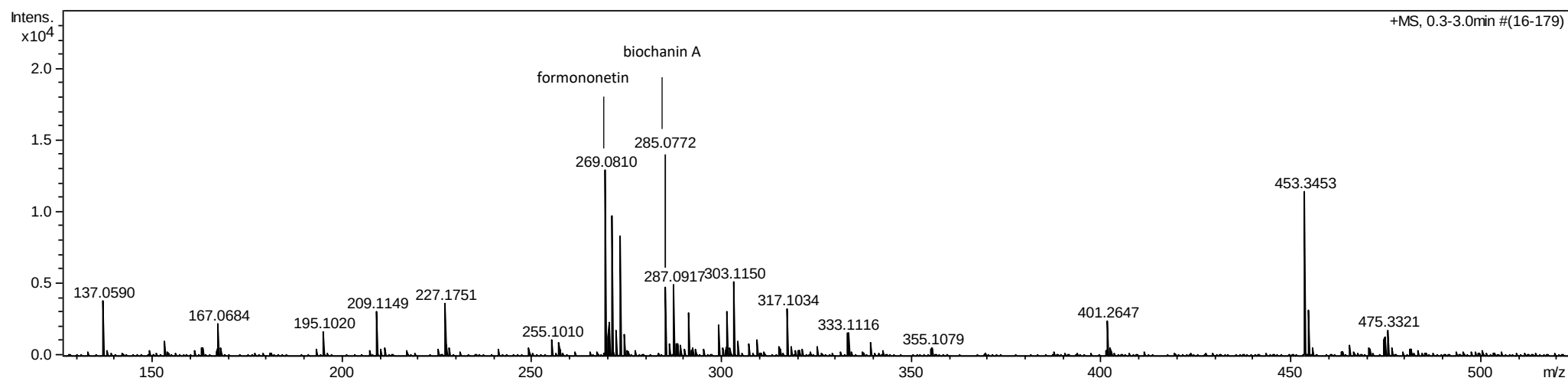


Figure 1- Total chromatogram of ethyl acetate total extract (EAE)

Table 1- Chemical characterization of fractions (04-11) and ethyl acetate total extract (EAE)

Entry	Sample	Identification	Elem. Comp. (+H)	Precursor ion <i>m/z</i>	Diff. ppm	Fragmentation ions (%) [MS-MS]	Ref.
1	EAE	Methoxyeugenol	C ₁₁ H ₁₅ O ₃	195.1017	2.15	-	(ALENCAR et al., 2007)
2	EAE	Elemicin (a) or cis-asarone (b) or trans-isoelemicin (c)	C ₁₂ H ₁₇ O ₃	209.1168	4.64	209.1164 (58); 194.0982 (100); 168.0807 (85) 168.0807 (85) 166.0531 (23).	(TRUSHEVA et al., 2006; RIGHI et al., 2011)
3	EAE; 04	(2S)-7-hydroxyflavanone	C ₁₅ H ₁₃ O ₃	241.0877	4.69	241.0913 (29); 226.0722 (7); 223.0804 (9); 195.0847 (13); 171.0802 (4); 163.0446 (8); 137.0262 (100); 131.0513 (52); 103.0573 (14)	(AWALE et al., 2008)
4	EAE	Chrysin	C ₁₅ H ₁₁ O ₄	255.0650	2.88	255.0736(100); 151.0422 (98); 131.0598 (12).	(LÓPEZ et al., 2014)
5	EAE; 08; 09; 10; 11	Liquiritigenin	C ₁₅ H ₁₃ O ₄	257.0820	2.40	257.0852 (33); 239.0802 (7); 229.0910 (16); 211.0780 (9); 147.0483 (49); 137.0271 (100); 133.0689 (10); 119.0528 (9) 269.0853 (100); 254.0599 (55); 253.0578 (44); 237.0573 (42); 226.0674 (83); 225.0564 (18); 213.0949 (51); 197.0643 (38); 181.0668 (12); 170.0758(9); 154.0320 (10); 137.0277 (14); 118.0457 (22); 107.0511 (6)	(DAUGSCH et al., 2008; FROZZA, et al., 2013)
6	EAE; 07; 08; 09; 10	Formononetin	C ₁₆ H ₁₃ O ₄	269.0861	4.30	271.1071 (30); 243.1059 (2); 161.0618 (8); 147.0516 (2) 137.0663 (100); 123.0458 (8).	(AWALE et al., 2008)
7	EAE; 04; 05; 06; 11	Medicarpin	C ₁₆ H ₁₅ O ₄	271.0981	3,93	273.1102 (44); 163.0845 (20); 149.0622 (21); 137.0640 (100); 123.0493 (97)	(ALENCAR et al., 2007; FROZZA, et al., 2013)
8	EAE; 07; 08; 09	Vestitol; or Isovestitol; or Neovestitol	C ₁₆ H ₁₇ O ₄	273.1116	3.97	285.0790 (100); 270.0593 (39); 253.0613 (8); 242.0586 (8); 229.0921 (14); 170.0275 (7); 137.0620 (17)	(Awale et al. 2008; Piccinelli et al. 2011)
9	EAE; 05; 06; 07; 08; 09; 10; 11	Biochanin A	C ₁₆ H ₁₃ O ₅	285.0772	3.16	285.1105 (72); 177.0615 (44); 163.0770 (16); 161.0699 (24); 149.0628 (15); 137.0623 (100) 287.0925 (41); 167.0375 (17); 163.0735 (24); 153.0548 (19); 137.0637 (100).	(AWALE et al., 2008)
10	EAE; 04	Homopterocarpin	C ₁₇ H ₁₇ O ₄	285.1128	0.41	287.1164 (21); 163.0770 (16); 137.0625 (100).	(PICCINELLI et al., 2011)
11	EAE; 07	(3S)-Vestitone	C ₁₆ H ₁₅ O ₅	287.0920	1.92	317.1130 (12); 299.1045 (30); 289.1074 (15); 271.0968 (35); 256.2742 (11); 243.1057 (23); 165.0588 (99); 151.0409 (24); 137.0635 (100)	(AWALE et al., 2008)
12	EAE; 04; 05; 06;	7-O-Methylvestitol	C ₁₇ H ₁₉ O ₄	287.1295	4.06	603.3758 (91); 411.1859 (100); 343.1183 (66); 137.0287 (15)	(Trusheva et al., 2006; Novak et al., 2014)
13	EAE; 04	(3S)-violanone	C ₁₇ H ₁₇ O ₆	317.1037	3.74		
14	EAE; 06; 07; 08	Guttiferone E or Xanthochymol	C ₃₈ H ₅₁ O ₆	603.3673	2.10		

Table 2 –IC50 in cells (HT-29, HCT-116, Vero) after exposition to EAE and fractions

Treatments	Cell lines		
	Vero	HT-29	HCT-116
Ethyl acetate extract	68.52±4.72 a	75.15±3.35 a	70.81±4.18 a
Fraction 04	81.68±6.18 a	68.33±5.97 b	90.38±5.63 a
Fraction 05	103.25±5.35 a	73.58±1.00 b	83.42±4.41 a
Fraction 06	107.61±6.91 a	93.80±7.56 b	72.45±6.57 b
Fraction 07	78.35±6.93 a	69.04±7.56 a	70.83±4.02 a
Fraction 08	92.96±6.00 a	77.67±6.76 b	83.86±2.26 a
Fraction 09	95.37±9.21 a	105.23±3.61 b	82.71±3.96 a
Fraction 10	98.25±4.70 a	81.14±2.02 b	99.17±8.10 a
Fraction 11	40.32±5.64 a	49.88±6.83 a	41.68±3.25 a

Results of IC50 expressed as mean $\pm$ standard deviation of three independent methods. Different letters in the same line indicate statistical difference in relation to control cell (Vero) ($p < 0.05$, ANOVA One-way).

Table 3- Polyphenol quantification by Folin-Ciocalteu Colorimetric assay

Samples	Quantification mg/g EAG
EAE	202.74±9.93
fraction 04	111.46±6.71
fraction 05	110.38±5.68
fraction 06	183.81±9.00
fraction 07	204.40±8.51
fraction 08	224.50±9.54
fraction 09	250.48±13.83
fraction 10	200.97±11.06
fraction 11	185.77±8.34

Results expressed as mean $\pm$ standard deviation of three independent experiments

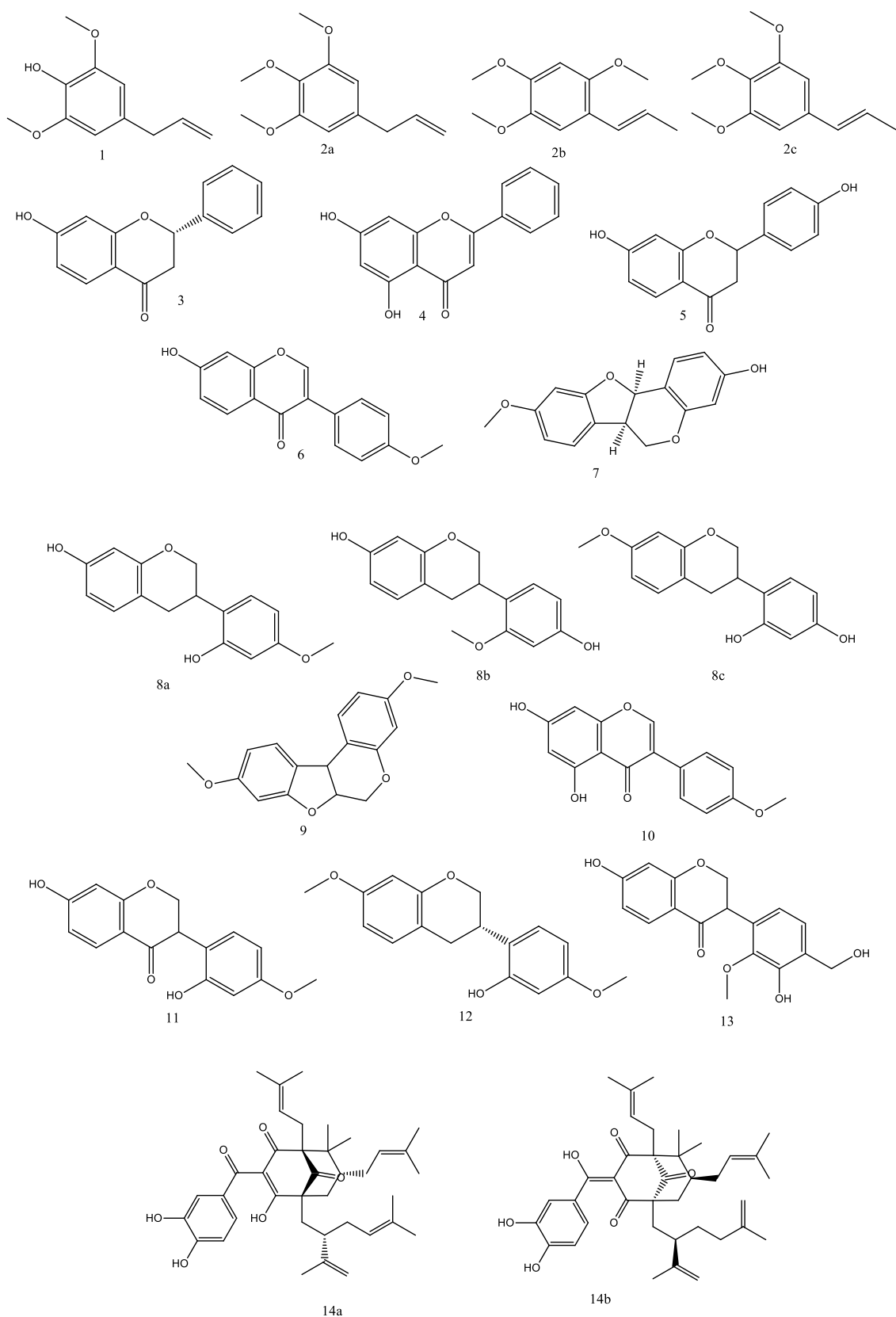


Figure 2- Structure of chemical compounds identified in the EAE and fractions.

Numbers under each structure are listed in **Table 1** before each chemical identification.

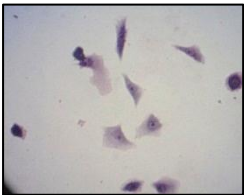
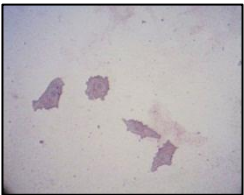
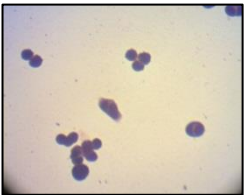
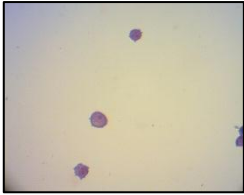
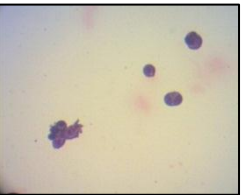
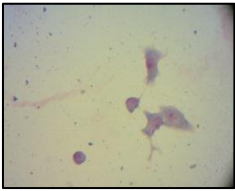
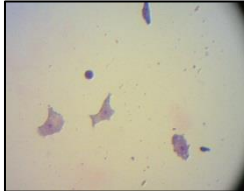
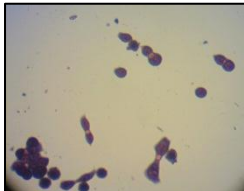
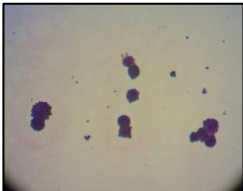
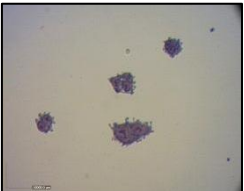
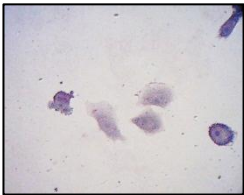
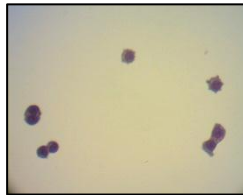
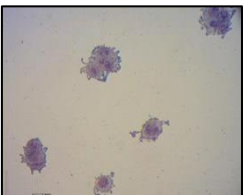
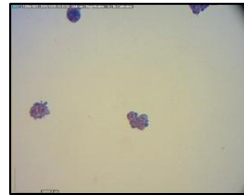
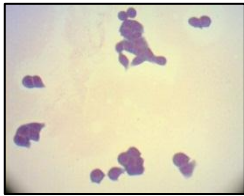
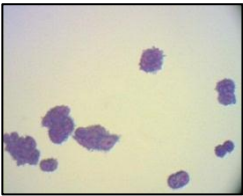
Samples	Cell lines					
	VERO		HT-29		HCT-116	
	35µg/mL	70µg/mL	35µg/mL	70µg/mL	35µg/mL	70µg/mL
EAE						
FRACTION 05						
FRACTION 06						
Control treatment (DMSO 0.5%)						

Figure 3 – Morphological changes in cell lines with May-Grouwald staining. Fractions 05 and 06 were selected as most selective against tumor cells

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4. DISCUSSÃO GERAL

Os dados obtidos com este estudo confirmam as possibilidades terapêuticas para o uso da própolis vermelha brasileira como fonte de novos compostos bioativos com potencial ação antitumoral. O processo de fracionamento desenvolvido neste estudo foi eficaz em produzir frações com menor diversidade química, apresentando frações ricas em compostos fenólicos e de polifenóis.

O uso de extratos totais para o *screening* fitoquímico apresenta dificuldades relacionadas à grande diversidade química presentes nos extratos, dificultando também a correta análise dos efeitos biológicos. Nesse contexto, as técnicas de fracionamento contribuem para reduzir a diversidade química das frações, permitindo tanto uma melhor caracterização química das amostras quanto uma melhor compreensão dos efeitos biológicos, que podem estar relacionados a compostos específicos presentes nas amostras testadas (Harvey et al., 2015).

Estudos utilizando própolis vermelha têm revelado que as técnicas de fracionamento são capazes de produzir frações mais ativas, possibilitando assim maiores perspectivas de aplicações terapêuticas (Awale et al., 2008; Anexo I). Dados da literatura confirmam que frações obtidas a partir da própolis vermelha produziram maior atividade sobre células tumorais em relação a extratos etanólicos brutos, bem como foram capazes de inibir o crescimento tumoral em modelo xenográfico em camundongos (Novak et al., 2014). Outros estudos também indicam que a partir do fracionamento de amostras de própolis da região nordeste do Brasil, obtidas inicialmente por extração metanólica, seguida de fracionamento em coluna, foi possível obter o composto natural 3,8-dihydroxy-9-methoxypterocarpan, que possui alta seletividade em

células tumorais de pâncreas (Awale et al., 2008; Anexo I).

Neste estudo, as frações 05 e 06 apresentaram maior atividade biológica em relação às células HT-29 e HCT-116, respectivamente, mostrando menor citotoxicidade nas células normais. A fração 05 apresentou o composto identificado como guttiferone E/xanthochymol. Estudos recentes definem esses dois compostos como uma mistura inseparável e que apresentam um grande potencial antitumoral. Dados da literatura indicam que a molécula Guttiferone E promove inibição celular em câncer de cólon através de vias que regulam o retículo endoplasmático e ativam cascatas de caspases relacionadas a eventos de apoptose. A molécula xanthochymol também atua promovendo parada de ciclo celular e apoptose, o que corrobora com os efeitos também observados nas linhagens tumorais (Fasolo et al., 2016).

A molécula medicarpina, presente nas frações 04, 05, 06 e 11 tem sido relacionada a atividades antiproliferativas e pode conduzir células à apoptose mesmo em moléculas resistentes à terapias (Gatouillat et al., 2015). Efeitos protetivos em células normais também são reportados para essa molécula, como efeitos de regeneração e cicatrização (Dixit et al., 2015).

O composto biochanina A é considerado um dos marcadores químicos para a própolis vermelha, auxiliando na diferenciação entre distintos tipos de própolis. Diferentes atividades biológicas tem sido descritas em linhagens HEPG2, HEP-2 e HeLa após exposição à biochanina A (Frozza et al., 2013; Mendonça et al., 2015). Em células tumorais de câncer de próstata, biochanina A ativa vias importantes de apoptose, inibindo a proliferação celular desse tipo de tumor (Szliszka & Krol, 2013).

Alterações morfológicas em células de câncer de cólon também são verificadas após exposição à biochanina A, como retração citoplasmática, sendo relacionadas a eventos de morte celular. Em relação às vias moleculares a biochanina A promove a

ativação de cascatas moleculares que levam a eventos de apoptose, assim como contribui para a efetividade de outras terapias combinadas (Puthli et al., 2013). Também são atribuídos efeitos de indução de apoptose em células HCT-116 e SW-480, bem como de alteração no ciclo celular destas linhagens de câncer de cólon (Zhang et al., 2013).

A fração 08, que apresentou maior citotoxicidade na linhagem HT-29, tem em sua composição compostos conhecidos como vestitol, isovestitol ou neovestitol. Vestitol apresentou grandes efeitos inibitórios sobre células de leucemia, com atividade superior à doxorrubicina, droga tradicionalmente utilizada para o tratamento do câncer (Shults et al., 2016). Essa molécula pode modular o sistema imunológico, atuando na migração de neutrófilos e diminuindo processos inflamatórios (Franchin et al., 2016), atuando na prevenção de tumores (Gharpure et al., 2008). O isômero isovestitol mostrou atividades citotóxicas, levando à apoptose células de câncer de cólon (Li et al., 2008). Também tem sido reportados efeitos antiproliferativos, bem como atuação antioxidante após exposição ao neovestitol, utilizando a molécula isolada em relação a extratos brutos da própolis (FREIRES et al., 2016).

Flavonoides e isoflavonoides têm um papel crucial para a saúde humana, assim como diversos efeitos para a redução de tumores (Hedelin et al., 2016). O isoflavonoide formononetina que apresenta ampla ocorrência no reino vegetal e também identificado nas frações, apresenta atividades inibitórias em células de câncer de mama, inibindo a migração e a invasão em modelos *in vitro*, contribuindo também para a redução da ocorrência de metástase em modelos murinos (Zhou et al., 2014). Frações enriquecidas com formononetina apresentaram atividades inibitórias, assim como contribuíram para a parada de ciclo celular, além de produzir fragmentação de DNA em células tumorais (Novak et al., 2014). Em células de câncer de cólon HCT-116, a formononetina foi capaz

de induzir apoptose, bem como alterações morfológicas fortemente relacionadas a eventos de morte celular (Auyeung & Ko, 2010).

Neste estudo, foram identificados grandes concentrações de fenólicos totais, tanto no extrato total como nas frações. A própolis tem uma composição rica em polifenóis que têm sua origem relacionada aos recursos botânicos da flora do local de coleta. As concentrações de compostos fenólicos podem variar devido à fenologia da vegetação e fatores relacionadas ao clima e sazonalidade (Daleprane et al., 2012).

Compostos fenólicos exibem uma grande gama de efeitos benéficos à saúde humana, como propriedades anti-inflamatórias, antibacterianas e anticâncer. Estudos tem comprovado que o consumo de produtos ricos em polifenóis diminui o risco de câncer, dando indícios de que esse grupo de compostos pode atuar também na supressão de células tumorais, em especial para a redução de tumores na região do trato gastro-intestinal (Roleira et al., 2015). Métodos de fracionamento podem produzir frações com altas concentrações de compostos fenólicos, potencializando as suas atividades biológicas (Alencar et al., 2007; Anexo I). Diversos mecanismos de ação têm sido identificados para os polifenóis, que explicam o seu grande potencial farmacológico, entre eles a indução de parada do ciclo celular e apoptose, efeitos antiproliferativos e anti-inflamatórios, assim como mudanças na sinalização celular (Pandey & Rizvi, 2009).

Estudos com frações enriquecidas e compostos isolados da própolis vermelha confirmam a existência de seletividade, onde cada composto químico pode atuar de maneira diferencial sobre diferentes linhagens de tumores (Awale et al., 2008; Novak et al., 2014; Anexo I).

Neste estudo, observou-se que a seletividade de frações enriquecidas em compostos varia de forma expressiva na dependência da linhagem celular avaliada. Em

conclusão, a própolis apresenta uma composição química complexa, com mais de 300 compostos químicos. Muitos destes compostos podem estar em baixas concentrações e serem mascarados quando presentes em extratos totais. A produção de frações gera extratos com menor diversidade química, o que torna mais evidente a diferenciação de seus efeitos biológicos. Neste sentido, ensaios com frações podem auxiliar no entendimento da atividade isolada de cada molécula natural, o que também pode contribuir para o desenvolvimento de terapias alvo, com a busca de moléculas com maior especificidade e menores efeitos colaterais.

5. CONCLUSÕES

Os dados obtidos com esse estudo permitem concluir que:

- O fracionamento da própolis vermelha é uma metodologia que pode auxiliar nas pesquisas que utilizam amostras da própolis vermelha brasileira, reduzindo a diversidade química presente em extratos brutos e auxiliando na identificação de possíveis novos fármacos de origem natural;
- As frações obtidas apresentaram diferentes atividades citotóxicas nas linhagens testadas, como redução na proliferação celular e promoção de alterações celulares associadas a processos apoptóticos.
- As frações 05 e 06, com composição química baseada em compostos como medicarpina, biochanina A e 7-O-Methylvestitol, foram as mais seletivas, com menores efeitos citotóxicos em linhagem não-tumoral;
- Os efeitos observados indicam seletividade dos compostos da própolis, sendo os efeitos diretamente relacionados à composição química de cada fração.

6. PERSPECTIVAS FUTURAS

Como continuidade deste trabalho seria importante:

- Realizar quantificação dos compostos químicos presentes em cada fração;
- Avaliar a expressão de proteínas diferencialmente expressas relacionadas à apoptose por metodologia de *Western Blot*;
- Realizar *screening* buscando verificar atividade das frações em uma bateria de linhagens tumorais e não-tumorais;
- Realizar isolamento dos principais compostos ativos;
- Fazer estudo comparativo entre amostras de própolis vermelha de outras regiões, buscando novas atividades biológicas e compostos bioativos.

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Anexo I

Red propolis: chemical composition and pharmacological activity

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Abstract

Propolis has been used worldwide for years in folk medicine and currently marketed by the pharmaceutical industry. In Brazil, propolis was classified into 13 groups based on their organoleptics and physicochemical characteristics. The 13th type named red propolis has been an important source of investigation since late 90s. Their property comes from the countless compounds, including terpenes, pterocarpanes, prenylated benzophenones and especially the flavonoids. This last compound class has been indicated as the responsible for its potent pharmacological actions, highlighting the antimicrobial, anti-inflammatory, antioxidant, healing and antiproliferative activities. The red propolis can also be found in other countries, especially Cuba, which has similar features as the Brazilian. Therefore, with the compilation of 80 papers, this review aims to provide a key reference for researchers interested in natural products and discovery of new active compounds, such as from propolis.

Keywords: activity, compounds, propolis, review.

1. Introduction

Natural products arising from the Brazilian flora have been attributed as valuable sources of substances used for the discovery and development of new therapeutic agents. Propolis is one of these products which have attracted the researchers' attention. Recently, the red propolis type, found in the Northeast of Brazil, has been highlight due its features. This variety is found in the states of Alagoas, Sergipe, Paraíba, Pernambuco and Bahia, from mangroves regions. The main botanical origin was identified as *Dalbergia ecastophyllum* (L) Taub. (Fabaceae), popularly known as “rabo-de-bugio” (Clardy and Walsh, 2004; Silva et al., 2007; Dausch et al., 2008).

Propolis is a complex natural resin collected by bees (*Apis mellifera*) from different parts of plants as branches, buds, exudates, among others. Salivary secretions and enzymes are added, and this substract is used mainly for protection against insects, invading microorganisms and in beehives repair (Marcucci, 1996; Pereira et al., 2002). In general, it is composed of 50-60% resins and balms, 30-40% waxes, 5-10% essential oils, 5% pollen grain, microelements and vitamins (Park et al., 2002; Menezes, 2005; Funari and Ferro, 2006).

The red propolis is classified as the 13th group and has shown several biological properties, e.g. antimicrobial, anticancer, antioxidant, which are related to its complex and variable chemical composition. Its main constituents are phenolic compounds, especially flavonoids, which have broad therapeutic range (Park et al., 2000; Cushine and Lamb, 2005; Cabral et al., 2009; Oldoni et al., 2011). In this way, the presence of two flavanols pigments named Retusapurpurin B and Retusapurpurin A (**12**), give its red identity feature (Piccinelli et al., 2011).

The chemical composition and pharmacological activities of this specific propolis class, has been intensely explored since the 90s, which is evidenced by the

publication of over 100 papers between scientific articles and patents. Thus, this review aims to compile these informations of the red propolis being a guide for future research related to this special type of propolis.

2. Chemical composition

The red propolis chemical composition is very complex and largely depends on the geographical origin and specific flora at the site of collection. Therefore, the compounds are directly related to the plant origin (Castro et al., 2007). More than 300 components have been reported in red propolis samples, which have been analyzed by divers methods. Table 1 shows the compounds most frequently mentioned, which are representatives of terpenes, flavonoids, aromatic acids and fatty acids class. Furhthermore, there are inorganic elements such as copper, manganese, iron, calcium, aluminum, vanadium and silicon also present (Marcucci, 1996; Pereira et al., 2002).

Table 1. The main compounds found in red propolis and some identification methods.

Entry	Compound	Identification Method	Reference
1	Formononetin	ESI/MS HPLC-PDA- ESI/MS	(Frozza et al., 2013) (Piccinelli et al., 2011)
2	Biochanin A	ESI/MS HPLC-PDA- ESI/MS	(Frozza et al., 2013) (Piccinelli et al., 2011)
3	Medicarpin	ESI/MS HPLC-PDA- ESI/MS	(Frozza et al., 2013) (Piccinelli et al., 2011)
4	Vestitol	GC/MS HPLC-PDA- ESI/MS GC/MS, NMR, HPLC	(Alencar et al., 2007) (Piccinelli et al., 2011) (Oldoni et al., 2011)
5	Neovestitol	HPLC-PDA- ESI/MS GC/MS; NMR; HPLC	(Piccinelli et al., 2011) (Oldoni et al., 2011)
6	Daidzein	RP-HPLC	(Alencar et al., 2007)
7	Elemicin	GC/MS	(Trusheva et al., 2006)
8	Guttiiferone E	HPLC-PDA- ESI/MS GC/MS, NMR	(Piccinelli et al., 2011) (Trusheva et al., 2006)
9	Xanthochymol	HPLC-PDA- ESI/MS GC/MS; NMR	(Piccinelli et al., 2011) (Trusheva et al., 2006)
10	Isoliquiritigenin	HPLC-PDA- ESI/MS GC/MS, NMR, HPLC	(Piccinelli et al., 2011) (Oldoni et al., 2011)
11	Liquiritigenin	ESI/MS HPLC-PDA- ESI/MS	(Frozza et al., 2013) (Piccinelli et al., 2011)
12	Retusapurpurin A and B	ESI/MS HPLC-PDA- ESI/MS	(Frozza et al., 2013) (Piccinelli et al., 2011)
13	10-Octadecenoic acid, methyl ester	GC/MS	(Alencar et al., 2007)

2.1 Volatile compounds - Terpenes

Volatile compounds are among the most widely secondary metabolites found in plants, animals and insects. These can be disperse in the air and related with pollinators attraction and seed dispersers, protect plants through repulsion or intoxication, among other functions. They are typically classified into four major categories: terpenes, fatty acid derivatives, amino acid derivatives and phenylpropanoid/benzenoid compounds (Rosenkranz and Schnitzler, 2016). Several of these compounds have been identified in red propolis, Figure 1.

Terpenes, which are biosynthetically derived from units of isoprene, are a large and diversified class of volatile compounds present in propolis. Limonene (**14**), α -Cubebene (**15**), β -Caryophyllene (**16**), which were identified by GC/MS are some representatives (Nunes et al., 2009).

The ester of oleic acid, 10-Octadecenoic acid, methyl ester (**13**), was recently identified in red propolis by GC-MS (Alencar et al., 2007). Among all volatile compounds found by the author, this was one of the most prevalent. These esters are usually used by plants to attract insects during pollination, which should happen with bees.

These volatile compounds are applied as flavors, fragrances, spices and used in perfumery, as well as food additives. Meanwhile, they have been reported also by the broad range of the biological activity including analgesic, anti-inflammatory, cancer chemopreventive effects, antimicrobial, antifungal, antiviral and antiparasitic activities (Paduch et al., 2007; Guimarães et al., 2014).

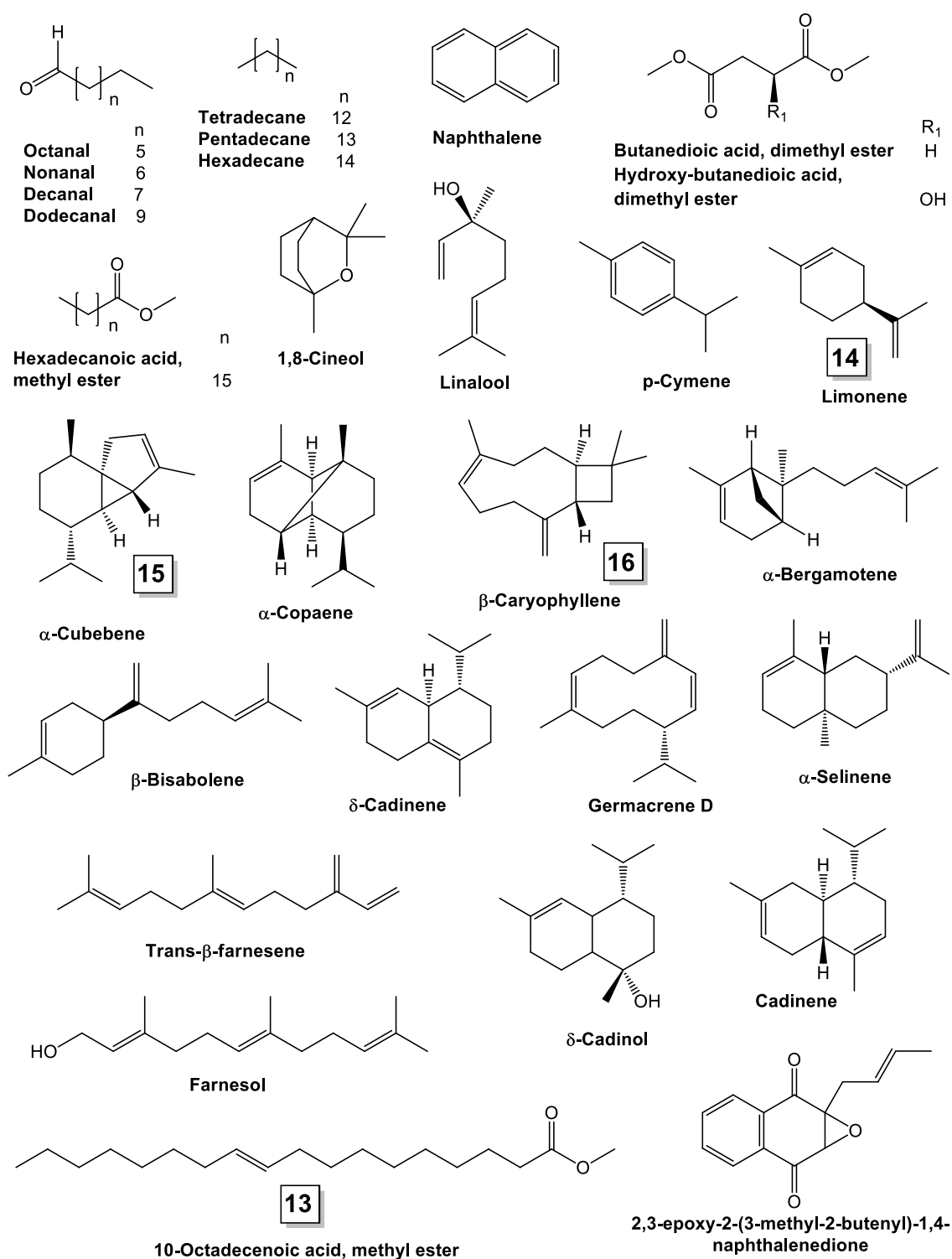


Figure 1. Chemical structures of volatile compounds, terpenes and derivatives from red propolis.

2.2 Phenolic compounds

Phenolic compounds are a large class of plant secondary metabolites, showing a diversity of structures including phenolic acids, flavonoids, lignans, quinones, tannins, coumarins and others (Huang et al., 2010). With ecological functions ranging from defense against microbial pathogens or herbivorous animals until sunlight protection, they can have simple or complex structures, as shown in fruits, vegetables, bark, roots and leaves.

In red propolis, several of these compounds have been found, as Elemicin (**7**), *trans*-anethole (**17**), Methyl eugenol (**18**), Figure 2 (Trusheva et al., 2006; Piccinelli et al., 2011). Also, these can play a important role in cancer prevention, anti-inflammatory and antioxidant activities (Balasundram et al., 2006; Cheynier, 2012). The Isoliquiritigenin (**10**) is considered a red propolis marker. In a comparative study between Brazilian and Cuban red propolis, this compound was among the major constituents in both samples (Piccinelli et al., 2011). The benzopyran know as Dalbergin (**19**) is a *Dalbergia ecastophyllum* marker, and its presence in Brazilian red propolis confirms the botanical origen (Daugusch et al., 2008).

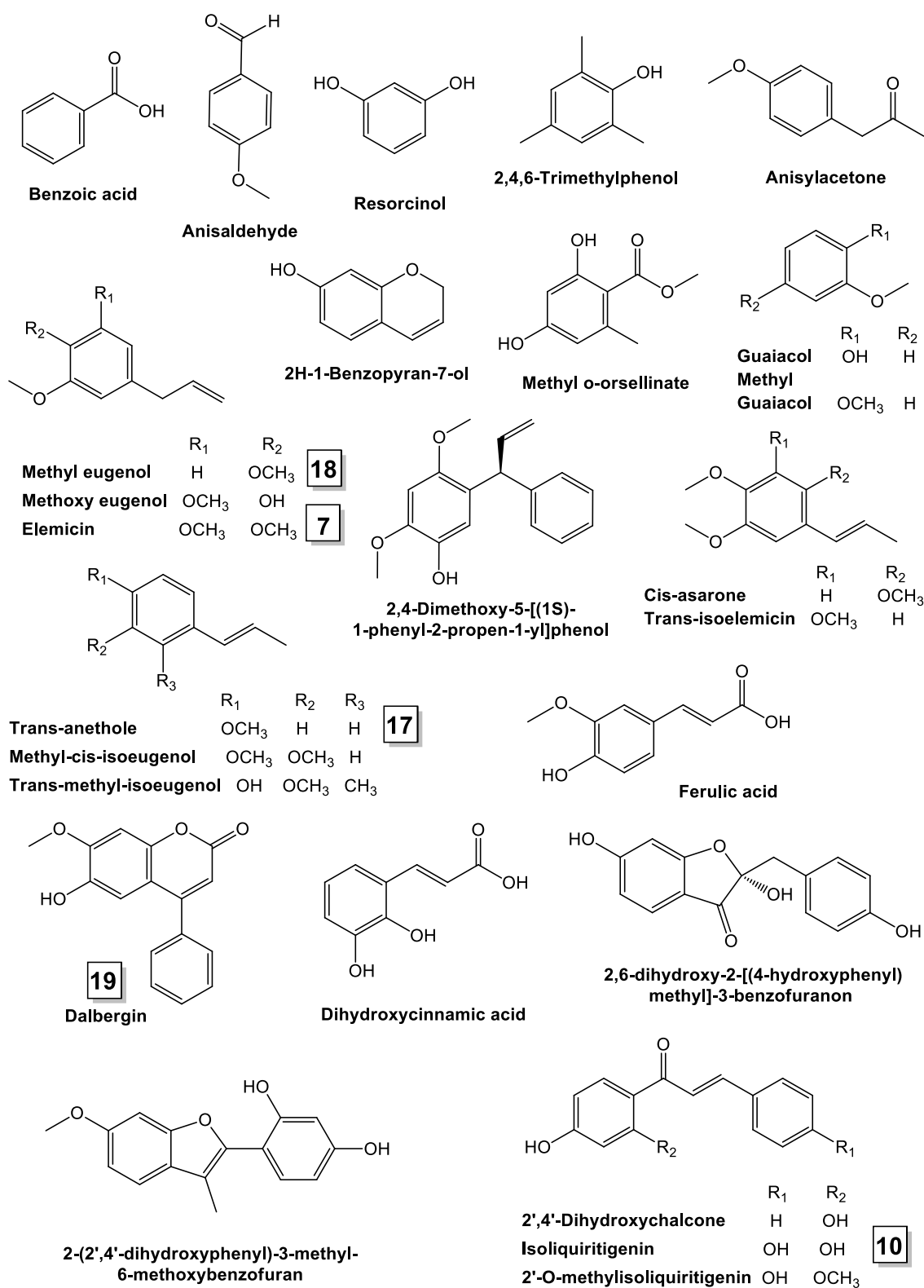


Figure 2. Chemical structures of some phenolics derivatives found in red propolis.

2.3 Flavonoids

Flavonoids represent the most common and widely distributed group of phenolics in red propolis. These are among the most active compounds in this resin, which act in different physiological processes, and perform various functions, including antimicrobial (Barbosa et al., 2009), as Quercetin (**20**) and Chrysin (**21**) Figure 3 (Silva et al., 2007). The Formononetin (**1**), an isoflavonoid with estrogenic, antiradical, cytotoxic and antifungal activities, was found in red propolis samples from Paraíba state (Frozza et al., 2013). In mammals it is metabolized to Daidzein (**6**), which has been reported efficient against breast and prostate cancer cells (Moraes, 2009). Another important flavonoid is the Biochanin A (**2**), which is a relevant chemical marker identified in red propolis (Piccinelli et al., 2011; Frozza et al., 2013) and has important activities such as inhibitory effects on cancer cells, anti-inflammatory action and others (Kole et al., 2011).

According to Hernandez et al. (2010), studies dealing with chemical composition of propolis can help establishing criteria for the quality control of the samples. The quality of propolis is checked by the Ministry of Agriculture, in Brazil, using parameters standardized (Brasil, 2001), since the biological properties of propolis are linked directly to its chemical composition.

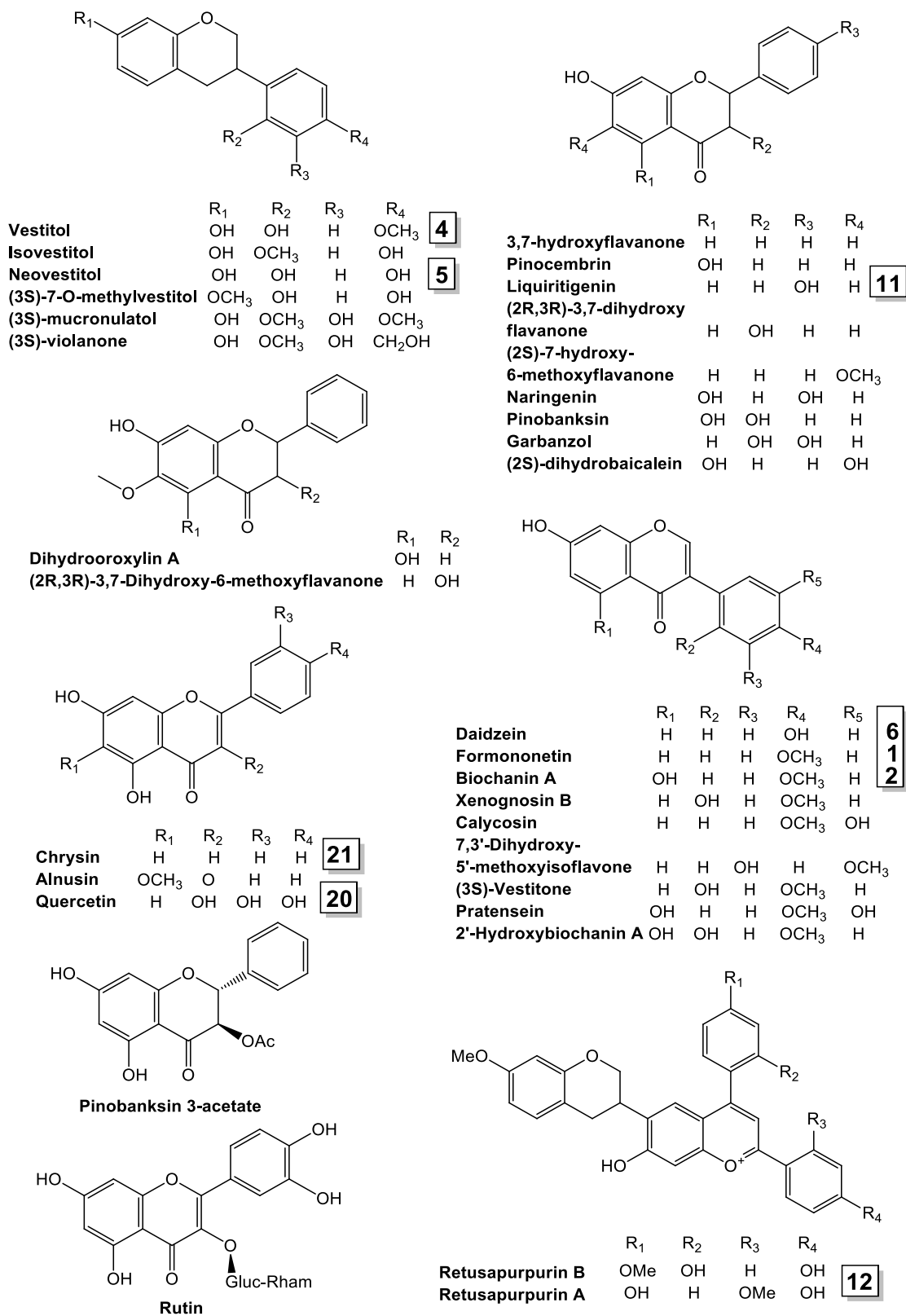


Figure 3. Chemical structures of flavonoids derivatives from red propolis.

2.4 Pterocarpan

Pterocarpan are isoflavonoids derivatives that can be described as benzo-pyrano-furano-benzenes. The Medicarpin (**3**) is well-known in this resin, which was identified using techniques such as ESI/MS, HPLC-PDA-ESI/MS and GC/MS (Alencar et al., 2007; Piccinelli et al., 2011; Frozza et al., 2013). Another important compound of this class is the Homopterocarpin (**22**), also identified in red propolis by GC/MS and HPLC-PDA-ESI/MS (Alencar et al., 2007; Piccinelli et al., 2011).

The pterocarpan have shown potent cytotoxic activity over a panel of tumor cell lines, highest antifungal activity and also play an important role as phytoalexins (Jiménez-González et al., 2008). The Figure 4 shows the derivatives found in red propolis.

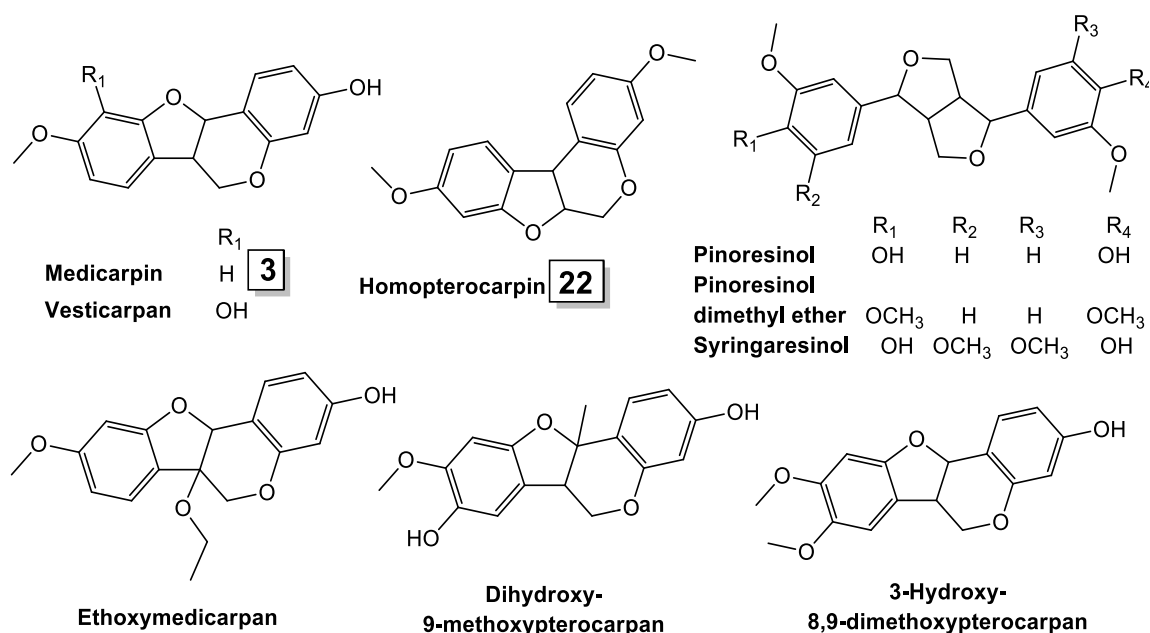


Figure 4. Chemical structures of pterocarpan derivatives from red propolis.

2.5 Xanthenes

The xanthone comprises important complex phenolics class. The structures are related to that of flavonoids and their chromatographic behaviours are similar. A number of xanthenes have been isolated from natural sources of higher plants, ferns, fungi and lichens. Some xanthone derivatives such as Guttiferone E (**8**), Xanthochymol (**9**) and Oblongifolin A (**23**), present in red propolis (Trusheva et al., 2006; Piccinelli et al., 2011) are shown in Figure 5. They have gradually increased their importance because of the medicinal properties as potent inhibitors of tumour growth, antioxidant, antibacterial, topoisomerase II inhibitory and antifungal activity (Lin et al., 1996; Luo et al., 2013; Negi et al., 2013).

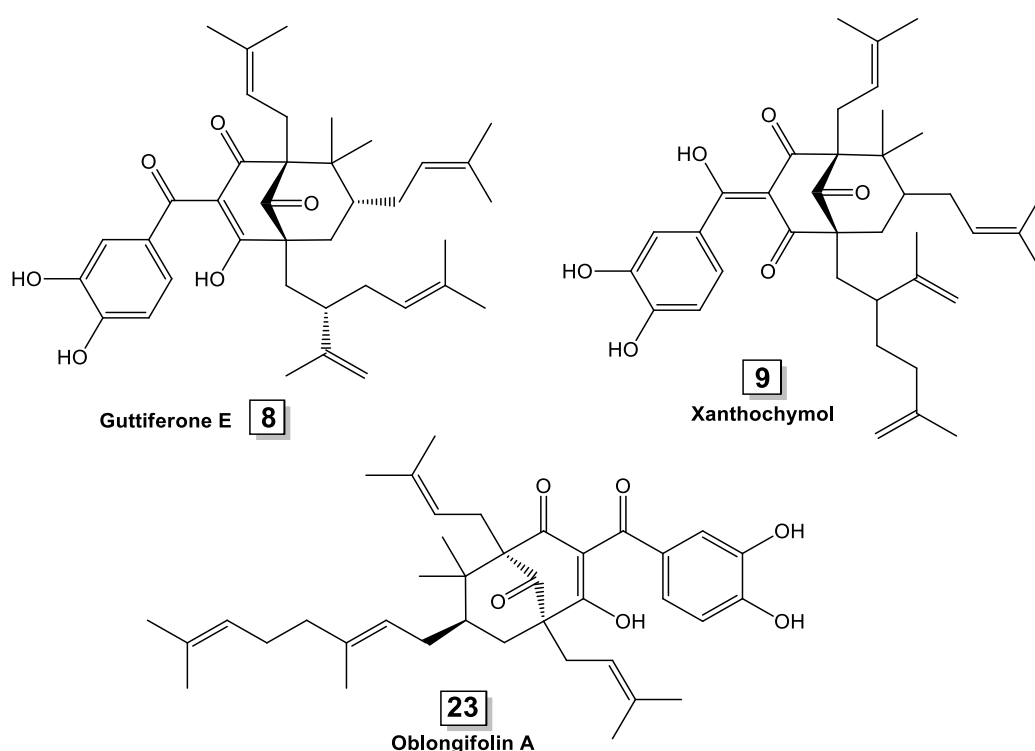


Figure 5. Chemical structure of Xanthenes derivatives isolated from red propolis.

2.6 Sterols

Sterols are a subgroup of the steroids, also known as steroid alcohols. They occur in plants, animals and fungi, with the most familiar type of animal sterol being cholesterol. Phytosterols are plant sterols structurally similar to cholesterol that act in the intestine to lower cholesterol absorption (Ostlund Jr., 2004). They can be isolated from vegetable oils, such as soybean oil, corn oil and others (Cantrill, 2008). Some sterol derivatives such as Lupeol (24) and β -Amyrin (25) can be identified by GC-MS and found in red propolis (Figure 6) (Trusheva et al., 2006).

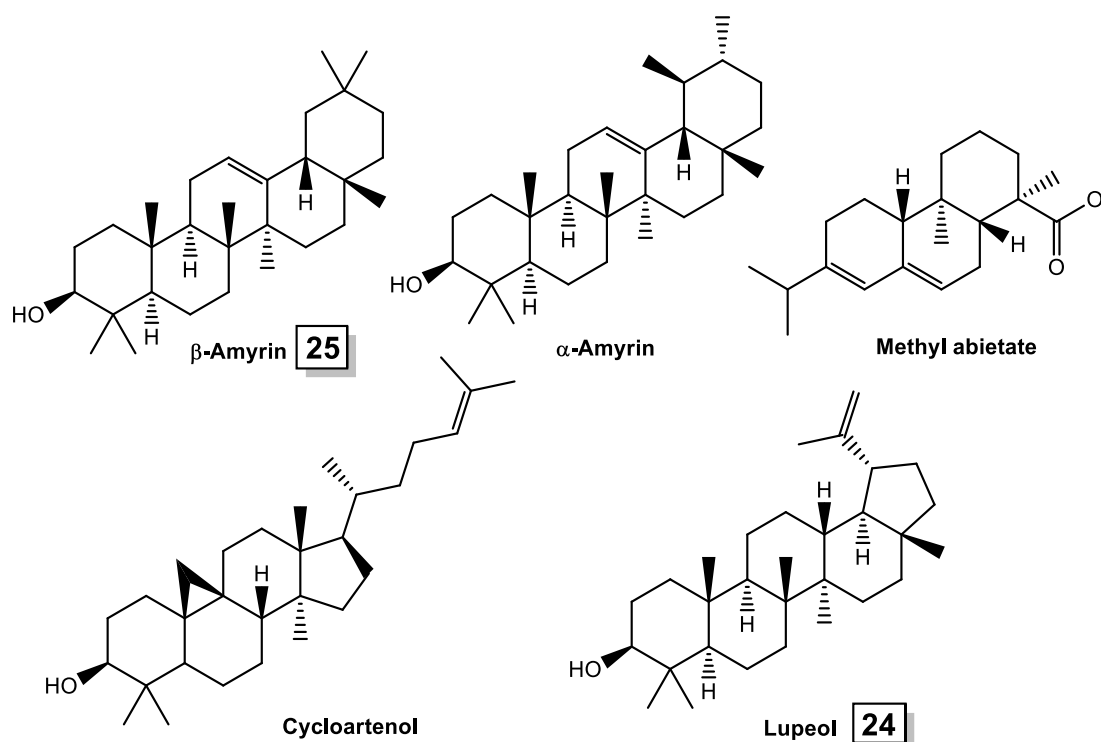


Figure 6. Chemical structures of Sterols derivatives in red propolis.

3. Pharmacological activities

Propolis has been systematically used in folk medicine by different civilizations over centuries. Studies confirm that propolis has a good therapeutic potential, especially antimicrobial, anticancer and antioxidant activities. The biological features are directly linked with the chemical composition, which can be a problem because of the variety of conditions, including the flora and harvest time, the processing technique, as well as the bee species (Castaldo and Capasso, 2002). The aim here is to highlight the pharmacological experiments and studies reported with red propolis, mainly from Brazil.

3.1 Antimicrobial Activity

Red propolis demonstrated a notable antimicrobial activity against many microorganisms such as bacteria, fungi and protozoa. The antimicrobial activity was evaluated against *Staphylococcus aureus* and *Staphylococcus mutans*, where the chloroform fraction was the most active with lower minimum inhibitory concentration (MIC) ranging from 25 to 50 $\mu\text{g.mL}^{-1}$ (Alencar et al., 2007). Cabral et al. (2009) also verified antibacterial properties against *S. aureus*. The best activity was obtained from the sub-fraction 4, obtained from an ethanolic extract of red propolis, with minimum bactericidal concentration (MBC) between 31.7-62.5 $\mu\text{g.mL}^{-1}$. Dausch et al. (2008) also described the antimicrobial activities of six samples of red propolis against *S. aureus*, four of them demonstrated higher inhibition of bacterial growth. The red propolis ethanol extract from Sergipe, Brazil, showed the highest antimicrobial activity *in vitro* for the three tested strains (*Staphylococcus aureus* ATCC 33951, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922), and the MIC were 400-100 $\mu\text{g.mL}^{-1}$, 50-25 $\mu\text{g.mL}^{-1}$ and 400 $\mu\text{g.mL}^{-1}$, respectively (Machado et al., 2016).

In a study developed by Oldoni et al. (2011), chloroform fraction (Chlo-fr) presented MIC values ranging from 31.2 to 62.5 $\mu\text{g.mL}^{-1}$ for *S. aureus* and from 62.5 to 125 $\mu\text{g.mL}^{-1}$ for *S. mutans* and *A. naeslundii*. Isolated compound vestitol presented MIC ranging from 31.2 to 62.5 $\mu\text{g.mL}^{-1}$, showing no distinction among the microorganisms assessed. Isoliquiritigenin was more potent than vestitol and exhibited MIC values ranging from 15.6 to 31.2 $\mu\text{g.mL}^{-1}$ for the three bacterial strains tested.

Bispo Junior et al. (2012) verified that the ethanol extract showed antimicrobial activity against gram-positive (100%) and gram-negative (62.5%) strains. The ethyl acetate fraction showed the best antimicrobial activity with efficiency at 100% for all species analyzed (*Shigella flexneri*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Escherichia coli*). Righi et al. (2011) verified that methanol extract inhibited the growth of all tested microorganisms. The MIC (256 $\mu\text{g.mL}^{-1}$) and MMC (512 $\mu\text{g.mL}^{-1}$) were observed against *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans*. This extract showed a higher MMC (1024 $\mu\text{g.mL}^{-1}$) against *Klebsiella pneumoniae*. Others MIC were: *Salmonella typhimurium* (512 $\mu\text{g.mL}^{-1}$), *Enterococcus faecalis* (512 $\mu\text{g.mL}^{-1}$), *Escherichia coli* (512 $\mu\text{g.mL}^{-1}$), *Proteus mirabilis* (512 $\mu\text{g.mL}^{-1}$) and *Streptococcus pyogenes* (512 $\mu\text{g.mL}^{-1}$). In addition, the ethanol extract of Brazilian red propolis showed larger inhibition zones and significantly different according to the type of propolis (green and brown), with inhibition zones of 27.25 ± 0.25 mm and 19.33 ± 0.94 mm to *Streptococcus mutans* and *Streptococcus sanguinis*, respectively (Da Silva et al., 2013).

Lopez et al. (2015) found that Brazilian red propolis (from Sergipe, Alagoas and Paraíba) when compared to the Cuba variety presented a similar chemical profile and showed antimicrobial activity against Gram-positive and Gram-negative (a better

activity with MIC between 6.2 to 500 $\mu\text{g.mL}^{-1}$) bacteria. The antimicrobial tests for some of the samples presented a MIC below the cytotoxic concentration of 50 $\mu\text{g.mL}^{-1}$ for HaCaT (human keratinocytes) and BALB/c 3T3 (murine fibroblast).

Virulent biofilms are responsible for a range of infections, including those occurring in the mouth. Dental caries is one of the most common and costly biofilm-dependent oral diseases, which afflicts children and adults worldwide (Dye et al., 2007). A fraction containing neovestitol and vestitol (NV) was isolated from red propolis and topical applications (800 $\mu\text{g.mL}^{-1}$) impaired the accumulation of biofilms of *S. mutans*. Also, the red propolis showed so effective as fluoride in reducing the development of carious lesions *in vivo* (Bueno-Silva et al., 2013).

Regarding orthopaedic implants, the four most prevalent bacterial species, accounting for over 75% of infections, are *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Nanohydroxyapatite (nanoHA)-based surfaces containing Brazilian extracts of propolis (green and red) to prevent bacterial growth and biofilm formation was investigated. The results obtained showed a reduction of *S. aureus* activity in a concentration-dependent way, which was significant at 6 $\mu\text{g.mL}^{-1}$ (Grenho et al., 2015).

Siqueira et al. (2014) evaluated the antimicrobial activity of red propolis extract against strains of *Enterococcus faecalis*. The extract promoted inhibition zone compared to results from solution of sodium hypochlorite (2.5%), showing values between 12 and 16 mm. There was no bacterial growth with solutions of red propolis at 7.5% and the MIC was 18000 mg.mL^{-1} and the MBC was 34090 mg.mL^{-1} .

Apart from vestitol and neovestitol aforementioned, other isolated molecules have also been tested for its antibacterial activity. In a recent study, the compound (6aS,11aS)-medicarpin exhibited the most potent antibacterial activity against *S. aureus*,

B. subtilis and *P. aeruginosa*, with MIC values of 16, 32 and 32 $\mu\text{g.mL}^{-1}$, respectively (Inui et al., 2014). Trusheva et al. (2006) observed that isosativan and medicarpin are important antimicrobial compounds, especially concerning the activity against *C. albicans*, showing inhibitory zone of 15 ± 1 and 26 ± 0 mm, respectively. Also, the mixture of prenylated benzophenones demonstrated good activity against *S. aureus* (19 ± 1 mm).

Red propolis containing high concentration of prenylated and benzophenones compounds showed to be the most active extract against *Leishmania amazonensis*. Ethanolic extracts of propolis were capable to reduce parasite load as monitored by the percentage of infected macrophages and the number of intracellular parasites. The parasite load of macrophages was reduced by the extract ($25 \mu\text{g.mL}^{-1}$), presenting no direct toxic effects to promastigotes and extracellular amastigotes (Ayres et al., 2011).

The activity of red propolis against fungi has also been described in some studies. Oral candidiasis is an infection caused by *Candida albicans*. It is known that saprobes microorganisms depend on predisposing factors to become pathogenic. This type of infection is most common in immuno-compromised individuals and presented increasing incidence in recent years. Bezerra et al. (2015) demonstrated in their study the antifungal action of the red propolis extract at 25% against *Candida*.

The dermatophytes are filamentous fungi belonging to three genera *Trichophyton*, *Microsporum* and *Epidermophyton* that are able to cause infection of the skin, hair and nails. The fungistatic activity of the red propolis alcoholic extract was determined in the concentrations ranging from 8 to 128 $\mu\text{g.mL}^{-1}$ for *T. rubrum*, 32 to 128 $\mu\text{g.mL}^{-1}$ for *T. tonsurans* and 16 to 128 $\mu\text{g.mL}^{-1}$ for *T. mentagrophytes*. The fungicide activity of the same extract was observed in the concentrations of 128-256,

128-1024 and 256-512 $\mu\text{g.mL}^{-1}$, respectively, for the same species (Siqueira et al., 2009).

Recently, Neves et al. (2016) analyzed the antimicrobial activity of Brazilian red propolis against the following bacteria and yeasts: *Staphylococcus aureus* ATCC 13150, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermides* ATCC 12228, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas aeruginosa* ATCC P-12, *Pseudomonas aeruginosa* ATCC P-03, *Candida albicans* ATCC 76645, *Candida albicans* LM P-20, *Candida tropicalis* ATCC 13803, *Candida tropicalis* LM 6, *Cryptococcus neoformans* ICB 59, *Cryptococcus neoformans* LM 2601. The hexane, acetate and methanol fractions of a variety of propolis inhibited all strains with MIC values ranging from 128 to 512 $\mu\text{g.mL}^{-1}$ for the six bacteria and from 32 to 1024 $\mu\text{g.mL}^{-1}$ for the yeasts tested. Similarly, hexane and acetate fractions of another variety of propolis inhibited all microorganisms, with MIC values of 512 $\mu\text{g.mL}^{-1}$ for bacteria and 32 $\mu\text{g.mL}^{-1}$ for yeasts.

3.2 Antioxidant Activity

The occurrence of many diseases is related to increases in the levels of free radicals, including cardiovascular, neurological diseases, cancer, osteoporosis, inflammation and diabetes (Devasagayam et al., 2004). In recent years, plants containing polyphenols showing antioxidant properties are target products used to control and prevent several diseases. In addition to the polyphenols, propolis contains an extensive range of other antioxidant compounds that interact with free radicals in body (Urquiaga and Leighton, 2000; Marquele et al., 2005).

Many studies have reported antioxidant activity for flavonoids that is due to their ability to reduce free radical formation and to scavenge free radicals (Heim et al., 2002;

Ahn et al., 2004). The hexane fraction of red propolis presented the highest concentration of total flavonoids and showed the best sequestering activity for the free radical DPPH (Alencar et al., 2007). Cabral et al. (2009) also found that the hexane fraction obtained from red propolis showed the highest antioxidant activity (74.4%), sequestering the free radical DPPH. In addition, Frozza et al. (2013) demonstrated that the hydroalcoholic extract of red propolis has important DPPH scavenging ability (IC₅₀ 270.13 µg.mL⁻¹). Also, Trusheva et al. (2006) observed that the mixture of prenylated benzophenones showed significant radical scavenging activity against DPPH (49% inhibition). DPPH free radical scavenging activity has also been tested by Righi et al. (2011) and the antioxidant activity of methanol extract of Brazilian red propolis (at maximum concentration 25 µg.mL⁻¹) was 39.12%. In the β-carotene oxidation method, the methanol extracts at concentrations 1.0, 1.5 and 2.0 mg.mL⁻¹ gave 84.5%, 85.3% and 85.7% of antioxidant activity, respectively, in relation to rutin. Oldoni et al. (2011) found that chloroform fraction (Chlo-fr) presented 57% activity, which was higher than that found for the ethanolic extract of propolis (26%). Isolated vestitol presented higher antioxidant activity (39.5%), neovestitol (21.4%) and isoliquiritigenin (8.7%).

More recently, the highest quantity of total phenols, flavonoids and the best antioxidant activity by ABTS was identified in the extract of red propolis (Sergipe), with values of 300.36 ± 0.01mg EAG/g, 57.60 ± 0.01 mg EQ/g and 98.50 ± 1.40%, respectively (Machado et al., 2016).

3.3 Anti-inflammatory activity

Inflammation is a natural response to a variety of hostile agents including parasites, pathogenic microorganism, toxic chemical substances, physical damage to tissue, among others (Agnihotri et al., 2010). The red propolis has also attracted interest

for its anti-inflammatory properties, as observed by Cavendish et al. (2015). The pretreatment with the hydroalcoholic extract of red propolis (10 and 30 mg.Kg⁻¹) and formononetin (10 mg.Kg⁻¹) produced reduction in the number of abdominal writhes and the extract was more effective. All the extract doses (3, 10 and 30 mg.Kg⁻¹) inhibited the late phase (inflammatory pain) of formalin-induced licking. All doses of extract (3, 10 and 30 mg.Kg⁻¹) and formononetin inhibited the carrageenan-induced leukocyte migration. Also, Bueno-Silva et al. (2013) verified the ethanolic extract, neovestitol and vestitol inhibition activity against neutrophil migration at 10 mg.Kg⁻¹ dose in Male Balb/c mice.

In a recent study, Franchin et al. (2016) investigated the mechanism of action of vestitol on the modulation of neutrophil migration in the inflammatory process. Pre-treatment with vestitol at 1, 3, or 10 mg.Kg⁻¹ reduced LPS- or mBSA-induced neutrophil migration and the *in vivo* release of CXCL1/KC and CXCL2/MIP-2.

Likewise, the *in vitro* levels of CXCL1/KC and CXCL2/MIP-2 in macrophage supernatants were reduced by vestitol (1, 3, or 10 µM). Moreover, the administration of vestitol (10 mg.Kg⁻¹) reduced leukocyte rolling and adherence in the mesenteric microcirculation of mice. The pre-treatment with vestitol (10 mg.kg⁻¹) in iNOS^{-/-} mice did not block its activity concerning neutrophil migration. With regard to the activity of vestitol (at 3 or 10 µM) on neutrophils isolated from the bone marrow of mice, there was a reduction on the chemotaxis of CXCL2/MIP-2 or LTB₄-induced neutrophils and on calcium influx.

3.4 Healing Activity

Propolis is an apitherapy product widely employed in natural medicine. Among the various therapeutic properties against a variety of conditions, its ability to heal tissues

has been discussed in some studies. Albuquerque-Júnior et al. (2009) observed that the incorporation of Brazilian red propolis into collagen-based films was able to improve wound healing, probably by modulating the dynamics of the inflammatory evolution and collagen deposition process.

In another study, Almeida et al. (2013) observed that the extract of red propolis provided decrease of the inflammatory severity of rodents, induced earlier replacement of type-III for type-I collagen, improved the epithelization rates and the myofibroblastic count was significantly increased in 14 and 21 days, as well as grosser interlacement of the collagen bundles.

3.5 Cytotoxic Activity

The search for new drugs against various types of cancer has led researchers to fractionate extracts and isolate compounds contained in propolis samples from different sources. Awale et al. (2008) observed that the methanol extract ($10 \mu\text{g.mL}^{-1}$) of Brazilian red propolis was cytotoxic against human pancreatic cancer cells. Human bladder cancer cells were tested with Brazilian red propolis ethanolic extract, which showed cytotoxicity (IC_{50} of $95 \mu\text{g.mL}^{-1}$) and also inducing apoptosis-like mechanisms (Beghini et al., 2014). Franchi Jr. et al. (2012) demonstrated that red propolis was cytostatic in human cell lines of leukemia and induced apoptosis.

Ethanolic extract of red propolis showed cytotoxic activity for the human cervical adenocarcinoma (HeLa) cells with an IC_{50} of $7.45 \mu\text{g.mL}^{-1}$ (Alencar et al., 2007). Frozza et al. (2013) analyzed the hydroalcoholic extract activity on human laryngeal epidermoid carcinoma cell (Hep-2), HeLa and human normal epithelial embryonic kidney (Hek-283) cell lines, with IC_{50} $63.48 \mu\text{g.mL}^{-1}$, $81.40 \mu\text{g.mL}^{-1}$ and $>150 \mu\text{g.mL}^{-1}$, respectively. A study conducted by Kamiya et al. (2012) showed that

ethanol extract of Brazilian red propolis reduced human breast cancer (MCF-7) cell viability through the induction of mitochondrial dysfunction, DNA fragmentation, caspase-3 activity and induces apoptosis through endoplasmic reticulum stress-related signaling.

The red propolis ethanol extracts (50 and 100 $\mu\text{g.mL}^{-1}$) from Sergipe, Brazil, showed the lowest contents of viable cells against melanoma murine (B16F10) models (Machado et al., 2016). Novak et al. (2014) performed a study where the antiproliferative effect of BRP-IV fraction was assayed using melanoma tumour xenografts in mice and acute promyelocytic leukaemia (HL-60), human chronic myelogenous leukaemia (K562), human multiple myeloma (RPMI 8226) and murine melanoma (B16F10) cell lines. This fraction inhibited growth of tumour cell lines with IC50 values of 20.5 ± 2.4 to $32.6 \pm 2.6 \mu\text{g.mL}^{-1}$, inhibiting the proliferation of B16F10 cells by blocking cell cycle progression in the G2/M phase and inducing apoptosis. Already, the ethanolic extract induced cytotoxic effect with IC50 of 29.7 ± 1.5 to $42.1 \pm 8.7 \mu\text{g.mL}^{-1}$.

Li et al. (2008) tested isolated compounds of red propolis against a variety of cell lines, among them 7-hydroxy-6-methoxyflavanone exhibited the most potent activity against Lewis lung carcinoma - LLC (IC50 9.29 μM), murine B16-BL6 melanoma (IC50 6.66 μM), human lung A549 adenocarcinoma (IC50 8.63 μM) and human HT-1080 fibrosarcoma (IC50 7.94 μM) cancer cell lines. Other compound, the mucronulatol, was potent against LLC (IC50 8.38 μM) and A549 (IC50 9.9 μM) cell lines.

Oral carcinogenesis is a highly complex multi-focal process that occurs when squamous epithelium is affected by several genetic alterations. Hydroalcoholic extract (50 and 100 mg.kg^{-1}) of Brazilian red propolis inhibited 40% of DMBA-induced oral

squamous cell carcinomas growth and promoted a 3-week delay in development of clinically detectable tumours in murine models (adult Swiss male mice, *Mus musculus*) (Ribeiro et al., 2015).

4. Other pharmacological potential uses

Table 2 describes other pharmacological applications reported in literature of Brazilian red propolis.

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Table 2. Potential uses of red propolis

SAMPLE	ACTIVITY	REFERENCE
Methanolic extract	The Brine shrimp bioassay was used and the extract demonstrated a DL_{50} of $18.9 \mu\text{g.mL}^{-1}$, suggesting an antitumor activity.	(Nunes et al., 2009)
Ethanolic extracts	The differentiation of 3T3-L1 preadipocytes into adipocytes was induced by the extracts. Also, enhanced the PPAR γ transcriptional activity, adiponectin promoter activity, attenuated the inhibitory effect of TNF- α on adipocyte differentiation and adiponectin production in mature adipocytes. So, the ethanolic extracts can be used as a diet supplement for prevention and treatment of obesity.	(Iio et al., 2010)
Ethanolic extracts	The ApoA-I-mediated cholesterol efflux in THP-1 macrophages was enhanced by EERP extracts and induction of ABCA1 gene. The effect of EERP on ABCA1-dependent cholesterol efflux was explained by its potency of induction of PPAR γ and LXR α expression. Thus, EERP have a potential as a diet supplement for prevention/treatment of cardiovascular disease.	(Iio et al., 2012)
Ethanolic extracts	The underlying molecular mechanisms of the potential anticancer effects of red propolis on Hep-2 and Hek-293 cells were evaluated. A total of 1336 and 773 proteins were identified for Hep-2 and Hek-293, of which 16 were regulated in the Hep-2 and 4 in Hek-293 cell. The biological process most prominent was associated to cell metabolism and the predominant molecular function was catalytic activity.	(Frozza et al., 2016)
Hydroalcoholic extract	Alterations in the protein profile of Hep-2 treated with red propolis were investigated. 177 proteins were identified using LC-MS-MS and most were down-regulated in presence of extract (IC_{50} $120 \mu\text{g.mL}^{-1}$): GRP78, PRDX2, LDHB, VIM, TUBA1A. Only two up-regulated proteins were identified in the non-cytotoxic ($6 \mu\text{g.mL}^{-1}$) red propolis treated group: RPLP0 and RAD23B. It was observed an increase in the mid- to late-stage apoptosis of Hep-2 cells induced by red propolis (60 and $120 \mu\text{g.mL}^{-1}$). Late apoptosis in a dose-dependent manner was induced by the extract.	(Frozza et al., 2014)
Hydroalcoholic extract	Treatment of rats with renal ablation using red propolis extract (150 mg/kg/day in drinking water) for 60 days reduced hypertension, proteinuria, oxidative stress, renal macrophage infiltration, serum creatinine retention and glomerulosclerosis. The renoprotective effects might be related to the reduction of oxidative stress and renal inflammation.	(Teles et al., 2015)

5. Red propolis worldwide

Propolis is a natural product widely used by the world population due to its interesting properties and this has generated distinct research lines in several countries. The red type is found in Brazil, but also in countries such as Cuba, Mexico, China and Nigeria.

Some studies are being developed in Cuba with respect to chemical composition and biological activity of this propolis. Fernández et al. (2008) analyzed seven red Cuban propolis samples by GC-MS and some compounds were identified: Formononetin (**1**); Medicarpin (**3**); Vestitol (**4**); Neovestitol (**5**); Isoliquiritigenin (**10**); Liquiritigenin (**11**); Homopterocarpin (**22**); 3-Hydroxy-8,9-dimethoxypterocarpan; 7-*O*-Methylvestitol; 3,10-Dihydroxy-9-methoxypterocarpan; 3,4-Dihydroxy-9-methoxypterocarpan and 3,8-Dihydroxy-9-methoxypterocarpan.

Piccinelli et al. (2011) verified that red propolis samples from different tropical zones have a similar chemical composition.

Some isoflavones, isoflavans, pterocarpan and compounds as isoliquiritigenin, liquiritigenin and naringenin were detected in Brazilian red propolis, Cuban red propolis and *Dalbergia ecastophyllum* exudates. However Guttiferone E (**8**), Xanthochymol (**9**) and Oblongifolin A (**23**) were detected only in Brazilian red propolis.

In another study, Cuesta-Rubio et al. (2007) prepared methanolic extracts of sixty-five samples of Cuban propolis (red, yellow and brown) and observed that the red propolis presented a more complex composition, containing isoflavonoids as the main constituents, and Formononetin (**1**) and Medicarpin (**3**) were considered marker compounds.

Ledón et al. (1997) studied the antipsoriatic, anti-inflammatory and analgesic effects of Cuban red propolis ethanolic extract. The extract induced the formation of

granular layer in the mouse tail test and also, it showed anti-inflammatory activity in the peritoneal capillary permeability test in mice (10 mg.kg⁻¹), in cotton-pellet granuloma assay in rats (50 mg.kg⁻¹) and in croton oil-induced edema in mice (at 25%). The extract showed analgesic effect in the model of acetic acid-induced writhings (25 mg.kg⁻¹), whereas 40 mg.kg⁻¹ was effective in the hot plate test. Also, the effect of Cuban red propolis against hepatitis induced by 1000 mg.kg⁻¹ of galactosamine in rats was studied by Rodríguez et al. (1997). An ethanolic extract (10, 50 and 100 mg.kg⁻¹) was given to rats and it was able to prevent hepatocytes alterations induced by galactosamine. Propolis extract induced reversion of the increased activity of alanine aminotransferase and malondialdehyde concentration in the serum of rats treated with galactosamine.

In another study with Cuban red propolis, the antibacterial, antiprotozoal and antifungal properties were evaluated and can be associated with the chemical composition. The samples showed the following IC₅₀: 4.4 to 25.9 µg.mL⁻¹ (*Staphylococcus aureus*), > 64 µg.mL⁻¹ (*Escherichia coli*), > 64 µg.mL⁻¹ (*Candida albicans*), 1.2 to 8.3 µg.mL⁻¹ (*Trypanosoma brucei*), 1.2 to 6.4 µg.mL⁻¹ (*Plasmodium falciparum*), 2.5 to 9 µg.mL⁻¹ (*Trypanosoma cruzi*), 14.9 to 39.4 µg.mL⁻¹ (*Trichophyton rubrum*) and 3.3 to 16.1 µg.mL⁻¹ (*Leishmania infantum*) (Monzote et al., 2012).

The red Mexican propolis was studied by Lotti et al. (2010). They verified the chemical composition and isolated three new compounds: 1-(3',4'-dihydroxy-2'-methoxyphenyl)-3-(phenyl)propane, (Z)-1-(2'-methoxy-4',5'-dihydroxyphenyl)-2-(3-phenyl)propene and 3-hydroxy-5,6-dimethoxyflavan, together with seven known compounds: Arizonicanol A; Vestitol; Pinocembrin; Mucronulatol; Melilotocarpan A; Melilotocarpan D and 7-Hydroxyflavanone.

Hatano et al. (2012) studied the red propolis from Shandong, China. Ethanol extracts (EE) showed strong antioxidant activity. The total polyphenol content, the

flavonoid content, DPPH and ABTS radical scavenging activity, ferric reducing activity power (FRAP assay) and the oxygen radical absorbance capacity (ORAC) value were $433.8 \pm 1.7 \text{ mg.g}^{-1}$ of EE, $129.6 \pm 1.1 \text{ mg.g}^{-1}$ of EE, $98.8 \pm 1.0\%$, $90.9 \pm 0.6\%$, $89.2 \pm 3.8 \text{ }\mu\text{g.mL}^{-1}$ and $14900 \pm 443 \text{ }\mu\text{mol Trolox equivalents/g}$ of EE, respectively. It was also possible to identify the major components in the EE sample, by HPLC: Apigenin ($15.4 \pm 0.8 \text{ mg.g}^{-1}$ of EE); Benzyl caffeate ($21.1 \pm 2.1 \text{ mg.g}^{-1}$ of EE); Caffeic acid ($3.8 \pm 0.4 \text{ mg.g}^{-1}$ of EE); Chrysin ($47.2 \pm 3.7 \text{ mg.g}^{-1}$ of EE); Cinnamic acid ($4.2 \pm 0.6 \text{ mg.g}^{-1}$ of EE); Cinnamyl caffeate ($7.6 \pm 0.6 \text{ mg.g}^{-1}$ of EE)/ *p*-Coumaric acid ($6.8 \pm 0.7 \text{ mg.g}^{-1}$ of EE)/ 3,4-Dimethoxycinnamic acid ($18.8 \pm 1.2 \text{ mg.g}^{-1}$ of EE); Ferulic acid ($9.8 \pm 0.5 \text{ mg.g}^{-1}$ of EE); Galangin ($101.6 \pm 4.5 \text{ mg.g}^{-1}$ of EE); Phenethyl caffeate ($32.7 \pm 2.3 \text{ mg.g}^{-1}$ of EEP); Pinobanksin ($3.0 \pm 0.3 \text{ mg.g}^{-1}$ of EEP); Pinobanksin 3-acetate ($85.7 \pm 3.4 \text{ mg.g}^{-1}$ of EEP); Pinobanksin 5-methyl ether ($17.2 \pm 1.1 \text{ mg.g}^{-1}$ of EEP); Pinocembrin ($38.2 \pm 2.8 \text{ mg.g}^{-1}$ of EEP); Pinostrobin ($4.0 \pm 0.5 \text{ mg.g}^{-1}$ of EEP); and Tectochrysin ($10.6 \pm 1.1 \text{ mg.g}^{-1}$ of EEP).

The Nigerian red propolis was evaluated with respect the activity against *Trypanosoma brucei* and its chemical composition. Some compounds were identified: Vestitol; Calycosin; Pinocembrin; Macarangin; Medicarpin; Liquiritigenin; 8-Prenylnaringenin; 6-Prenylnaringenin; Propolin D and Riverinol. The compounds showed anti-trypanosomal activity with EC_{50} values from $4.2 \text{ }\mu\text{g.mL}^{-1}$ for the crude extract to $16.6 \text{ }\mu\text{g.mL}^{-1}$ for Riverinol. The compound 8-Prenylnaringenin ($6.1 \text{ }\mu\text{g.mL}^{-1}$) was the most active against the wild-type strain, but vestitol and macarangin displayed similar activities (Omar et al., 2016).

6. Conclusion

This review highlighted the chemical composition and the biological features of red propolis. The potential of this special bee resin have been demonstrated by its broad spectrum of therapeutic properties. However, due to its distinct and complex chemical constitution, the development of further research is important, ensuring its safe use.

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