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Estudo bioguiado da Própolis Vermelha Brasileira visando à atividade antibacteriana

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

Orientador: Dr. Sidnei Moura e Silva

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LUCIANE CORBELLINI RUFATTO

ESTUDO BIOGUIADO DA PRÓPOLIS VERMELHA BRASILEIRA VISANDO À ATIVIDADE ANTIBACTERIANA

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

Orientador: Prof. Dr. Sidnei Moura e Silva

Co-orientadora: Profa. Dra. Mariana Roesch Ely

TESE APROVADA EM 20 DE DEZEMBRO DE 2016.

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Orientador: Prof. Dr. Sidnei Moura e Silva

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RESUMO

O desenvolvimento de fármacos eficientes no combate às infecções bacterianas foi um processo que revolucionou áreas voltadas à prevenção e terapêutica de doenças infecciosas. Entretanto, o uso indiscriminado destes agentes antibacterianos, fez com que as bactérias desenvolvessem inúmeras defesas, tornando-se resistentes. Esta resistência é um problema cada vez mais grave para a saúde pública mundial, impondo limitações preocupantes no tratamento das doenças causadas por bactérias. Desta forma, os produtos de origem natural emergem como importantes provedores de compostos bioativos, servindo para o desenvolvimento e a síntese de um grande número de novos fármacos. A própolis é um destes produtos, a qual vem sendo bastante utilizada na medicina popular devido as suas inúmeras propriedades biológicas, entre elas a antibacteriana, que estão associadas à presença de compostos como flavonoides, terpenos, derivados fenólicos, entre outros. Desta forma, este trabalho teve como objetivo a realização de um estudo bioguiado com o extrato hidroalcoólico da própolis vermelha de Alagoas (Brasil), sendo possível obter subfrações com significante atividade bacteriostática. A subfração SC2 demonstrou os melhores resultados, evidenciando-se pela sua potente ação frente *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* e *Pseudomonas aeruginosa*, com CIM de 56,75 $\mu g.mL^{-1}$, 28,37 $\mu g.mL^{-1}$, 454 $\mu g.mL^{-1}$ e 227 $\mu g.mL^{-1}$, respectivamente. Todavia, o estudo também revelou um efeito citotóxico frente à linhagem celular não tumoral Vero. Além disso, a caracterização química realizada por Espectrometria de Massas de Alta Resolução (EMAR), Cromatografia a Líquido de Alta Eficiência com detector UV (CLAE-UV) e Cromatografia a Gás acoplada com Espectrometria de Massas (CG-EM) permitiu a identificação de importantes compostos químicos marcadores desta própolis, como a Formononetina (*m/z* 267.0663), Biochanina A (*m/z* 283.0601) e Liquiritigenina (*m/z* 255.0655), além de outros compostos relevantes com

comprovada atividade antibacteriana. Portanto, a própolis vermelha brasileira mostra-se como uma alternativa terapêutica promissora para prevenir e combater doenças infecciosas, sendo fonte de compostos biologicamente ativos contra micro-organismos patogênicos.

ABSTRACT

The development of efficient drugs to combat bacterial infections was a process that revolutionized areas for the prevention and treatment of infectious diseases. However, the indiscriminate use of these antibacterial agents, made many bacteria develop defenses, becoming resistant. This resistance is an increasingly serious problem for global public health, imposing worrying limitations on the treatment of diseases caused by bacteria. In this way, products of natural origin emerge as important suppliers of bioactive compounds, serving for the development and synthesis of a large number of new drugs. Propolis is one of these products, which has been widely used in popular medicine due to its innumerable biological properties, including the antibacterial, which are associated with the presence of compounds such as flavonoids, terpenes, phenolic derivatives, among others. Thus, this work aimed to carry out a bioguided study with the hydroalcoholic extract of red propolis of Alagoas (Brazil), being possible to obtain subfractions with significant bacteriostatic activity. The SC2 subfraction showed the best results, evidenced by its potent action against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*, with MIC of 56.75 $\mu g.mL^{-1}$, 28.37 $\mu g.mL^{-1}$, 454 $\mu g.mL^{-1}$ and 227 $\mu g.mL^{-1}$, respectively. However, the study also revealed a cytotoxic effect against the Vero non-tumor cell line. In addition, the chemical characterization performed by High Resolution Mass Spectrometry (HRMS), High Performance Liquid Chromatography with UV detection (HPLC-UV) and Gas Chromatography coupled to Mass Spectrometry (GC-MS) allowed the identification of important compounds chemical markers of this propolis, such as Formononetin (*m/z* 267.0663), Biochanin A (*m/z* 283.0601) and Liquiritigenin (*m/z* 255.0655), as well as other relevant compounds with proven antibacterial activity. Therefore, Brazilian red propolis is a

promising therapeutic alternative for preventing and treating infectious diseases, being a source of biologically active compounds against pathogenic microorganisms.

1. INTRODUÇÃO

As plantas representam um rico depósito natural de substâncias químicas, as quais têm sido utilizadas pelo homem para os mais diversos fins. A partir delas, é possível obter diferentes produtos, como por exemplo, os extratos vegetais, os quais são utilizados com notável eficácia frente a diversas enfermidades na medicina popular. Nas últimas décadas, o uso de produtos naturais tem crescido e ganhando credibilidade em decorrência de importantes pesquisas farmacêuticas, que priorizam a identificação de novos compostos bioativos, bem como a compreensão dos seus mecanismos de ação.

Em meio à enorme diversidade de produtos de origem natural existentes no Brasil, os produtos apícolas têm se destacado pela fácil obtenção e por apresentarem inúmeras propriedades farmacológicas. Dentre eles está a própolis, uma substância resinosa produzida por abelhas a partir de substâncias vegetais. No Brasil, há 13 tipos distintos desta resina, que possuem diferentes colorações, composição química complexa e relevantes atividades biológicas, com destaque para a própolis encontrada na região nordeste do Brasil, denominada própolis vermelha.

Por muitos anos tem se estudado as propriedades medicinais da própolis, a qual vem sendo usada para várias finalidades, especialmente como antibacteriana. Esta atividade tem sido relatada em alguns estudos, porém ainda em pequena proporção, necessitando de maiores investigações. Além disso, grande parte dos trabalhos encontrados na literatura refere-se à própolis verde, sendo que apenas nos últimos anos a própolis vermelha tem se tornado objeto de estudo.

A descoberta dos antibióticos constituiu um progresso inquestionável na medicina, porém a eficácia destes agentes foi superada pela capacidade das bactérias de se oporem à sua ação. Esta resistência também está ligada a escassez de novos antibióticos, e pode levar ao

sofrimento prolongado do paciente, maior risco de complicações, aumento da mortalidade, além de um custo mais elevado no diagnóstico e tratamento.

As infecções bacterianas provocam em torno de 25% das mortes no mundo e, de acordo com pesquisas, caso não sejam tomadas providências, estima-se que em 2050 as bactérias resistentes causarão mais mortes do que enfermidades crônicas como o câncer. Assim, devido à alta incidência de processos infecciosos, muitos associados ao uso indiscriminado de antibióticos e a consequente resistência bacteriana, e diante do número crescente de mortes relacionadas, são necessárias pesquisas para a busca e desenvolvimento de novas estratégias preventivas e terapêuticas, sendo os produtos naturais, como a própolis, uma importante alternativa.

Neste contexto, é importante avaliar a atividade antibacteriana da própolis vermelha através de estudos bioguiados, os quais são processos racionalizados que permitem somente a seleção de amostras ativas a uma determinada função desejada, sendo estas usadas no seguimento do estudo. Além disso, a identificação da complexa diversidade de compostos químicos presentes na própolis vermelha é primordial, visto que, as propriedades biológicas estão ligadas diretamente a sua composição química. E, sobretudo, é indispensável à avaliação de sua seletividade e citotoxicidade antes de sugerir sua utilização para fins terapêuticos, garantindo o seu uso seguro.

2. OBJETIVOS

Objetivo geral

Realizar um estudo bioguiado com a própolis vermelha brasileira, a fim de se obter frações e/ou subfrações que apresentem atividade antibacteriana.

Objetivos específicos

- Elaborar uma ampla revisão, compilando informações sobre a própolis vermelha encontrada no Brasil e também em outros países do mundo;
- Identificar o método mais adequado (com maior rendimento de extrato bruto e menor quantidade de solvente) para extração dos compostos ativos da própolis vermelha;
- Realizar o fracionamento do extrato bruto da própolis vermelha, bem como de suas frações, visando à seleção da fração e/ou subfração mais ativa segundo o teste biológico *in vitro* frente às bactérias;
- Avaliar a atividade antibacteriana do extrato bruto e das frações/subfrações de própolis vermelha frente a diferentes bactérias como *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* e *Pseudomonas aeruginosa*;
- Avaliar a citotoxicidade das frações e/ou subfrações, que apresentaram melhor atividade antibacteriana, frente à célula não tumoral Vero;
- Qualificar a composição química do extrato bruto e das frações/subfrações mais ativas, através de metodologias sensíveis, seletivas e com boa reprodutibilidade (EMAR, CLAE-UV, CG-EM);

- Quantificar os compostos marcadores de interesse (Formononetina, Liquiritigenina e Biochanina A) na própolis vermelha através de CLAE-UV;
- Relacionar os possíveis compostos identificados com a atividade biológica apresentada.

3. REVISÃO BIBLIOGRÁFICA

3.1 Plantas medicinais: fonte de moléculas bioativas

Desde os primórdios da existência humana, os homens buscam na natureza recursos para melhorar sua própria condição de vida, aumentando suas chances de sobrevivência. Tal interação é evidenciada na relação entre seres humanos e plantas, uma vez que os usos dos recursos vegetais são dos mais diversos, como é o caso da alimentação, construção de moradias, confecção de vestimentas e finalidades medicinais. O emprego de plantas medicinais para a saúde tem ocorrido ao longo dos tempos desde as formas mais simples de tratamento local até as formas mais sofisticadas de produção de medicamentos (Hamilton, 2004; Giraldi & Hanazaki, 2010).

As práticas relacionadas ao uso tradicional de plantas medicinais são o que muitas comunidades têm como alternativa para tratar doenças. Por outro lado, a introdução da medicina moderna traz uma opção para as práticas de saúde locais já estabelecidas e, em muitas instâncias, os procedimentos da medicina moderna e da medicina popular podem se tornar complementares (Amorozo, 2004).

Uma das características dos seres vivos é a presença de atividade metabólica. No caso dos vegetais, o metabolismo costuma ser dividido em primário e secundário. Os metabólitos primários são frequentemente citados como compostos diretamente ligados à sobrevivência da planta, tais como: proteínas, lipídeos, aminoácidos, açúcares, etc (Kerbauy, 2004; Simões *et al.*, 2010). Já os metabólitos secundários são compostos pouco abundantes e têm um papel importante na adaptação ao ambiente, contribuindo para que as mesmas possam ter uma boa interação com os diferentes ecossistemas. Ainda, eles aumentam a probabilidade de sobrevivência de uma espécie, pois são responsáveis por diversas atividades biológicas com

este fim como, por exemplo, podem atuar como antibióticos, antifúngicos e antivirais para proteger as plantas dos patógenos, e também por apresentar atividades antigerminativas ou tóxicas para outras plantas. Alguns constituem importantes compostos que absorvem a luz ultravioleta evitando que as folhas sejam danificadas (Li *et al*., 1993).

Estes compostos são usualmente classificados, de acordo com a sua rota biossintética (Figura 1), em três famílias principais: alcaloides, compostos fenólicos e terpênicos/esteroides.

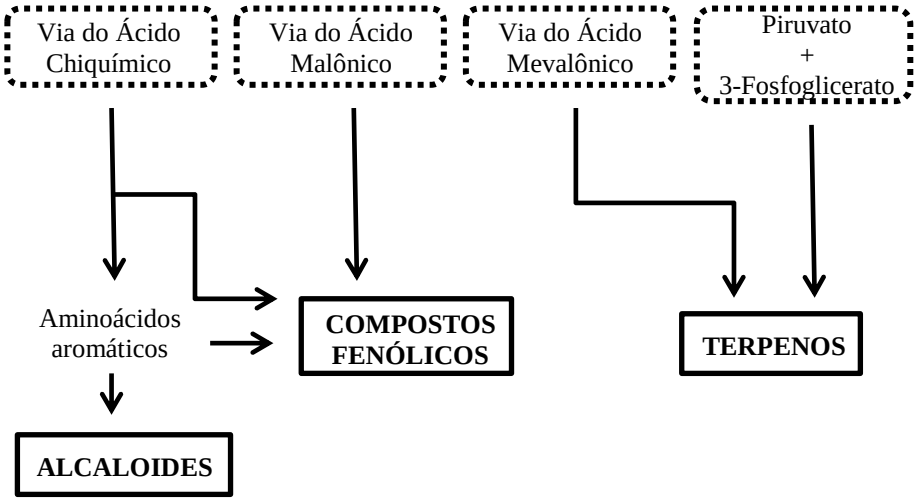

Figura 1. Rota biossintética simplificada dos metabólitos secundários.

(Fonte: adaptado de Taiz & Zeiger, 2004)

Devido à produção de inúmeros metabólitos com propriedades farmacológicas relevantes, as plantas têm contribuído para o desenvolvimento de novas estratégias terapêuticas e obtenção de vários fármacos até hoje amplamente utilizados na clínica. Estes têm as mais diversas funções, que vão desde o combate ao câncer até micro-organismos patogênicos, como é o caso da morfina, vincristina, colchichina, emetina e muitos outros, os quais se destacam por suas propriedades (Cechinel Filho & Yunes, 1998).

Porém, apesar do seu uso estar apoiado em um conhecimento consolidado por séculos de observação, planta medicinal não é sinônimo de inocuidade. Ao contrário do senso comum de que "medicamento natural se não fizer bem, mal não faz", a planta medicinal é um xenobiótico, ou seja, um produto estranho ao organismo. Desta forma, alguns compostos, ou mesmo seus metabólitos, ao serem introduzidos no organismo humano podem ter ação diferente da desejada, apresentando atividade tóxica (Veiga Junior *et al*., 2005; Nicoletti *et al*., 2007). Por isso, é imprescindível a investigação do potencial tóxico de plantas medicinais para elucidar aspectos farmacológicos e permitir uma utilização segura, respeitando os riscos toxicológicos.

3.2 Estudos bioguiados e obtenção de compostos ativos

Na busca de alternativas que visem substituir ou complementar o uso de substâncias sintéticas na medicina contemporânea, tem-se verificado que muitos extratos de plantas apresentam atividades biológicas interessantes, muitas vezes devido à presença dos metabólitos secundários que os compõem.

Os extratos vegetais são preparações concentradas, que possuem consistências diversas, obtidas de matérias-primas vegetais, tratadas ou não previamente (moagem, secagem, etc.) e preparadas por processos que envolvem a utilização de solventes. O processo de separação dos produtos naturais bioativos corresponde a três fases principais: extração a partir da matéria vegetal, fracionamento do extrato ou óleo e purificação do princípio ativo (Lima Junior, 2011).

As vantagens relacionadas ao uso de extratos vegetais quando comparados aos produtos sintéticos, se devem ao fato de gerarem novos compostos, os quais os patógenos não se tornaram capazes de inativar. Além disso, na maior parte dos casos, são menos tóxicos,

degradados rapidamente pelo ambiente e possuem um amplo modo de ação, sendo derivados de recursos renováveis (Ferraz, 2008). Entretanto, os mesmos também apresentam algumas limitações, como a falta de controle de qualidade, baixa estabilidade dos compostos orgânicos presentes nas soluções e o não monitoramento de possíveis substâncias tóxicas presentes nas plantas ou resultantes da decomposição dos produtos durante sua manipulação (Silva *et al*., 2005).

A análise de substâncias ativas em material vegetal é muito complexa. Normalmente, os compostos presentes em menor concentração são os que possuem melhores efeitos biológicos, porém não são os mais estudados e isolados. Desta forma, a análise de frações e/ou compostos isolados é indispensável, visando identificar o composto responsável pela atividade biológica.

Quando está em causa a análise de uma dada atividade farmacológica num número elevado de amostras de material vegetal ou de uma matriz que apresenta complexidade química, a estratégia de pesquisa normalmente adotada começa pelo estudo *in vitro* da sua interação com um ou mais sistemas biológicos, que sirvam de modelo para uma determinada via biomolecular associada com a atividade farmacológica em estudo (Oldoni, 2010).

A realização de um isolamento bioguiado tem sido a melhor opção quando se deseja isolar componentes com atividade biológica. O estudo de produtos naturais usando esta técnica tem sido muito empregado, devido os benefícios a partir de uma racionalização do processo de isolamento de substâncias biologicamente ativas de extratos quimicamente complexos (Ho *et al.*, 2009; Teke *et al.*, 2011).

A principal vantagem é que conforme o fracionamento é realizado e as frações/subfrações são obtidas, estas podem ser submetidas a ensaios biológicos e/ou químicos para se identificar onde a atividade está concentrada e, desta forma, seguir trabalhando somente com as frações de interesse. Outras vantagens são: evitar o descarte de

compostos bioativos durante os processos de fracionamento e isolamento cromatográfico em extrato bruto cuja diversidade química seja grande; economia de solvente e rapidez no isolamento a partir de critérios biológicos de inclusão e exclusão de frações e subfrações; viabilidade do estudo de substâncias bioativas em produtos naturais que possuem alta complexidade química, uma vez que permite a exclusão de grande número de substâncias, sem atividade biológica, presentes em frações e subfrações (Oldoni, 2010).

Assim, para a obtenção de compostos isolados de uma amostra, alguns passos são importantes, como: preparação do material, procedimento de extração, processos cromatográficos, quantificação dos compostos, isolamento e análise da estrutura química.

Na preparação de extratos vegetais, para a busca de compostos ativos isolados, é adequado iniciar com a preparação de um extrato hidroalcoólico. Caso o extrato apresente efeitos biológicos de interesse, procede-se com um método sistemático de estudo. Neste caso, o solvente mais adequado para obtenção do extrato bruto é o metanol, pois possibilita a extração de um maior número de compostos. Após, este extrato deve ser submetido a um processo de partição (líquido-líquido ou em sílica gel) com solventes de polaridades crescentes, para uma semi-purificação das substâncias através de suas polaridades. Além disso, todos os extratos semi-puros devem ser testados e aquele que apresentar efeito biológico de interesse, deverá ser submetido aos procedimentos cromatográficos para isolamento, purificação e elucidação estrutural. Para isto, a cromatografia de coluna (CC) aberta com sílica gel como fase estacionária é comumente utilizada, sendo eluída com uma mistura de solventes que deve ser previamente determinada por cromatografia em camada delgada (CCD) (Cechinel Filho & Yunes, 1998; Pinto, 2005).

As frações obtidas devem ser reunidas segundo seu perfil cromatográfico. Essas frações são analisadas farmacologicamente e, se apresentarem atividade, devem ser novamente submetidas à CC ou, dependendo da complexidade da mistura, à técnicas

cromatográficas especiais, como por exemplo, cromatografia líquida de alta eficiência (CLAE). Uma vez isolados os compostos ativos, procede-se a elucidação estrutural dos mesmos. Para a identificação rápida e eficiente, pode-se fazer uso de cromatografia gasosa (CG) ou cromatografia gasosa acoplada ao espectrômetro de massa (CG-EM). Também, o uso associado de técnicas como espectroscopia no ultravioleta (UV), espectroscopia no infravermelho (IV), ressonância magnética nuclear (RMN), espectrometria de massa (EM) tem permitido identificar e propor a estrutura molecular de substâncias naturais (Silverstain *et al.*, 1994; Latif & Sarker, 2012; Breton & Reynolds, 2013).

Metodologias analíticas qualitativas e quantitativas são de grande relevância em diversas áreas e é evidente que não há uma técnica de análise perfeita, sendo apenas mais aplicável a algumas condições e amostras. Assim, é de extrema importância otimizar o processo analítico para identificar e quantificar os compostos de interesse.

3.3 Seletividade de compostos bioativos

Para investigação de novos fármacos de origem vegetal, é comum o uso de técnicas fitoquímicas de isolamento associadas à *screenings* biológicos. Muitas substâncias apresentam potencial atividade frente a um determinado agente agressor (bactéria, fungo, célula neoplásica, etc), mas é necessário verificar sua possível citotoxicidade em células normais. Uma das primeiras etapas para esse tipo de investigação é a avaliação *in vitro* da atividade citotóxica. A citotoxicidade significa causar efeito tóxico a nível celular, sendo que o efeito pode ser representado como morte celular, alterações na permeabilidade da membrana, inibição enzimática, entre outros. A cultura de células é uma ferramenta valiosa para a investigação de efeitos biológicos, pois consiste na manutenção e multiplicação *in vitro* de células vivas, possibilitando a análise do metabolismo e do comportamento celular frente a

um componente a ser testado. Também é possível observar se a substância em análise atua de forma seletiva e específica no metabolismo celular (Luisi, 2006; Montagner, 2010).

Fármacos clinicamente eficientes devem exibir toxicidade seletiva, atingindo o agente agressor e não o hospedeiro. Na terapia antimicrobiana, a seletividade varia dependendo do antibiótico. Quando a seletividade é elevada os antibióticos são normalmente não tóxicos. Entretanto, mesmo antibióticos altamente seletivos podem ter efeitos colaterais como diarreia, náuseas, alergias, vômitos, toxicidade renal, entre outros efeitos (Cunha, 2001; Mota *et al*., 2010).

Outra área em que a seletividade é bastante discutida é na terapia antitumoral. A quimioterapia continua a ser um desafio ao tentar destruir as células neoplásicas e preservar as células normais. Até certo ponto, dispomos de terapias alvo específico que atuam bloqueando os sinais de crescimento das células tumorais, reduzindo o fornecimento de nutrientes para estas células ou estimulando o sistema imunológico a reconhecer e destruir as células tumorais (De David, 2013). Independente da origem, as classes de quimioterápicos afetam, por diferentes mecanismos de ação, a proliferação das células, o que, devido às semelhanças existentes entre células tumorais e normais, causa grande dificuldade em encontrar alvos de ação seletivos. As estruturas normais que se renovam constantemente, como a medula óssea, os pelos e a mucosa do tubo digestivo, são também atingidas pela ação desses fármacos (Fernandes & Mello, 2008).

Portanto, a busca de fármacos que venham suprir uma ou mais falhas do arsenal terapêutico atual é incessante, visando aumentar de forma significativa a seletividade para o alvo desejado, evitando, sobretudo, possíveis efeitos colaterais e resistência celular.

3.4 Atividade antibacteriana: do antibiótico à resistência

Diversas propriedades de substâncias produzidas por plantas como consequência do seu metabolismo secundário são descritas, sendo que a antibacteriana é reconhecida há séculos, porém comprovada de forma científica recentemente. Uma vez que as plantas medicinais produzem uma variedade de substâncias com propriedade antibacteriana (ex. compostos fenólicos, terpenos), é esperado que programas de triagem possam descobrir compostos candidatos para o desenvolvimento de novos antibióticos e que estes atinjam alvos diferentes daqueles utilizados pelos antibióticos conhecidos, sendo ativos contra patógenos resistentes (Ahmad & Beg, 2001; Duarte, 2006).

As bactérias são parte integral e inseparável da vida na terra. São organismos unicelulares, procariontes, encontrados de forma isolada ou em colônias, sendo capazes de viver na presença de ar (aeróbios), na ausência de ar (anaeróbios) ou, ainda, serem anaeróbios facultativos. Podem ser divididas em dois grandes grupos, as bactérias Gram-positivas e as Gram-negativas, através da técnica de coloração denominada Técnica de Gram. As Gram-positivas apresentam parede celular com uma única e espessa camada de peptidoglicanos, e pelo emprego da coloração de Gram, coram-se na cor púrpura ou azul. Já, as Gram-negativas possuem uma parede celular mais delgada e uma segunda membrana lipídica no exterior desta parede celular, sendo visualizadas na coloração rosa-avermelhada (Purves *et al*., 2002; Pádua, 2009).

As bactérias foram inicialmente identificadas por Antonie van Leeuwenhoek por volta de 1670, após a invenção do microscópio. Porém, apenas no século XIX a possibilidade destes micro-organismos serem causadores de processos infecciosos começou a ser aventada. Esta hipótese surgiu após os experimentos de Louis Pasteur, o qual demonstrou que algumas bactérias eram primordiais em processos de fermentação e largamente distribuídas no meio

ambiente (Guimarães *et al*., 2010). Elas podem ser encontradas em qualquer lugar, revestindo a pele, as mucosas e cobrindo o trato intestinal dos homens e dos animais. Estão intrinsecamente ligadas à vida de organismos e aos amplos ambientes em que habitam. Muitas são inofensivas e algumas são benéficas para seu hospedeiro (homem, animal, planta), fornecendo nutrientes ou proteção contra patógenos e doenças, e limitando a habilidade de colonização de bactérias nocivas (Santos, 2004). Entretanto, várias bactérias são responsáveis por causar importantes infecções, afetando a saúde de milhares de pessoas e representando um percentual significativo na mortalidade mundial.

Para o tratamento das doenças infecciosas bacterianas existem os antibióticos. Estes podem ser definidos como compostos naturais ou sintéticos, os quais são classificados como bactericidas, quando causam a morte da bactéria, ou bacteriostáticos, quando promovem a inibição do crescimento microbiano (Walsh, 2003). Há vários alvos de ação onde os mesmos podem atuar, como na inibição da síntese da parede celular e da síntese de ácidos nucléicos, enfraquecimento da membrana citoplasmática, inibição da síntese proteica e perturbação do metabolismo bacteriano. No entanto, existem mecanismos pelos quais as bactérias podem escapar da ação destes compostos: expulsão do antibiótico por mecanismos de efluxo, alteração do local de ação do fármaco, diminuição da permeabilidade (menor concentração de fármaco no alvo) e presença de enzimas que destroem ou modificam as moléculas responsáveis pela ação antibacteriana, entre outros (Fernandes *et al*., 2013).

A todos esses possíveis mecanismos, dá-se o nome de resistência bacteriana, que é o conjunto de mecanismos de adaptação das bactérias contra os efeitos nocivos ou letais aos quais estão sendo submetidas. Esse processo de resistência é um dos casos mais bem documentados de evolução biológica e um sério problema tanto em países desenvolvidos como em desenvolvimento. O consumo de uma grande quantidade diária de antibióticos tem

resultado na resistência de populações bacterianas, causando um sério problema de saúde pública (Marccuci, 1995; Baquero & Blázquez, 1997; Arantes *et al*., 2013).

Todavia, algumas estratégias podem ser adotadas para evitar o desenvolvimento destes mecanismos de resistência, como: uso racional de antibióticos, prevenção de infecções bacterianas com o uso de vacinas, controle e prevenção da disseminação de micro-organismos resistentes, descoberta e desenvolvimento de novos antibióticos. Ainda, a caracterização dos genes responsáveis pela resistência, assim como sua localização e diversidade são de grande importância para o entendimento dos fatores envolvidos neste processo (Guimarães *et al*., 2010).

Em vista do presente cenário, a busca por novas substâncias antimicrobianas a partir de fontes naturais tem ganhado importância nas indústrias farmacêuticas, demonstrado uma alternativa eficaz e econômica.

3.4.1 *Staphylococcus aureus*

Staphylococcus aureus é uma bactéria coco Gram-positiva comumente encontrada na microbiota humana (Figura 2). A maioria dos portadores são assintomáticos e o processo de infecção normalmente está associado a algum fator que diminui a resposta imunológica do indivíduo, como doenças, tratamentos mais agressivos ou procedimentos médicos invasivos, que abrem uma via de acesso para o micro-organismo (Gordon & Lowy, 2008).

O *S. aureus* é responsável por uma grande variedade de infecções, como infecções na pele e região subcutânea, pneumonias, osteomielites, abscessos, infecções pós-cirúrgicas, endocardites e bacteremia. É umas das causas mais comuns de infecções nosocomiais, bem como de infecções comunitárias que podem apresentar altos índices de morbidade e mortalidade. Além disso, em muitos países é considerado o segundo ou terceiro patógeno

mais comum causador de intoxicação alimentar, perdendo em número apenas para *Salmonella* sp. e competindo com o *Clostridium perfringens* (Bean *et al*., 1996; Lowy, 1998; Gelatti *et al*., 2009).

A eficiência da disseminação de *S. aureus* se deve, em parte, à grande versatilidade desse micro-organismo. A capacidade de se adaptar rapidamente a diferentes ambientes, muitas vezes hostis devido ao pH, pressão osmótica, umidade ou deficiência de nutrientes, possibilita a colonização do homem e do ambiente ao seu redor, criando reservatórios de células aptas a colonizar outros indivíduos (Cepeda *et al*., 2005; Kniehl *et al*., 2005).

A terapia antimicrobiana para infecções por esse micro-organismo inicialmente era simples. A primeira vez que um antimicrobiano foi utilizado clinicamente foi contra uma amostra de *S. aureus*, a partir da descoberta da penicilina, que funcionou muito bem até a década de 1960, quando começaram a aparecer isolados resistentes a esse antimicrobiano. Para contornar o problema, foi criado o beta-lactâmico sintético meticilina, que era resistente a ação de beta-lactamases que o *S. aureus* produzia, mas logo surgiram relatos de amostras resistentes também a esse antimicrobiano, além da expressão de multirresistência. Essas cepas foram denominadas de MRSA (*S. aureus* resistente a meticilina) e são resistentes a todos os antimicrobianos beta-lactâmicos. O crescente aumento das infecções hospitalares causadas por MRSA tornou a vancomicina uma das poucas alternativas terapêuticas eficazes no tratamento de infecções causadas por este tipo de cepa, porém o surgimento de cepas com resistência intermediária a vancomicina limitam a possibilidade de uso deste antibacteriano (Lowy, 1998; Arantes *et al.*, 2013).

Estudos vêm demonstrando a atividade antibacteriana promissora de produtos naturais contra *S. aureus*. Cabral *et al.* (2009) verificaram a atividade de subfrações de própolis vermelha, onde a subfração 4 apresentou a melhor atividade frente *S. aureus*, com Concentração Bactericida Mínima (CBM) de 31,7 a 62,5 $\mu g.mL^{-1}$. Oldoni *et al.* (2011)

também observaram a ação da fração clorofórmica de própolis vermelha, a qual exibiu Concentração Inibitória Mínima (CIM) de 31,2 a 62,5 µg.mL^{-1}.

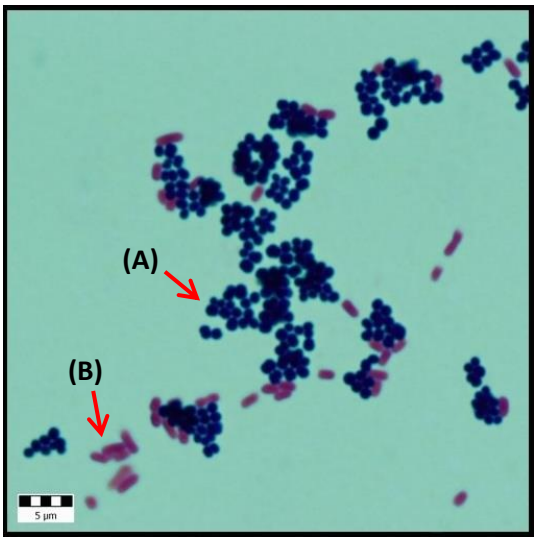

Figura 2. *Staphylococcus aureus* (A) e *Escherichia coli* (B).

(Fonte: www.bacteriainphotos.com)

3.4.2 *Escherichia coli*

Escherichia coli é uma bactéria bastonete Gram-negativa (Figura 2), encontrada predominantemente no trato intestinal dos mamíferos saudáveis, coexistindo sem causar danos ao hospedeiro. Porém, várias cepas ao adquirirem fatores de virulência específicos tornaram-se potencialmente danosas, especialmente em condições de imunossupressão (Nataro & Kaper, 1998).

Aproximadamente 10 % são patogênicas, podendo causar infecções e levar à doença no trato intestinal, como a gastroenterite. Em alguns casos, pode ocorrer disseminação e colonização para o sistema nervoso central, trato urinário e sangue (Kaper *et al*., 2004; Nakazato *et al*., 2009). As linhagens associadas às gastroenterites estão subdivididas em grupos patogênicos: *E. coli* enteropatogênica (EPEC), *E. coli* enterotoxigênica (ETEC), *E.*

coli enterohemorrágica (EHEC) - subgrupo da *E. coli* produtora da toxina de Shiga (STEC), *E. coli* enteroinvasiva (EIEC), *E. coli* enteroagregativa (EAEC) e *E. coli* que adere difusamente (DAEC) (Vidal *et al*., 2005; Shelton *et al*., 2006).

Entre as escolhas terapêuticas para o tratamento de infecções causadas por *E. coli* estão os antibióticos β-lactâmicos e as fluoroquinolonas, mas, os isolados de *E. coli* podem desenvolver resistência a estes e a outros antibióticos (Bergogne-Bérézin, 2006; Oteo *et al*., 2010).

Diversos produtos naturais, como a própolis, vêm sendo estudados como possíveis alternativas terapêuticas no tratamento de infeções causadas por *E coli*. Righi *et al.* (2011) verificaram que o extrato metanólico de própolis vermelha inibiu o crescimento de *E coli*, apresentando CIM de 512 $\mu g.mL^{-1}$. Em recente estudo, Machado *et al.* (2016) observaram a atividade do extrato etanólico de própolis vermelha, com CIM de 400 $\mu g.mL^{-1}$.

3.4.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa é um bacilo Gram-negativo (Figura 3), isolado de água, solo, plantas e animais, incluindo o homem. É considerada uma bactéria oportunista, capaz de crescer em água com baixos níveis de sólidos dissolvidos e compostos orgânicos, adaptando-se a ambientes nutricionalmente pobres (Koneman *et al*., 2001; Guerra *et al*., 2006).

Está frequentemente envolvida nas infecções com ampla localização e severidade, como aquelas do trato respiratório, trato urinário e da corrente sanguínea, porém raramente afeta indivíduos sadios (Pitten *et al*., 2001). Ainda, posiciona-se entre as principais bactérias causadoras de infecções hospitalares, principalmente em pacientes predispostos, que

apresentam quebra de barreiras físicas e imunossupressão, perdendo apenas para o *Staphylococcus coagulase* negativo e o *Staphylococcus aureus* (Sader *et al*., 2001).

Apesar de todos os avanços médicos dos últimos anos e da alta tecnologia no suporte de doentes críticos, as infecções por *P. aeruginosa* continuam associadas a elevados índices de morbimortalidade e são de difícil tratamento, em virtude das limitadas opções terapêuticas. Um dos principais problemas é a possível resistência a vários antibióticos, incluindo os beta-lactâmicos, macrolídeos, sulfametoxazol-trimetoprim, tetraciclinas e a maioria das fluroquinolonas (Zavascki, 2003). Entretanto, existem alguns antibióticos disponíveis para o tratamento de infecções por *P. aeruginosa*, especialmente ciprofloxacino, levofloxacino e as polimixinas B e E (Lopes, 2009).

Além dos antibióticos atualmente utilizados, alguns extratos e compostos isolados de produtos naturais vêm demonstrando interessante ação contra esta bactéria. Righi *et al.* (2011) verificaram a atividade do extrato de própolis vermelha frente *P. aeruginosa*, o qual apresentou CIM de 256 $\mu g.mL^{-1}$. Ainda, um composto isolado da própolis vermelha, a (6aS,11aS)-medicarpina, exibiu CIM de 32 $\mu g.mL^{-1}$ (Inui *et al.*, 2014).

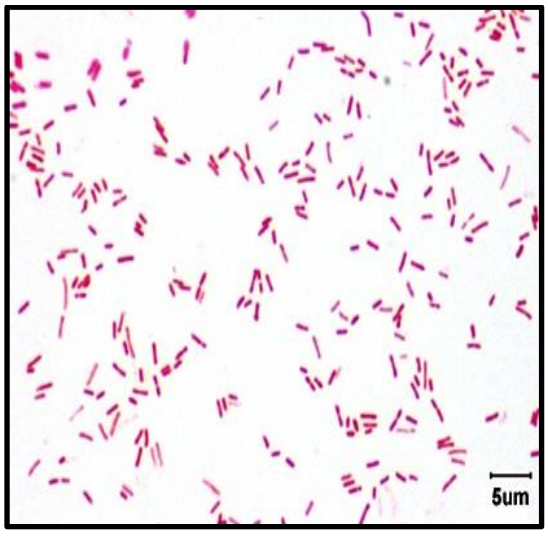

Figura 3. *Pseudomonas aeruginosa.*

(Fonte: http://faculty.ccbcmd.edu)

3.4.4 *Bacillus subtilis*

Bacillus subtilis é uma bactéria em forma de bastonete Gram-positiva (Figura 4). É comumente encontrado no solo e existem evidências de que é um comensal normal de intestino em humanos. São micro-organismos altamente resistentes em condições adversas presentes no meio ambiente, devido à capacidade de formação de esporos, que suportam extremos de temperatura, pH, radiação UV, dessecação e diversos produtos químicos (Driks, 2004; Piggot & Hilbert, 2004; Hong *et al*., 2009).

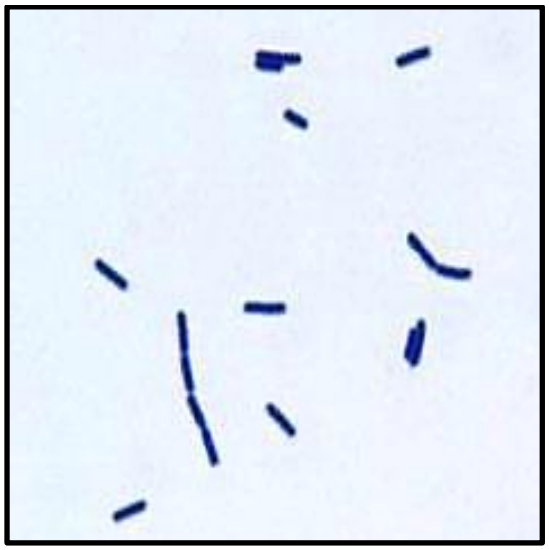

Figura 4. *Bacillus subtilis.*
(Fonte: Moretti, 2007)

Tem sido usado para o biocontrole de enfermidades de plantas, assim como para aumentar a produtividade de culturas, uma vez que produz antibióticos, fitormônios e enzimas benéficas para as plantas (Yao *et al*., 2006; Araujo, 2008).

Também é utilizado como probiótico em indivíduos saudáveis e seu potencial patogênico é geralmente descrito como baixo ou ausente, mas pode causar doença em pacientes imunocomprometidos. Ainda, é capaz de produzir uma toxina extracelular, a

subtilisina, que causa alergia e hipersensibilidade em indivíduos expostos repetidamente. Porém, os dados sobre infecções causadas por *B. subtilis* são incompletos e apenas alguns casos são relatados, uma vez que é uma prática da maioria dos laboratórios descartar essas cepas (Oggioni *et al*., 1998; Yassin & Ahmad, 2012).

Além disso, vem sendo observado um aumento da taxa de incidência de *B. subtilis* em ambientes hospitalares e alguns destes isolados demostram boa susceptibilidade à alguns antibióticos (ex. gentamicina, ciprofloxacino), mas também uma resistência significativa à múltiplas drogas, sendo, portanto, necessário o monitoramento da propagação desta bactéria nestes ambientes, a fim de compreender a sua distribuição em diferentes casos (Yassin & Ahmad, 2012).

Algumas pesquisas vêm evidenciando os produtos de origem natural e sua atividade contra *B. subtilis*. Em estudo desenvolvido por Righi *et al.* (2011), o extrato de própolis vermelha inibiu o crescimento de *B. subtilis*, com CIM de 256 $\mu g.mL^{-1}$. Inui *et al.* (2014) verificaram a atividade de um composto isolado de própolis vermelha, (6aS,11aS)-medicarpina, o qual exibiu CIM de 32 $\mu g.mL^{-1}$.

3.4.5 *Métodos antimicrobianos*

Atualmente, existe uma vasta quantidade de métodos que podem ser empregados para verificar a sensibilidade de micro-organismos aos agentes antimicrobianos. Estes podem ser classificados em três grupos: métodos de diluição, difusão em ágar (poço, cilindro, disco) e método bioautográfico. São indicados para qualquer organismo que provoque um processo infeccioso e necessite de terapia antimicrobiana (CLSI, 2003).

Os testes de difusão e de bioautografia são qualitativos, pois apenas demonstram a presença ou ausência de substâncias antimicrobianas. Já, os testes de diluição são

quantitativos, visto que possibilitam a determinação da Concentração Inibitória Mínima (CIM), ou seja, a menor concentração do antimicrobiano capaz de inibir o crescimento do organismo avaliado (Valgas *et al*., 2007; Madigan *et al*., 2010).

Para determinação da CIM, há os ensaios de macro e microdiluição, onde são preparadas diluições sucessivas do antimicrobiano (em meios de cultura sólidos ou líquidos), semeia-se o micro-organismo e após a incubação determina-se, através de leitura visual direta ou turbidimétrica, a menor concentração do antimicrobiano que inibiu o crescimento do micro-organismo (CLSI, 2003). Algumas vantagens deste método são: requerer uma pequena quantidade de amostra, possuir baixo custo, ter reprodutibilidade e ser mais sensível que outros métodos (Ostrosky *et al*., 2008).

Esta avaliação é comparada em relação a um padrão de referência, porém, para compostos de plantas, não existe um consenso sobre os níveis de inibição aceitáveis quando comparados com antibióticos padrões (Duarte, 2006).

3.5 Própolis

Os produtos de origem natural sempre foram muito utilizados por diversas civilizações como poderosos recursos terapêuticos, a fim de prevenir e curar enfermidades. Entre eles estão os produtos apícolas, como a própolis, a qual foi reconhecida por suas interessantes propriedades medicinais por médicos gregos e romanos. Além disso, era empregada pelo povo egípcio, principalmente no processo de embalsamar cadáveres e devido a sua ação cicatrizante foi amplamente utilizada na segunda guerra mundial (Castaldo & Capasso, 2002; Pereira *et al*., 2002).

Já no Brasil, o interesse pela própolis ocorreu somente na década de 80, com o livro "Abelhas e saúde" de Ernesto Ulrich Breyer, o qual descrevia as suas propriedades terapêuticas e utilização como antibiótico natural (Lima, 2006).

A própolis é uma substância resinosa complexa, gomosa e balsâmica, coletada pelas abelhas, de diversas partes da planta como broto, botões florais e exsudados resinosos, as quais são acrescentadas de cera, pólen, secreções salivares e enzimas para a elaboração do produto final. Segundo sua origem, a palavra *própolis* é derivada do grego, *pro-* (em defesa) e *polis-* (cidade ou comunidade), ou seja, em defesa da comunidade, por isso seu uso na construção e adaptação da colmeia, proteção contra insetos, garantindo um ambiente asséptico (Marcucci, 1996; Pereira *et al*., 2002; Funari & Ferro, 2006).

A coloração da própolis depende de sua procedência e pode variar de amarelo-esverdeado, passando pelo marrom-avermelhado até marrom escuro e negro. Possui odor característico, que pode alterar de uma amostra para outra (Marcucci, 1996).

Sua composição química é complexa e pode variar de acordo com a origem geográfica, tempo de colheita, espécie de abelha, flora local, etc (Castaldo & Capasso, 2002). No geral, é composta por cerca de 50-60% de resinas e bálsamos, 30-40% de ceras, 5-10% de óleos essenciais, 5% de grãos de pólen, microelementos e vitaminas (Park *et al*., 2002; Menezes, 2005; Funari & Ferro, 2006). Entre os inúmeros compostos identificados tem-se os flavonoides, ácidos aromáticos, fenóis, ácidos graxos, terpenos, aminoácidos e outros. Ainda, elementos inorgânicos como o cálcio, alumínio, cobre, ferro, manganês, silício e vanádio podem ser encontrados (Marcucci, 1996; Pereira *et al.*, 2002).

No Brasil, a própolis foi inicialmente classificada em 12 tipos de acordo com suas características físico-químicas. Após, uma nova própolis descoberta e denominada "própolis vermelha" foi classificada como o 13º tipo (Park *et al*., 2000).

3.5.1 *Própolis Vermelha*

A própolis vermelha Brasileira (Figura 5-A) pode ser encontrada na região Nordeste do Brasil, sobretudo nos estados de Alagoas, Sergipe, Bahia, Pernambuco e Paraíba, em regiões de manguezais. A principal origem botânica foi identificada como *Dalbergia ecastophyllum* (L) Taub. (Fabaceae) (Figura 5-B), conhecida popularmente como "rabo-de-bugio" (Clardy & Walsh, 2004; Silva *et al.*, 2007; Daugsch *et al*., 2008).

Figura 5. Própolis vermelha obtida no Estado de Alagoas - Brasil **(A)** e *Dalbergia ecastophyllum* (L) Taub. **(B)**.

(Fonte: Foto **A** - Luciane C. Rufatto, 2016; Foto **B** - Lustosa, 2007)

Este novo tipo de própolis tem sido uma importante fonte de novos compostos bioativos, devido sua composição química distinta e sua potente ação. Diversas atividades são relatadas e cientificamente comprovadas, como a antimicrobiana, anti-inflamatória, anticariogênica, antioxidante, antitumoral e outras (Alencar *et al*., 2007; Bueno-Silva *et al*., 2013; Frozza *et al*., 2013; Cavendish *et al*., 2015).

Uma atividade relevante e bastante descrita é a antibacteriana, visto que nas últimas décadas, juntamente com o aumento significativo de antibióticos, ocorreu o desenvolvimento da resistência bacteriana, que levou a intensa procura por novos agentes antimicrobianos,

particularmente os de origem natural. O mecanismo de ação é complexo e pode estar relacionado com a inibição da RNA-polimerase bacteriana, inibição da formação de biofilme ou danos na membrana citoplasmática, podendo resultar de um efeito sinérgico entre os flavonoides (em especial os isoflavonoides), sesquiterpenos, ácidos aromáticos, ésteres e diversos outros compostos presentes na própolis (Marcucci, 1995; Bosio *et al*., 2000; Freires *et al.*, 2016).

Alguns estudos constataram resultados positivos, tanto do extrato bruto quanto de frações e compostos isolados da própolis, frente a várias bactérias, sendo as Gram-positivas mais sensíveis que as Gram-negativas (Kosalec *et al.,* 2005; Trusheva *et al*., 2006; Alencar *et al.*, 2007; Inui *et al*., 2014; Lopez *et al*., 2015).

As diferentes atividades observadas são devidas, principalmente, a presença dos compostos fenólicos, como os flavonoides (Figura 6), que são citados como os principais responsáveis pelas ações farmacológicas e efeitos benéficos.

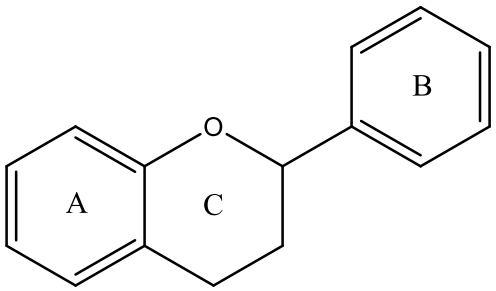

Figura 6. Estrutura básica de um flavonoide.
(Fonte: Kumar & Pandey, 2013)

Dentre a complexidade de compostos existentes, alguns têm se destacado na própolis vermelha Brasileira (Figura 7). Entre eles está a Formononetina (**1**), uma isoflavona com propriedades estrogênica, citotóxica, anti-inflamatória, antifúngica, sendo também um importante marcador químico (Frozza *et al*., 2013; Cavendish *et al*., 2015).

Outros compostos relevantes encontrados nesta resina são a Biochanina A (**2**), Isoliquiritigenina (**3**), Medicarpina (**4**) e Vestitol (**5**), os quais apresentam várias atividades como antitumoral, anti-inflamatória, antibacteriana, antioxidante, entre outras (Trusheva *et al*., 2006; Kole *et al*., 2011; Oldoni *et al*., 2011). Além disso, dois pigmentos derivados de flavonoides, a Retusapurpurina A e a Retusapurpurina B (**6**), são indicados como responsáveis pela sua coloração específica (Piccinelli *et al.*, 2011).

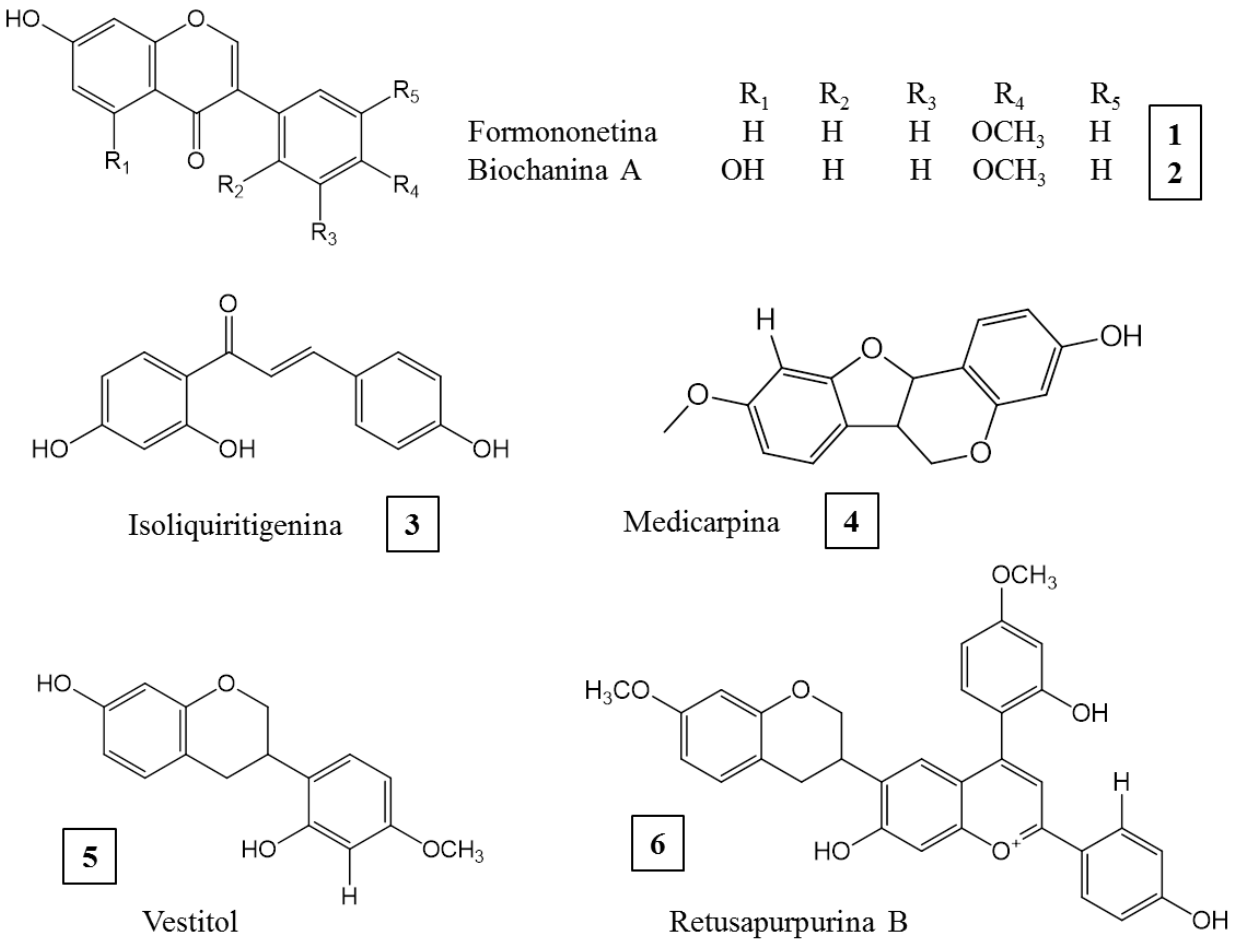

Figura 7. Importantes compostos presentes na própolis vermelha.

Assim, apesar de a própolis ser aceita por órgãos regulatórios como produto terapêutico, é necessário padronizá-la quimicamente para garantir a sua qualidade, eficácia e

segurança. No entanto, muitos fatores podem interferir na sua composição, dificultando esse processo. A maioria dos produtos à base de própolis, comercializados no Brasil, têm registro no Ministério da Agricultura, que preconiza os limites para fixação de identidade e qualidade da própolis na Instrução Normativa nº 3, de 19 de janeiro de 2001 (Brasil, 2001).

Além disso, são necessários estudos que relacionem a composição química com a atividade biológica, pois assim é possível correlacionar o tipo de própolis com a sua aplicação terapêutica.

4. RESULTADOS E DISCUSSÃO

Este item será apresentado na forma de artigos científicos sendo subdividido em dois Capítulos.

CAPÍTULO 1

Artigo:

Red propolis: chemical composition and pharmacological activity

Phytochemistry letters

Autores: Luciane Corbellini Rufatto, Denis Amilton Dos Santos, Flávio Marinho, João Antonio Pêgas Henriques, Mariana Roesch Ely, Sidnei Moura

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, pterocarpans, 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; Daugsch 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 diverses 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	(Frozza et al., 2013)
		HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
2	Biochanin A	ESI/MS	(Frozza et al., 2013)
		HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
3	Medicarpin	ESI/MS	(Frozza et al., 2013)
		HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
		GC/MS	(Alencar et al., 2007)
4	Vestitol	HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
		GC/MS, NMR, HPLC	(Oldoni et al., 2011)
5	Neovestitol	HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
		GC/MS; NMR; HPLC	(Oldoni et al., 2011)
6	Daidzein	RP-HPLC	(Alencar et al., 2007)
7	Elemicin	GC/MS	(Trusheva et al., 2006)
8	Guttiferone E	HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
		GC/MS, NMR	(Trusheva et al., 2006)
9	Xanthochymol	HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
		GC/MS; NMR	(Trusheva et al., 2006)
10	Isoliquiritigenin	HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
		GC/MS, NMR, HPLC	(Oldoni et al., 2011)
11	Liquiritigenin	ESI/MS	(Frozza et al., 2013)
		HPLC-PDA-ESI/MS	(Piccinelli et al., 2011)
12	Retusapurpurin A and B	ESI/MS	(Frozza et al., 2013)
		HPLC-PDA-ESI/MS	(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 compouds 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 indentified 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).

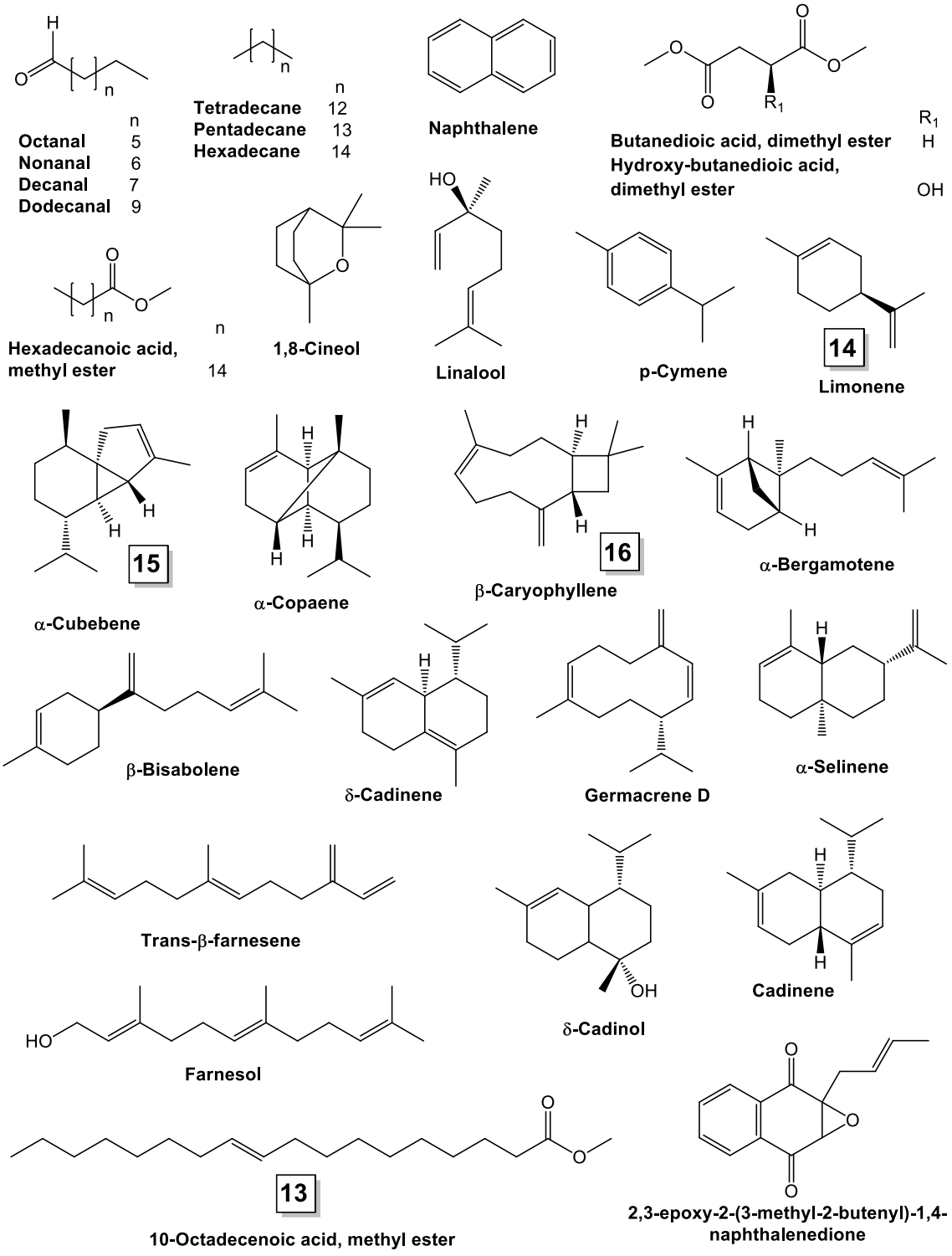


Figure 1. Chemical structures of volatile compounds, terpenes and derivatives from red propolis.

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 an 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 (Daugsch et al., 2008).

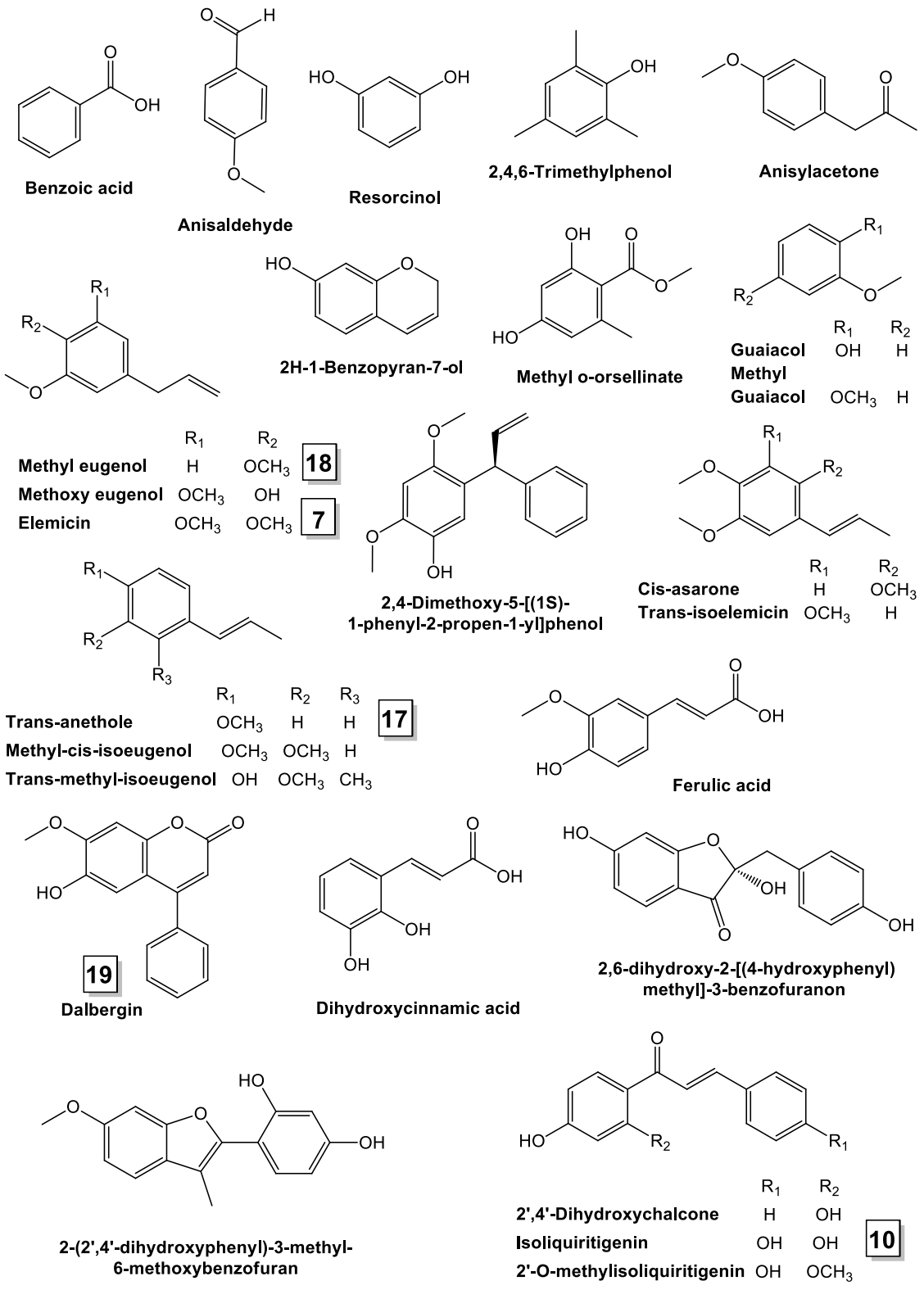
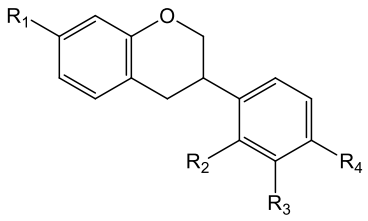


Figure 2. Chemical structures of some phenolics derivatives found in red propolis.

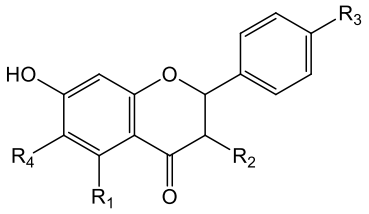
2.3 Flavonoids

Flavonoids represent the most common and widely distributed group of phenolics in red propolis. These are among the most actives 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).

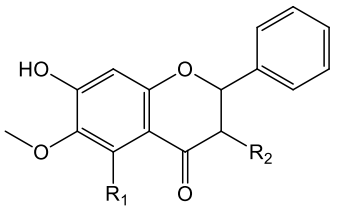
According to Hernandez et al. (2010), studies dealing with chemical composition of propolis can help establishing criteria for the quality control of the samples. The quality of propolis is checked by the Ministry of Agriculture, in Brazil, using parameters standardized (Brasil, 2001), since the biological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



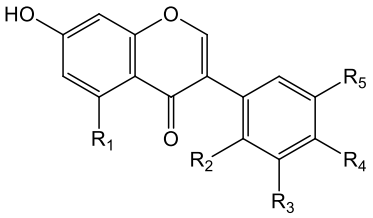
	R_1	R_2	R_3	R_4	
Vestitol	OH	OH	H	OCH_3	4
Isovestitol	OH	OCH_3	H	OH	
Neovestitol	OCH_3	OH	H	OH	5
(3S)-7-O-methylvestitol	OCH_3	OH	H	OH	
(3S)-mucronulatol	OH	OCH_3	OH	OCH_3	
(3S)-violanone	OH	OCH_3	OH	CH_2OH	



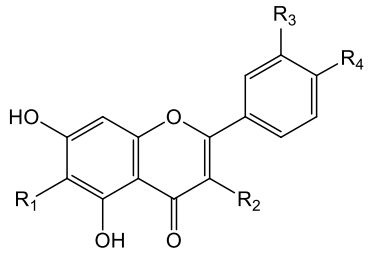
	R_1	R_2	R_3	R_4	
3,7-hydroxyflavanone	H	H	H	H	
Pinocembrin	OH	H	H	H	
Liquiritigenin	H	H	OH	H	11
(2R,3R)-3,7-dihydroxy flavanone	H	OH	H	H	
(2S)-7-hydroxy-6-methoxyflavanone	H	H	H	OCH_3	
Naringenin	OH	H	OH	H	
Pinobanksin	OH	OH	H	H	
Garbanzol	H	OH	OH	H	
(2S)-dihydrobaicalein	OH	H	H	OH	



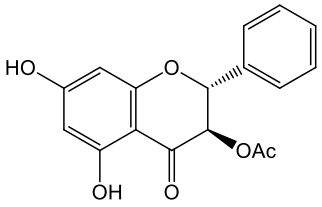
	R_1	R_2
Dihydrooroxylin A	OH	H
(2R,3R)-3,7-Dihydroxy-6-methoxyflavanone	H	OH



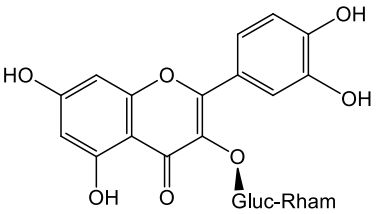
	R_1	R_2	R_3	R_4	R_5	
Daidzein	H	H	H	OH	H	6
Formononetin	H	H	H	OCH_3	H	1
Biochanin A	OH	H	H	OCH_3	H	2
Xenognosin B	H	OH	H	OCH_3	H	
Calycosin	H	H	H	OCH_3	OH	
7,3'-Dihydroxy-5'-methoxyisoflavone	H	H	OH	H	OCH_3	
(3S)-Vestitone	H	OH	H	OCH_3	H	
Pratensein	OH	H	H	OCH_3	OH	
2'-Hydroxybiochanin A	OH	OH	H	OCH_3	H	



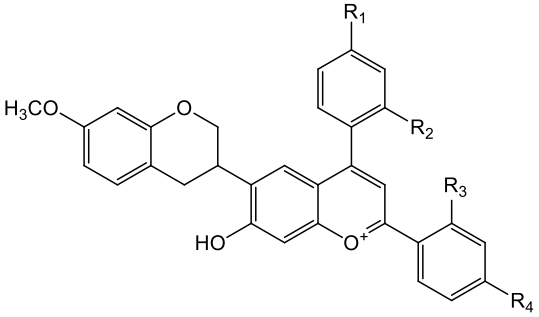
	R_1	R_2	R_3	R_4	
Chrysin	H	H	H	H	21
Alnusin	OCH_3	OH	H	H	
Quercetin	H	OH	OH	OH	20



Pinobanksin 3-acetate



Rutin



	R_1	R_2	R_3	R_4	
Retusapurpurin B	OCH_3	OH	H	OH	12
Retusapurpurin A	OH	H	OCH_3	OH	

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

2.4 Pterocarpans

Pterocarpans 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 pterocarpns 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.

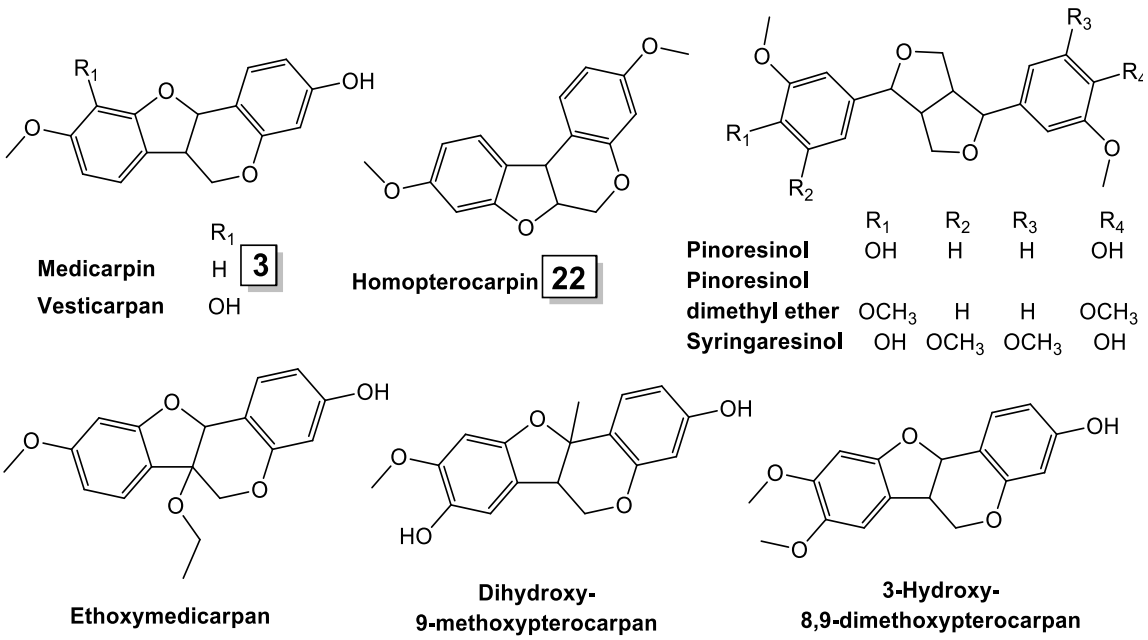


Figure 4. Chemical structures of pterocarpans derivatives from red propolis.

2.5 Benzophenones

Benzophenones are phenolic compounds of natural origin and restricted distribution. They have important biological properties such as antitumor, antibacterial, plasmodicidal, anti-HIV and others. They can be used in products such as perfumes, soaps, sunscreens, preventing against ultraviolet light (Silva, 2010). Some benzophenones 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.

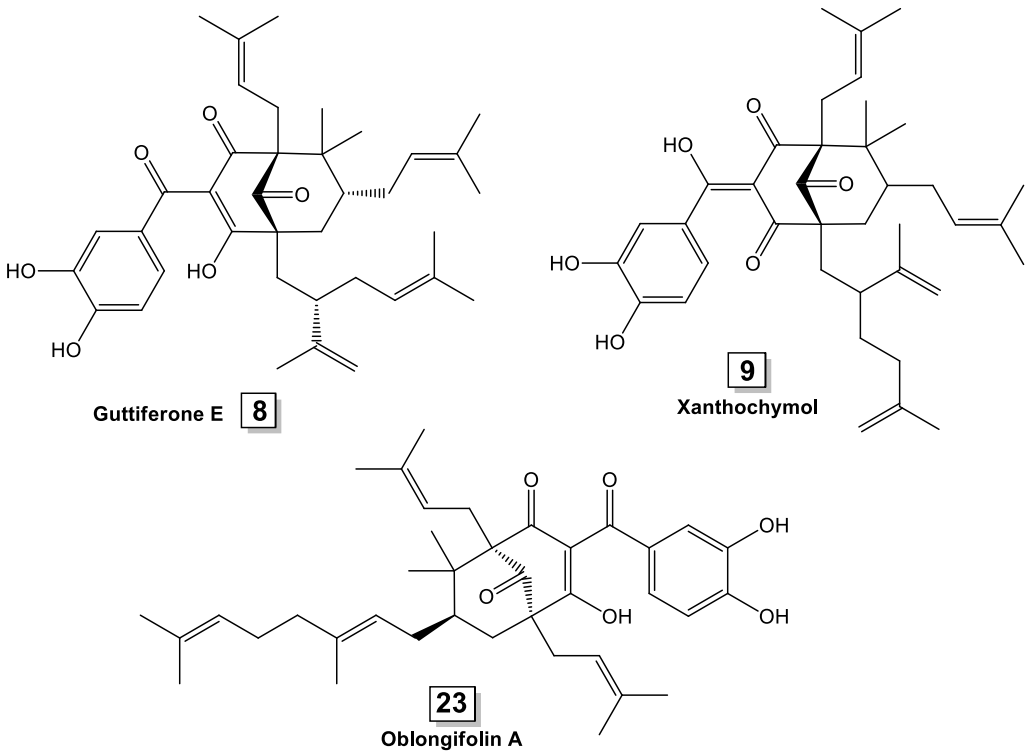


Figure 5. Chemical structure of benzophenones derivatives isolated from red propolis.

2.6 Triterpenes

The triterpenes are one of the largest classes of plant natural products. Simple triterpenes are components of surface waxes, specialized membranes and may

potentially act as signaling molecules. Complex glycosylated triterpenes provide protection against pests and pathogens, and also they have important activities such as antitumor, anti-inflammatory, antibacterial and others (Thimmappa et al., 2014). Some triterpenes 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).

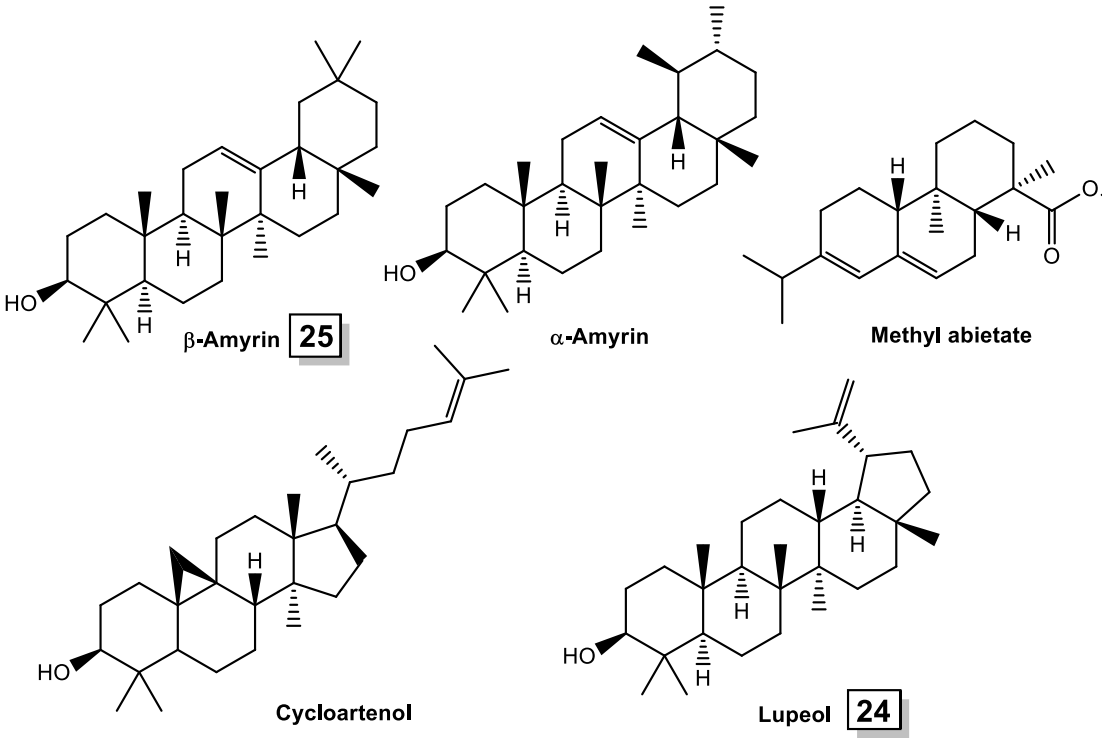


Figure 6. Chemical structures of triterpenes derivatives in red propolis.

3. Pharmacological activities

Propolis has been sistematically 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, manly 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 *Streptococcus mutans*, where the chloroform fraction was the most active with lower minimum inhibitory concentration (MIC) ranging from 25 to 50 $\mu g.mL^{-1}$ (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 $\mu g.mL^{-1}$. Daugsch 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 (*S. aureus* ATCC 33951, *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922), and the MIC were 400-100 $\mu g.mL^{-1}$, 50-25 $\mu g.mL^{-1}$ and 400 $\mu g.mL^{-1}$, 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 $\mu g.mL^{-1}$ for *S. aureus* and from 62.5 to 125 $\mu g.mL^{-1}$ for *S. mutans* and *A. naeslundii*. Isolated coumpond vestitol presented MIC ranging from 31.2 to 62.5 $\mu g.mL^{-1}$, showing no distinction among the microorganisms assessed. Isoliquiritigenin was more potent than vestitol and exhibited MIC values ranging from 15.6 to 31.2 $\mu g.mL^{-1}$ 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, S. aureus, Proteus vulgaris, Klebsiella pneumoniae, E. coli*). Righi et al. (2011) verified that methanol extract inhibited the growth of all tested microorganisms. The MIC (256 $\mu g.mL^{-1}$) and MMC (512 $\mu g.mL^{-1}$) were observed against *P. aeruginosa, Bacillus subtilis* and *Candida albicans*. This extract showed a higher MMC (1024 $\mu g.mL^{-1}$) against *Klebsiella pneumoniae*. Others MIC were: *Salmonella typhimurium* (512 $\mu g.mL^{-1}$), *Enterococcus faecalis* (512 $\mu g.mL^{-1}$), *E. coli* (512 $\mu g.mL^{-1}$), *P. mirabilis* (512 $\mu g.mL^{-1}$) and *Streptococcus pyogenes* (512 $\mu g.mL^{-1}$). 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 *S. mutans* and *Streptococcus sanguinis*, respectively (Da Silva et al., 2013).

Lopez et al. (2015) found that Brazilian red propolis (from Sergipe, Alagoas and Paraiba) 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 $\mu g.mL^{-1}$) bacteria. The antimicrobial tests for some of the samples presented a MIC below the cytotoxic concentration of 50 $\mu g.mL^{-1}$ 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 µg.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 *S. aureus*, *Staphylococcus epidermidis*, *P. 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 µg.mL^{-1} (Grenho et al., 2015).

Siqueira et al. (2014) evaluated the antimicrobial activity of red propolis extract against strains of *E. 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 µg.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 $\mu g.mL^{-1}$), 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 $\mu g.mL^{-1}$ for *T. rubrum*, 32 to 128 $\mu g.mL^{-1}$ for *T. tonsurans* and 16 to 128 $\mu g.mL^{-1}$ for *T. mentagrophytes*. The fungicide activity of the same extract was observed in the concentrations of 128-256, 128-1024 and 256-512 $\mu g.mL^{-1}$, 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: *S. aureus* ATCC 13150, *S. aureus* ATCC 25923, *S. epidermides* ATCC 12228, *P. aeruginosa* ATCC 9027, *P. aeruginosa* ATCC P-12, *P. aeruginosa* ATCC P-03, *C. albicans* ATCC 76645, *C. 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 $\mu g.mL^{-1}$ for the six bacteria and from 32 to 1024 $\mu g.mL^{-1}$ for the yeasts tested. Similarly, hexane and acetate fractions of another variety of propolis inhibited all microorganisms, with MIC values of 512 $\mu g.mL^{-1}$ for bacteria and 32 $\mu g.mL^{-1}$ 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, diabetes and others (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 sequestrating activity for the free radical 2,2-diphenyl-1-picrylhydrazyl (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 (IC_{50} 270.13 $\mu g.mL^{-1}$). 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 $\mu g.mL^{-1}$) 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 terapeutic 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 $\mu g.mL^{-1}$) 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 $\mu g.mL^{-1}$) and also inducing apoptosis-like mechanisms (Begnini 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 $\mu g.mL^{-1}$ (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 $\mu g.mL^{-1}$, 81.40 $\mu g.mL^{-1}$ and >150 $\mu g.mL^{-1}$, 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 $\mu g.mL^{-1}$) from Sergipe, Brazil, showed the lowest contents of viable cells agains 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 IC_{50} values of 20.5 ± 2.4 to 32.6 ± 2.6 $\mu g.mL^{-1}$, 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 IC_{50} of 29.7 ± 1.5 to 42.1 ± 8.7 $\mu g.mL^{-1}$.

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 (IC_{50} 9.29 µM), murine B16-BL6 melanoma (IC_{50} 6.66 µM), human lung A549 adenocarcinoma (IC_{50} 8.63 µM) and human HT-1080 fibrosarcoma (IC_{50} 7.94 µM) cancer cell lines. Other compound, the mucronulatol, was potent against LLC (IC_{50} 8.38 µM) and A549 (IC_{50} 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.

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 $\mu g.mL^{-1}$, 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 PPARγ 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 PPARγ and LXRα expression. Thus, EERP have a potential as a diet supplement for prevention/treatment of cardiovascular disease.	(Iio et al., 2012)
	The underlying molecular mechanisms of the potential anticancer effects of red propolis on Hep-2 and Hek-293 cells were evaluated. A	

Ethanolic extracts	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 $\mu g.mL^{-1}$): GRP78, PRDX2, LDHB, VIM, TUBA1A. Only two up-regulated proteins were identified in the non-cytotoxic (6 $\mu g.mL^{-1}$) 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 $\mu g.mL^{-1}$). 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-metoxypterocarpan 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, pterocarpans 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 IC_{50}: 4.4 to 25.9 $\mu g.mL^{-1}$ (*S. aureus*), > 64 $\mu g.mL^{-1}$ (*E. coli*), > 64 $\mu g.mL^{-1}$ (*C. albicans*), 1.2 to 8.3 $\mu g.mL^{-1}$ (*Trypanosoma brucei*), 1.2 to 6.4 $\mu g.mL^{-1}$ (*Plasmodium falciparum*), 2.5 to 9 $\mu g.mL^{-1}$ (*Trypanosoma cruzi*), 14.9 to 39.4 $\mu g.mL^{-1}$ (*Trichophyton rubrum*) and 3.3 to 16.1 $\mu g.mL^{-1}$ (*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 ± 1.7 $mg.g^{-1}$ of EE, 129.6 ± 1.1 $mg.g^{-1}$ of EE, 98.8 ± 1.0%, 90.9 ± 0.6%, 89.2 ± 3.8 $\mu g.mL^{-1}$ and 14900 ± 443 μmol Trolox equivalents/g of EE, respectively. It was also possible to identify the major components in the EE sample, by HPLC: Apigenin (15.4 ± 0.8 $mg.g^{-1}$ of EE); Benzyl caffeate (21.1 ± 2.1 $mg.g^{-1}$ of EE); Caffeic acid (3.8 ± 0.4 $mg.g^{-1}$ of EE); Chrysin (47.2 ± 3.7 $mg.g^{-1}$ of EE); Cinnamic acid (4.2 ± 0.6 $mg.g^{-1}$ of EE); Cinnamyl caffeate (7.6 ± 0.6 $mg.g^{-1}$ of EE)/ *p*-Coumaric acid (6.8 ± 0.7 $mg.g^{-1}$ of EE)/ 3,4-Dimethoxycinnamic acid (18.8 ± 1.2 $mg.g^{-1}$ of EE); Ferulic acid (9.8 ± 0.5 $mg.g^{-1}$ of EE); Galangin (101.6 ± 4.5 $mg.g^{-1}$ of EE); Phenethyl caffeate (32.7 ± 2.3 $mg.g^{-1}$ of EEP); Pinobanksin (3.0 ± 0.3 $mg.g^{-1}$ of EEP); Pinobanksin 3-acetate (85.7 ± 3.4 $mg.g^{-1}$ of EEP); Pinobanksin 5-methyl ether (17.2 ± 1.1 $mg.g^{-1}$ of EEP); Pinocembrin (38.2 ± 2.8 $mg.g^{-1}$ of EEP); Pinostrobin (4.0 ± 0.5 $mg.g^{-1}$ of EEP); and Tectochrysin (10.6 ± 1.1 $mg.g^{-1}$ 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 $\mu g.mL^{-1}$ for the crude extract to 16.6 $\mu g.mL^{-1}$ for Riverinol. The compound 8-Prenylnaringenin (6.1 $\mu g.mL^{-1}$) 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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CAPÍTULO 2

Artigo:

Antibacterial activity from bioguided fractionation of Brazilian Red Propolis

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Antibacterial activity from bioguided fractionation of Brazilian Red Propolis

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ABSTRACT

The need for new and efficient antibiotics is evident. The indiscriminate use is causing serious bacterial resistance complicating therapeutic planning. In this context, natural products emerge as major providers of bioactive compounds. Brazilian red propolis is a natural product that has gained prominence worldwide due to its biological properties, among them the antimicrobial. These are related to its chemical composition, which includes terpenes, pterocarpans, phenol derivatives, flavonoids and others. Thus, through the bioguided fractionation performed with the hydroalcoholic extract of red propolis from Alagoas, it was possible to obtain subfractions with remarkable bacteriostatic activity when compared to its precursor fractions. The SC2 subfraction can highlighted, which showed the best results with MIC of 56.75 µg.mL^{-1}, 28.37 µg.mL^{-1}, 454 µg.mL^{-1} and 227 µg.mL^{-1} against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa*, respectively. However, the study also revealed a cytotoxic effect against non-tumor cell line (Vero). Furthermore, through the chemical analyses by High Resolution Mass Spectrometry (HRMS), High Performance Liquid Chromatography with UV detection (HPLC-UV) and Gas Chromatography coupled to Mass Spectrometry (GC-MS), it was verified the presence of important marker compounds in red propolis, such as Formononetin (*m/z* 267.0663), Biochanin A (*m/z* 283.0601) and Liquiritigenin (*m/z* 255.0655). Other relevant compounds were also identified and contribute to the antibacterial activity observed, which makes propolis a very promissing natural product and a new therapeutic alternative against pathogenic microorganisms.

Keywords: Red propolis, Bioguided study, Chemical composition, Antibacterial, Vero cells.

1. INTRODUCTION

The research of natural products with antimicrobial activity has attracted the attention of many researchers, in particular, motivated by the increase in the bacterial resistance to traditional antimicrobial agents and the side effects caused by the indiscriminate and excessive use of drugs (Rates, 2001; Sheldon, 2003; Salatino et al., 2005; Auerbach et al.,2010). To this end, when it is necessary the identification of new active molecules, the use of natural products has always been a humanity pathway, which in most cases, came from the ethnopharmacological knowledge.

In the last decades an increase use of natural products has been highlighted for the prevention and treatment of various diseases. Given its enhanced pharmacological properties, natural products are the largest sources for discovery of new drugs (Pereira et al., 2002; Castilho et al., 2007). According to Newman and Cragg (2012), of all the therapeutic agents developed and approved between 1981 and 2010, 74% were of natural origin.

Among the recently investigated natural products, propolis appears in evidence, in particular the one found in the Northeast region of Brazil, known as red propolis. This substance with resinous, gummy and balsamic characteristics, is the result of compounds collected by bees from plants of the region, associated with salivary secretions, wax and pollen, which is used in the construction and protection of the hive (Ghisalberti, 1979; Brasil, 2001).

Propolis has been widely used in traditional medicine due to its antimicrobial, anti-inflammatory, healing, antioxidant, antitumor activities (Ghisalberti, 1979; Marcucci et al., 2001; Alencar et al., 2007; Frozza et al., 2013; Freires et al., 2016), proving to be an important therapeutic alternative. Its chemical composition is highly variable and directly related to compounds found in the original plant, *Dalbergia ecastophyllum* (L) Taub. The phenolic compounds, among them the flavonoids, have been considered the main biologically active constituents of this resin, together with the cinnamic acid derivatives, esters and some terpenes (Soares, 2002; Salatino et al., 2005; Daugsch et al., 2008).

However, despite the vast literature found for red propolis, few studies are reported with respect to bioguided fractionation processes and activity of fractions or isolated compounds, against pathogenic microorganisms and its possible cytotoxicity in normal cells, ensuring its selective action on desired target, without harming the host.

Thus, considering that the resistance to antimicrobials is an important global problem and knowing that the fractionation is the beginning for the isolation and identification of bioactive compounds from natural products (Hayacibara et al., 2005), the goal of this research was to conduct a bioguided study of this propolis, in order to identify compounds with antibacterial potential and also evaluate its cytotoxicity against non-tumor cells (Vero cells - kidney epithelial cells extracted from an African green monkey), aiming its safe use.

2. MATERIALS AND METHODS

2.1 Chemicals

Water was obtained by a Milli-Q system (TGI Pure Water Systems, USA). Dulbecco's Modified Eagle Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS) was purchased from Cultilab Lab Inc. (Campinas, SP, Brazil). Folin-Ciocalteu's phenol reagent was purchased from Merck.

2.2 Red propolis sample and extraction

The red propolis was collected in the state of Alagoas (9° 34' 12" S, 36° 33' 0" W), Northeast region of Brazil, in 2013. After crushed, it was protected from light and frozen at -20°C. The extraction followed the method indicated by Frozza et al. (2013) with some modifications, in which the crushed propolis was submitted to extraction by maceration with constant agitation (room temperature for 6 h), using ethanol 70% (1:3 w/v). After, the hydroalcoholic extract of propolis (EHP1) was filtered and concentrated in a rotary evaporator at 35 °C.

2.3 Fractionation

2.3.1 Fractionation at medium pressure

In order to remove the waxes, the EHP1 extract (21.47 g) was extracted with hexane (5 × 100 mL), resulting in EHP2 extract (10.50 g), which was fractionated by column chromatography (3.6 × 46 cm) using Silica Gel 60 (190 g; 40-63 µm; Merck) as stationary phase, in Preparative Chromatograph (Sepacore® Büchi) composed by the

following modules: Control Unit C-620, Fraction Collector C-660, Pump Module C-605 and UV Photometer C-640. The system was controlled by "SepacoreControl 1.2" software. The separation occurred at solvent flow of 10 mL/min, collecting volume of 10 mL/tube, maximum pressure of 30 bar and detection at 210, 254, 280 and 365 nm. The mobile phase was a gradient of 0 → 10% methanol/chloroform/0.1% acetic acid. After elution, 18 fractions were obtained, which had the residual solvents evaporated with the aid of a rotary evaporator: F1 (165 mg), F2 (95 mg), F3 (235 mg), F4 (644 mg), F5 (1932 mg), F6 (756 mg), F7 (540 mg), F8 (98 mg), F9 (328 mg), F10 (108 mg), F11 (137 mg), F12 (407 mg), F13 (1447 mg), F14 (234 mg), F15 (174 mg), F16 (577 mg), F17 (317 mg), F18 (687 mg).

2.3.2 Liquid-Liquid Extraction

The EHP1 extract (2.50 g) was also fractionated by liquid-liquid extraction, in a separate flask, with cyclohexane, ethyl acetate, butanol and water, separately. After evaporation of the solvents in a rotary evaporator (35°C), 4 fractions were obtained: 480 mg of cyclohexane fraction (FCHex), 1065 mg of ethyl acetate fraction (FAE), 340 mg of butanol fraction (FBut) and 392 mg of aqueous fraction (FH_2O).

2.4 Subfractionation

The FCHex fraction (0.20 g) was rechromatographed through silica column (Flash Column Silica-CS-Agela; 12 g; 40-60 µm; max. pressure 12.6 bar), using the CombiFlash Companion® Chromatograph, at a flow rate of 18 mL/min, detection at 254 nm, mobile phase gradient of 0 → 25% methanol/dichloromethane/0.1% acetic acid. The following subfractions were obtained: SH1 (26 mg), SH2 (14 mg), SH3 (34 mg), SH4 (6 mg), SH5 (8 mg).

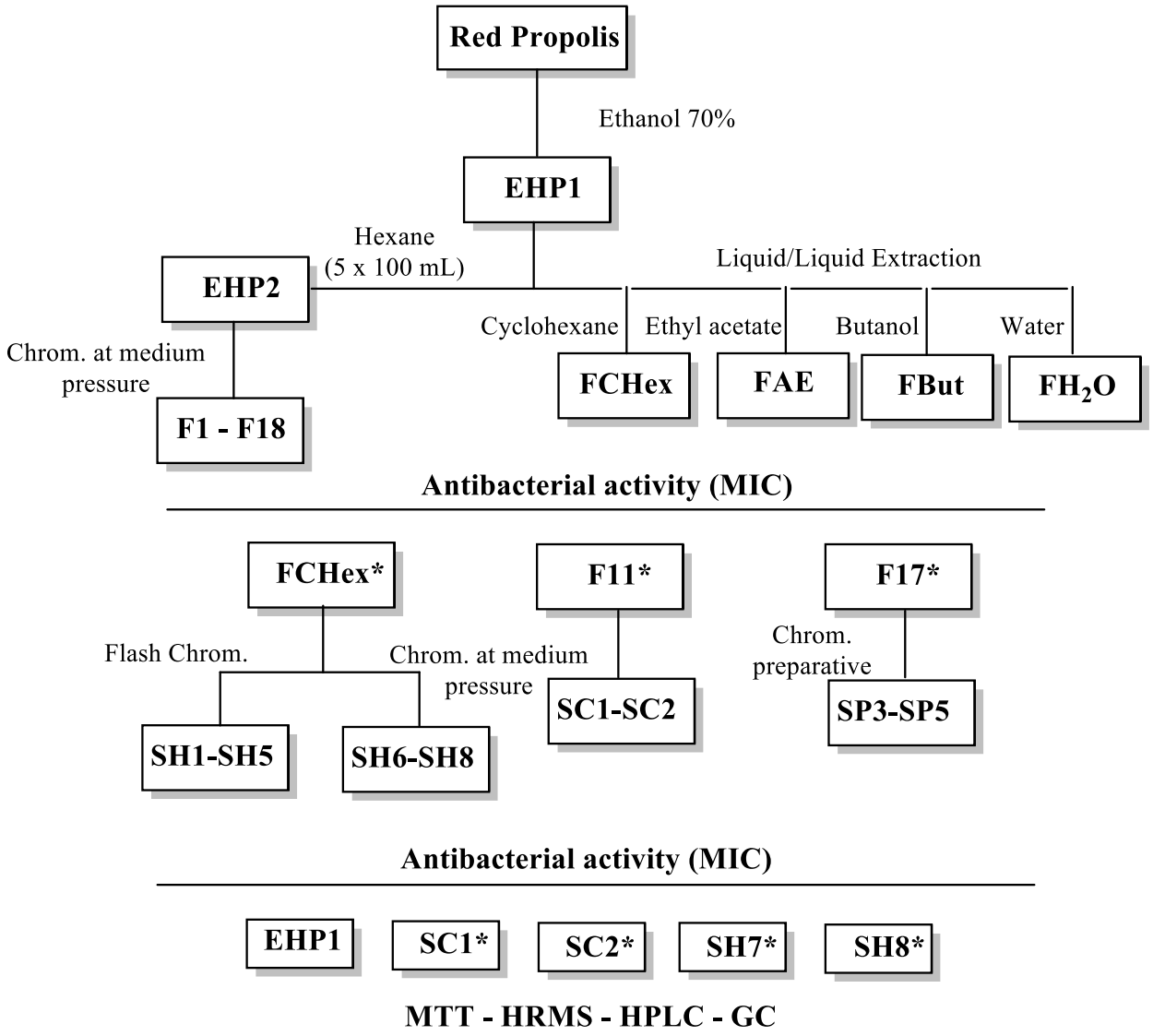
The FCHex fraction (0.095 g) was rechromatographed also by medium pressure column chromatography (2 × 40 cm) of silica (Lichroprep; Si60; 15-25 µm; Merck), with a mobile phase gradient of ethyl acetate/pentane (1:19), ethyl acetate/pentane (1: 9), 10% methanol/dichloromethane/0.1% acetic acid. The subfractions were: SH6 (11 mg), SH7 (16 mg), SH8 (28 mg).

The F11 fraction (0.08 g) was rechromatographed by medium pressure column chromatography (2 × 40 cm) of silica (Lichroprep; Si60; 15-25 µm; Merck). The mobile phase was a gradient of 3% methanol/dichloromethane/0.1% acetic acid, 10%

methanol/dichloromethane/0.1% acetic acid and methanol PA. After evaporation of the solvents, the subfractions were: SC1 (12 mg), SC2 (5 mg).

The F17 fraction (0.05 g) was rechromatographed by preparative silica chromatography with 3% methanol/dichloromethane/0.1% acetic acid to provide the subfractions: SP3 (20 mg), SP5 (19 mg).

A flowchart is representing the bioguided fractionation stages, from propolis maceration until acquisition of fractions/subfractions and preparation for biological and chemical tests (Scheme 1).



Scheme 1. Bioguided fractionation stages of red propolis.

(* Fractions/subfractions that presented better antibacterial activity)

2.5 Chemical characterization

2.5.1 Phenolic compounds

The quantitation of phenolic compounds in the EHP1 extract was performed by the Folin-Ciocalteu colorimetric method, which involves the reduction of the reagent by the phenolic compounds of the samples with concomitant formation of a blue complex. The total phenolics content was quantified using a standard curve prepared with gallic acid (2-22 $\mu g.mL^{-1}$; Chem. Service, Inc., USA) and expressed as mg gallic acid equivalents (GAE)/g of extract.

2.5.2 Electrospray ionization mass spectrometry (ESI-MS)

For chemical analysis by High Resolution Mass Spectrometry (HRMS) on a Bruker Daltonics micrOTOF-Q II instrument, the red propolis samples (EHP1 extract, SC1, SC2, SH7 and SH8) were diluted in specific solutions according with the analysis mode. In positive mode, it was added a solution of chromatographic grade acetonitrile/water (1:1, v/v) with 0.1% formic acid. In negative mode, it was used a solution of chromatographic grade acetonitrile/water (1:1, v/v) with 0.1% ammonium hydroxide. The solutions were infused directly into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 3 $\mu L.min^{-1}$. ESI(+)-MS, ESI(-)-MS and tandem ESI-MS/MS were acquired using quadrupole time-of-flight (QTOF) with the conditions: capillary voltage of +4500V, desolvation temperature of 180 °C, nebulization gas pressure of 0.4 bar and drying gas flow of 5 L/min (N_2). For MS/MS, the energy for the collision induced dissociations (CID) was optimized for each component. Diagnostic ions were identified by the comparison of their exact mass (*m/z*), isotopic ratio and fragmentation pathway with compounds identified in previous studies. For data acquisition and processing, Compass DataAnalysis 4.0 and Microtof Control were used. The data were collected in the *m/z* range of 50-1400 at the speed of two scans per second, providing the resolution of 50,000 (FWHM) at *m/z* 200.

2.5.3 Gas chromatography-mass spectrometry (GC-MS)

The analyses in GC-MS were performed on a Gas Chromatograph coupled to mass selective detector Hewlett Packard 6890/MSD5973 equipped with HP Chemstation software and Wiley 275 spectra library. An HP-5 column (30 m × 250 µm) was used, 0.25 µm film thickness (Hewlett Packard, Palo Alto, USA). The temperature

program used was 60 °C to 310 °C at 5 °C/min, injector and interface temperatures of 220 °C and 250 °C, split ratio 1:80, He carrier gas (56 kPa), flow of 1 mL/min, injection volume of 1 µL and ionization energy of 70 eV (adapted from Nunes et al., 2009).

2.5.4 High Performance Liquid Chromatography (HPLC)

The analyses were performed in HPLC equipment HP 1100 model, equipped with UV detector at 270 nm and quaternary pump system. The samples were filtered through Nylon membranes of 0.45 µm pore size. For the analysis on reverse phase was used a Lichrospher RP_{18} column (250 mm × 4 mm, 5 µm) and the solvent system consisted of: solvent A - Milli-Q water with 1% acetic acid and solvent B - acetonitrile, working in isocratic mode, with 50% solvent A and 50% solvent B for 25 min. The column temperature was maintained at 25 °C, the injection volume was 5 µL and the flow pattern was maintained at 0.6 $mL.min^{-1}$. The compounds were identified according to their elution order and by comparison of their retention times with the pure standards. The quantification was performed by external standardization method, using the correlation area (mAU*s) of the peak of the compound to the standard curve made with each evaluated pattern (Liquiritigenin, Formononetin and Biochanin A) (adapted from KüÇükboyaci et al., 2013).

2.6 Antibacterial activity

The microorganisms used were *Bacillus subtilis* CIP 52.62, *Escherichia coli* CIP 54127, *Pseudomonas aeruginosa* CIP 82118 and *Staphylococcus aureus* CIP 4.83. To prepare the inoculum, colonies were removed from a culture of 18-24 h at 37 ± 1 °C in TSA medium (*Trypticase Soy Agar* - Merck), in peptone saline solution, adjusted to 75% T at 620 nm. The bacteriostatic activity was determined by dilution method in solid medium using 24-well plates, where 200 µL of sample was added to 1.8 mL of TSA culture medium (50 °C) in a well. Then, successive dilutions, in geometric progression of ratio 2, were made with the culture medium. After solidify, 4 µL of the bacterial suspension were deposited on the surface and the plates incubated for 24 h at 37 °C. As positive control the antibiotic ciprofloxacin (20 $\mu g.mL^{-1}$) was used. The Minimum Inhibitory Concentration (MIC) was given by the lowest sample concentration able to inhibit bacterial growth.

2.7 Cell viability assay

Vero cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% inactivated fetal bovine serum and 1% antibiotic (penicillin/streptomycin). The cells were maintained in tissue culture bottles at 37 °C in humidified atmosphere with 5% CO_2. An inoculum of 8×10^4 cells/mL was prepared and incubated in 96-well plates. After cell plating, the cells were treated for 24 h with the crude extract and subfractions of red propolis, and 24 h with crude extract and subfractions + 24 h with DMEM medium supplemented, at different concentrations. It was determined the viability of Vero cells to the treatments with red propolis by the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), according to the methodology described by Denizot and Lang (1986). After, the medium was removed with the treatments and added 0.1 mL of DMEM without serum containing MTT (1 $mg.mL^{-1}$). The mixture was incubated at 37 °C with 5% CO_2 for 2 hours. The medium with MTT was removed and the precipitate was dissolved in dimethylsulfoxide (DMSO), with subsequent stirring for 30 minutes and protected from light. The spectrophotometric reading was performed at 570 nm and the results were expressed as percent of viability. The absorbance of the negative control (cells that did not receive treatment) represented 100% of viability and the values of the treated cells were calculated as a percentage of control.

3. RESULTS AND DISCUSSION

The bioguided fractionation has been an effective method and frequently employed in the discovery of new drugs, due to the possibility to obtain bioactive fractions or isolated compounds, through a rationalized process. Therefore, each fraction is further evaluated in a bioassay system and only the active fractions are fractionated again, continuing the study (Ode et al., 2011). Thus, through the bioguided study performed with red propolis from Alagoas, aiming the antibacterial activity, it was possible to obtain subfractions with relevant bacteriostatic action. The Minimum Inhibitory Concentration (MIC) presented by the crude extract (EHP1), fractions and subfractions of red propolis are represented in Tables 1 and 2.

Table 1.

Table 2.

Overall, it can be observed that the Gram-positive bacteria (*S. aureus* and *B. subtilis*) were more sensitive than Gram-negative bacteria (*E. coli* and *P. aeruginosa*). This is explained by the structural differences in the cell wall of these bacteria, because Gram-negative bacteria present a more chemically complex wall, providing a greater resistance (Araújo et al., 2010). In addition, *B. subtilis* was more sensitive than *S. aureus* when submitted to the activity of red propolis fractions and subfractions. Already, *E. coli* showed to be more sensitive to fractions and *P. aeruginosa* more sensitive to subfractions.

Also, it was found that the crude extract (EHP1) was more active against *S. aureus* (MIC = 125 $\mu g.mL^{-1}$), followed by *B. subtilis* (MIC = 1136 $\mu g.mL^{-1}$). The fractions that had a higher activity against *S. aureus* were: FCHex, F4 and F11, all with MIC of 62.50 $\mu g.mL^{-1}$. The most active fractions against *B. subtilis* were: F4, F11, F12 and F17, all with MIC of 62.50 $\mu g.mL^{-1}$. Regarding the Gram-negative bacteria, the fractions F12 and F17 (MIC = 1250 $\mu g.mL^{-1}$) were more active against *E. coli* and the fractions FBut, F6, F13, F14 and F18, all with MIC of 2500 $\mu g.mL^{-1}$, against *P. aeruginosa*. Among the subfractions, SC2 was the one that showed better activity against all bacteria tested, with the following MIC: 56.75 $\mu g.mL^{-1}$ (*S. aureus*), 28.37 $\mu g.mL^{-1}$ (*B. subtilis*), 454 $\mu g.mL^{-1}$ (*E. coli*) and 227 $\mu g.mL^{-1}$ (*P. aeruginosa*).

Therefore, the fractionation significantly reduced the MIC of some subfractions when compared to the precursor fractions and this may be associated with possible isolation of active compounds against bacteria, evidencing its activity. This was mainly observed in Gram-negative bacteria. Still, the differences observed in bacteriostatic activity may be due to interactions between different active compounds and their abundance.

The bacteria used in this study are often associated with various infections, because they act in an opportunistic way, especially in cases of immunosuppression. Also, they show resistance to many antibiotics available nowadays, fact that increases the importance in seeking for new antibacterial therapeutic alternatives (Lowy, 1998; Nataro and Kaper, 1998; Oggioni et al., 1998; Gelatti et al., 2009).

Among the various activities presented by red propolis, the antibacterial has been reported and scientifically proven (Alencar et al., 2007; Daugsch et al., 2008; Righi et al., 2011; Lopez et al., 2015). Many authors attribute this activity to flavonoids,

aromatic acids and esters present in its composition. However, its chemical composition varies according to many factors, for instance flora, seasonal variation, among others, affecting its biological properties (Cabral, 2008).

In a recent study conducted by Inui et al. (2014), the compound (6aS,11aS)-medicarpin isolated from red propolis taken from Alagoas, exhibited potent antibacterial activity against *S. aureus*, *B. subtilis* and *P. aeruginosa*, with MIC values of 16, 32 and 32 $\mu g.mL^{-1}$, respectively. Machado et al. (2016) evaluated the activity of the ethanol extract of red propolis from Sergipe (Brazil), which showed a high antimicrobial activity *in vitro*, with MIC of 100-400 $\mu g.mL^{-1}$ (*S. aureus* ATCC 33951), 25-50 $\mu g.mL^{-1}$ (*S. aureus* ATCC 25923) and 400 $\mu g.mL^{-1}$ (*E. coli* ATCC 25922). On the other hand, propolis from Brazil and Cuba presented a better activity against Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Proteus morganii*, *Enterobacter aerogenes*), with MIC between 6.25 to 500 $\mu g.mL^{-1}$, feasible for antimicrobial topical applications (Lopez et al., 2015).

An interesting classification for plant materials was proposed by Aligianis et al. (2001), which considered the following results for MIC: "strong inhibition" - MIC up to 500 $\mu g.mL^{-1}$, "moderate inhibition" - MIC between 600 and 1500 $\mu g.mL^{-1}$, and "weak inhibition" - MIC above 1600 $\mu g.mL^{-1}$. Thus, the crude extract (EHP1) showed strong inhibition against *S. aureus*, moderate inhibition against *B. subtilis* and weak inhibition against *E. coli* and *P. aeruginosa*. The most active subfractions (SC1, SC2, SH7, SH8) showed strong inhibition against all bacteria tested, with MIC up to 500 $\mu g.mL^{-1}$.

The traditional use of natural products, such as propolis, does not necessarily guarantee their safety. Therefore, the safety evaluation of bioactive natural products should always be considered together with the analysis of its biological activity (Lopez et al., 2015).

And for this, cytotoxicity tests in normal cells (non-tumor) are very important in order to verify the possible toxicity of the compound, as well as its selectivity to the desired target. A non-tumor cell line widely used is the Vero, kidney epithelial cells extracted from African green monkey. Investigations using Vero has demonstrated a wide range of applications, which extend from virology to diagnostic practice in hospitals and tests with bacterial toxins, including: *screening* for toxin of *Escherichia coli*, as host cells for the growth of virus and eukaryotic parasites, and use in human vaccines. In addition, these cells already have determined the sequence of its genome (Osada et al., 2014).

Through the evaluation of viability of Vero cells by the MTT method, a cytotoxic effect from both the crude extract (EHP1) and subfractions of propolis was observed (Table 3). The amount of viable cells varied according to each sample tested, from 14.5% to 46%. Thus, to ensure the safe use generating no cytotoxic effects of red propolis, the importance of using concentrations below the MIC should be highlighted.

Table 3.

There are few studies in the literature evaluating the cytotoxic activity of Brazilian red propolis in non-tumor cell lines, with the aim of verify the inherent toxicity of this product. Among these studies, there is Frozza et al. (2013), who used Hek-293 (*human normal epithelial embryonic kidney*) and obtained an IC_{50} > 150 $\mu g.mL^{-1}$, and Lopez et al. (2015), which also observed cytotoxic effect of red propolis against HaCaT cells (*human keratinocytes*) and BALB/c 3T3 (*murine fibroblast*). Therefore, further studies with non-tumor cells are necessary, exposing this resin to cells, with guarantee of a reliable and effective result.

As important as the bioactivity determination, is necessary the evaluation of chemical composition, qualifying and quantifying the possible responsible for the activity. For the evaluation of the extract and subfractions that showed higher antimicrobial potential, some precise and selective analytical techniques were selected. One of them is the High Resolution Mass Spectrometry (HRMS), a modern technique, which has gained importance in the characterization and identification of chemical compounds in complex mixtures, as extracts of natural products. This is possible in accordance with a set of information as exact mass (*m/z*), isotopic ratio and fragmentation pathway in tandem mass. Also, it is able to detect volatile and non-volatile compounds (Vessecchi et al. 2011; Murata et al., 2015).

The analysis of crude extract (EHP1) and subfractions of propolis by HRMS identified several compounds with interesting biological activities (Table 4). Figure 1 shows the chemical structures of some of the main compounds identified. In the full mass spectrum of the crude extract (EHP1), in positive mode (Figure 2), is possible to highlight some compounds commonly found in Brazilian red propolis that have remarkable antibacterial activity, such as: Vestitol (*m/z* 273.1123), Medicarpin (*m/z* 271.0981), Elemicin (*m/z* 209.1182) and Guaiacol (*m/z* 125.0600) (Rossi et al., 2007;

Cooper, 2013; Inui et al., 2014). The Retusapurpurin (*m/z* 523.1782), responsible for the color of propolis, was identified only in EHP1 extract (Piccinelli et al., 2011).

Table 4.

Figure 1.

Figure 2.

Furthermore, in the full mass spectrum, in negative mode, of the SC2 subfraction (Figure 3), which expressed the best bacteriostatic activity, some essential markers compounds of red propolis were observed, such as Liquiritigenin (*m/z* 255.0655), Formononetin (*m/z* 267.0663) and Biochanin A (*m/z* 283.0601). These compounds are responsible for a huge of biological activities such as antibacterial, cytotoxic, antifungal, anti-inflammatory and others (Kole et al., 2011; Frozza et al., 2013; Gaur et al., 2016).

Figure 3.

In addition, these compounds were quantified by High Performance Liquid Chromatography with UV detection (HPLC-UV) and the results are shown in Table 5. This method is widely applied in qualitative and quantitative purposes, especially in the pharmaceutical industry, environmental and other fields of science (Tonhi et al., 2002). Thus, through the quantification performed, it was possible to verify the presence of Formononetin in all samples, especially the SC1 subfraction with 128.13 $\mu g.mL^{-1}$. In their study, Daugsch et al. (2008) performed the quantification by Reversed Phase High Performance Liquid Chromatography (RPHPLC) of different Brazilian red propolis samples, highlighting the compounds Formononetin (10.2 to 10.9 $mg.g^{-1}$), Liquiritigenin (1.8 to 5.7 $mg.g^{-1}$) and Biochanin A (0.4 to 2.1 $mg.g^{-1}$).

Table 5.

In the analyzes performed by Gas Chromatography coupled to Mass Spectrometry (GC-MS), it was possible to characterize some compounds in the crude extract (EHP1), which are shown in Table 6. The Elemicin (8.75%), Medicarpin

(6.05%), Methyleugenol (4.96%) showed significant concentrations and Vestitol (17.71%) was the major compound. The GC-MS is suitable for the analysis of volatile compounds and has a broad application, used in the identification of numerous compounds present in natural and biological systems (Chauhan et al., 2014). In a study performed by Alencar et al. (2007), several compounds were identified in red propolis using GC-MS such as *m*-Guaiacol, Methyleugenol, Medicarpin, Benzoic acid, Homopterocarpin. The same technique was used by Righi et al. (2011) to characterize the chloroform extract of red propolis, where some compounds were identified: Methyleugenol (1.0%), Elemicin (1.0%), Medicarpin (10.0%), Lupeol (4.6%), α-Amyrin (5.0%) and others.

Table 6.

Propolis is a natural product that is distinguished by the presence of a wide variety of phenolic compounds, responsible for many of the activities of this resin. Some studies suggest that the antimicrobial activity is associated with the presence of phenolic compounds in propolis (Alencar et al., 2007), however, the activity can be obtained from the synergist effect of various substances found in this natural product (Krol et al., 1993; Marcucci, 1995).

Phenolic compounds are widely distributed in nature and are essential for growth and reproduction of plants, formed under stress conditions, such as infections, injuries and others (Naczk et al., 2004). The consumption of foods containing these compounds may interfere with various physiological processes of the human body, because they have antioxidant activity, act in healing processes and have important antimicrobial activity (Menezes, 2005).

The crude propolis and its extract need to follow certain specifications, according to the Ministry of Agriculture and Supply. In Brazil, the "Identity Regulation and Quality of Propolis Extract" present in the Instruction nº 03, of 19 January 2001, aims to establish the identity of Brazilian propolis extract and determine the minimum quality requirements for the domestic or international trade. One of these requirements is the content of phenolic compounds, establishing a minimum threshold of 0.50% (w/w). In this study, EHP1 extract showed a content of phenolic compounds of 7.33 mg GAE/g of extract, i.e., 0.73% (w/w), being above the accepted limit. Some studies have shown that the proportion of phenolic compounds in propolis is variable, depending on

location and time of collection. Oliveira et al. (2012) found in different propolis extracts a variation in total phenol content, from 3.36 to 4.81%. Already, Silva et al. (2006), analyzing propolis samples of Paraiba, collected at different times of the year, found phenols content between 2.93 to 8.13%.

All identified compounds related to red propolis are very important once they present different pharmacological activities. However, despite a large amount of antibiotics available at the present, an ideal compound is not feasible, given collateral and resistance effects. The frequent exposure of bacteria to commercial antibacterial agents leads to adaptation, which makes the strains resistant to many of these drugs. At this point, natural products emerge as an alternative treatment, as isolated compounds, complementary therapies or as nutraceuticals (Lam, 2007).

Thus, the search for substances with higher selectivity and fewer side effects, higher therapeutic potency and lower resistance is extremely relevant to the current therapy. In this way, the red propolis, inside the great diversity of natural products in Brazil, has emerged as an important alternative therapy against pathogenic microorganisms. Besides the use of propolis and its derivatives in the health field, an alternative would be the food area, as food preservatives by preventing the proliferation of pathogenic microorganisms (Tosi et al., 2007).

The results obtained in this study are promissing, which suggest an important antimicrobial potential of propolis subfractions, due to the ability to inhibit the proliferation of these microorganisms. Still, it is possible to suggest the influence of some identified compounds, in the significant activity presented by SC2 subfraction. Among them are the marker compounds Formononetin, Biochanin A and Liquiritigenin, flavonoids that have proven antimicrobial activity (Hanski et al., 2014; Kong et al., 2015). However, other compounds present in this subfraction also have activity against pathogenic bacteria, and thus, it is not possible to rule out the hypothesis of a synergism.

In this context, the bioguided fractionation has been an appropriate and very important process, because it potentiates biological activity, due to the ability to isolate and concentrate active compounds in a logical and rational way.

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Table 1. Bacteriostatic activity of the crude extract (EHP1) and fractions of red propolis from Alagoas - Brazil (MIC = $\mu g.mL^{-1}$).

Sample	Time	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeruginosa*
EHP1 extract [a]	24 h	125	1136	5000	5000
FCHex [b]	24 h	62.50	2272	> 5000	> 5000
FAE [b]	24 h	125	1136	5000	> 5000
FH_2O [c]	24 h	> 5000	> 5000	> 5000	> 5000
FBut [a]	24 h	1250	2500	2500	2500
F1 [b]	24 h	5000	183	5000	> 5000
F2 [b]	24 h	5000	312.50	> 5000	5000
F3 [b]	24 h	2500	125	> 5000	5000
F4 [b]	24 h	62.50	62.50	5000	5000
F5 [b]	24 h	2500	5000	5000	5000
F6 [b]	24 h	125	125	5000	2500
F7 [b]	24 h	625	250	5000	5000
F8 [b]	24 h	5000	312.50	5000	5000
F9 [a]	24 h	5000	625	5000	5000
F10 [b]	24 h	250	125	5000	5000
F11 [b]	24 h	62.50	62.50	5000	5000
F12 [b]	24 h	2500	62.50	1250	5000
F13 [b]	24 h	625	2500	2500	2500
F14 [b]	24 h	312.50	125	2500	2500
F15 [b]	24 h	2500	125	5000	5000
F16 [b]	24 h	312.50	312.50	2500	5000
F17 [b]	24 h	2500	62.50	1250	5000
F18 [b]	24 h	2500	5000	5000	2500
Ciprofloxacin*	24 h	0.5	0.0625	0.0156	0.25

Solvents used for the solubilization of the samples: [a] DMSO, [b] Ethanol, [c] H_2O; Negative controls: Ethanol, H_2O, DMSO; *Positive control.

Table 2. Bacteriostatic activity of subfractions of red propolis from Alagoas - Brazil (MIC = $\mu g.mL^{-1}$).

Sample	Time	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeruginosa*
SC1	24 h	115.25	57.62	461	230.50
SC2	24 h	56.75	28.37	454	227
SP3	24 h	250	250	500	250
SP5	24 h	125	125	500	250
SH1	24 h	250	62.50	500	250
SH2	24 h	250	125	500	250
SH3	24 h	250	62.50	500	250
SH4	24 h	500	500	500	250
SH5	24 h	250	125	500	250
SH6	24 h	> 500	500	500	250
SH7	24 h	62.50	31.25	500	250
SH8	24 h	62.50	62.50	500	250
Ciprofloxacin*	24 h	0.5	0.0625	0.0156	0.25

Solvent used for the solubilization of the samples: Ethanol; Negative control: Ethanol; *Positive control.

Table 3. The Minimum Inhibitory Concentration (MIC) of the crude extract (EHP1) and subfractions of red propolis from Alagoas and the viability of normal cells (Vero).

	MIC (µg.mL^{-1})	**Viability (%)**	**Viability (%)**
	S. aureus	**Vero cells 24 h TT***	**Vero cells 24 h TT* + 24 h medium**
EHP1 extract	125	21.75 ± 8.49	28.90 ± 6.56
SC1	115.25	46.02 ± 2.62	22.04 ± 2.61
SC2	56.75	25.96 ± 6.05	17.23 ± 0.88
SH7	62.50	30.32 ± 9.19	16.77 ± 0.29
SH8	62.50	34.87 ± 13.32	18.53 ± 3.73

	MIC (µg.mL^{-1})	**Viability (%)**	**Viability (%)**
	B. subtilis	**Vero cells 24 h TT***	**Vero cells 24 h TT* + 24 h medium**
EHP1 extract	1136	ND**	ND**
SC1	57.62	22.09 ± 1.51	16.02 ± 1.32
SC2	28.37	17.32 ± 2.47	14.51 ± 1.54
SH7	31.25	20.99 ± 5.51	14.78 ± 1.21
SH8	62.50	34.87 ± 13.32	18.53 ± 3.73

Results expressed as mean ± SD of two independent experiments performed in triplicate; Negative control: ethanol 80%; *TT: treatment; **ND: not determined; Not determined for *E. coli* and *P. aeruginosa* due to the high MIC values.

Table 4. Chemical compounds identified in the crude extract (EHP1) and subfractions of red propolis from Alagoas by ESI-MS, in positive and negative mode.

Sample	Precursor ion m/z	Identification	Diff. ppm	Fragmentation pathways	Reference
Positive mode					
EHP1 extract	123.0450	Benzoic acid	3.21		Alencar et al. 2007
	125.0600	Guaiacol	2.03		Alencar et al. 2007
	137.0603	Anisaldehyde	0.33		Nunes et al. 2009
	179.1074	Methyl eugenol	1.09		Righi et al. 2011
	195.1015	Methoxyeugenol	3.17		Righi et al. 2011
	205.1961	Cadinene	2.31		Nunes et al. 2009
	209.1182	Elemicin; Cis-asarone; Trans-isoelemicin	2.06	194.0969 $[M\text{-}CH_3]^+$; 178.0982 $[M\text{-}CH_3O]^+$; 168.0783 $[M\text{-}C_3H_5]^+$; 153.0571 $[M\text{-}C_4H_8]^+$	Trusheva et al. 2006
	257.0823	Liquiritigenin	3.56	153.0177 $[M\text{-}C_8H_8]^+$; 147.0448 $[M\text{-}C_6H_6O_2]^+$; 137.0216 $[M\text{-}C_8H_8O]^+$; 131.0450 $[M\text{-}C_6H_6O_3]^+$; 121.0483 $[M\text{-}C_8H_8O_2]^+$; 119.0471 $[M\text{-}C_7H_6O_3]^+$	Frozza et al. 2013
	269.0824	Formononetin	3.77	254.0636 $[M\text{-}CH_3]^+$; 253.0512 $[M\text{-}CH_4]^+$; 237.0592 $[M\text{-}CH_4O]^+$; 226.0692 $[M\text{-}C_2H_3O]^+$; 225.0571 $[M\text{-}C_2H_4O]^+$; 213.0974 $[M\text{-}C_2O_2]^+$; 197.0666 $[M\text{-}C_3H_4O_2]^+$; 137.0216 $[M\text{-}C_9H_8O]^+$; 133.0615 $[M\text{-}C_7H_4O_3]^+$; 118.0434 $[M\text{-}C_8H_7O_3]^+$	Frozza et al. 2013

	271.0981	Medicarpin	3.93	243.1078 $[M-CO]^+$; 177.0561 $[M-C_6H_6O]^+$; 163.0391 $[M-C_7H_8O]^+$; 161.0643 $[M-C_6H_6O_2]^+$; 151.0422 $[M-C_8H_8O]^+$; 149.0562 $[M-C_7H_6O_2]^+$; 147.0448 $[M-C_7H_8O_2]^+$; 131.0523 $[M-C_7H_8O_3]^+$; 123.0412 $[M-C_9H_8O_2]^+$	Frozza et al. 2013
	273.1123	Vestitol; Isovestitol; Neovestitol	1.41	163.0716 $[M-C_6H_6O_2]^+$; 151.0562 $[M-C_7H_6O_2]^+$; 123.0482 $[M-C_9H_{10}O_2]^+$	Piccinelli et al. 2011
	523.1782	Retusapurpurin	4.82		Frozza et al. 2013
	603.3724	Xanthochymol; Guttiferone E; Oblongifolin A	6.35	585.3631 $[M-H_2O]^+$; 479.2531 $[M-C_5H_{16}O_3]^+$; 467.2620 $[M-C_6H_{16}O_3]^+$	Piccinelli et al. 2011
SC1	123.0449	Benzoic acid	2.40		Alencar et al. 2007
	125.0602	Guaiacol	0.43		Alencar et al. 2007
	137.0604	Anisaldehyde	1.06		Nunes et al. 2009
	195.1022	Methoxyeugenol	0.41		Righi et al. 2011
	257.0822	Liquiritigenin	3.17		Frozza et al. 2013
	269.0824	Formononetin	3.77		Frozza et al. 2013
	271.0975	Medicarpin	1.72		Frozza et al. 2013
	287.0915	Vesticarpan	1.56		Piccinelli et al. 2011
	603.3713	Xanthochymol; Guttiferone E; Oblongifolin A	4.53		Piccinelli et al. 2011
	125.0606	Guaiacol	2.76		Alencar et al. 2007
	137.0603	Anisaldehyde	0.33		Nunes et al. 2009
	195.1017	Methoxyeugenol	2.15		Righi et al. 2011

	209.1166	Elemicin; Cis-asarone; Trans-isoelemicin	5.59		Trusheva et al. 2006
SC2	269.0832	Formononetin	6.74		Frozza et al. 2013
	271.0969	Medicarpin	0.49		Frozza et al. 2013
	287.0928	Vesticarpan	2.96		Piccinelli et al. 2011
	603.3725	Xanthochymol; Guttiferone E; Oblongifolin A	6.52		Piccinelli et al. 2011
	125.0597	Guaiacol	4.43		Alencar et al. 2007
	137.0602	Anisaldehyde	0.39		Nunes et al. 2009
	195.1018	Methoxyeugenol	1.63		Righi et al. 2011
SH7	209.1179	Elemicin; Cis-asarone; Trans-isoelemicin	0.62		Trusheva et al. 2006
	269.0831	Formononetin	6.37		Frozza et al. 2013
	271.0979	Medicarpin	3.19		Frozza et al. 2013
	273.1126	Vestitol; Isovestitol; Neovestitol	0.31		Piccinelli et al. 2011
	123.0449	Benzoic acid	2.40		Alencar et al. 2007
	125.0598	Guaiacol	3.63		Alencar et al. 2007
	137.0601	Anisaldehyde	1.12		Nunes et al. 2009
SH8	269.0827	Formononetin	4.89		Frozza et al. 2013
	271.0969	Medicarpin	0.49		Frozza et al. 2013
	273.1137	Vestitol; Isovestitol; Neovestitol	3.72		Piccinelli et al. 2011
	603.3728	Xanthochymol; Guttiferone E; Oblongifolin A	7.02		Piccinelli et al. 2011
Negative mode					
EHP1 extract	267.0663	Formononetin	2.12		Frozza et al. 2013

	121.0294	Benzoic acid	3.68		Alencar et al. 2007
	255.0663	Liquiritigenin	2.22		Frozza et al. 2013
	267.0663	Formononetin	2.12		Frozza et al. 2013
	269.0830	Medicarpin	6.00		Frozza et al. 2013
SC1	283.0621	Biochanin A	5.12		Frozza et al. 2013
	299.0572	Alnusin; Pratensein; 2'-hydroxybiochanin A	5.47		Awale et al. 2008
	601.3539	Xanthochymol; Guttiferone E; Oblongifolin A	1.64		Piccinelli et al. 2011
	253.0491	Daidzein	3.88		Awale et al. 2008
	255.0655	Liquiritigenin	0.91		Frozza et al. 2013
	267.0663	Formononetin	2.12		Frozza et al. 2013
	269.0820	Medicarpin	2.29		Frozza et al. 2013
SC2	283.0601	Biochanin A	1.93		Frozza et al. 2013
	285.0761	Vesticarpan	0.69		Piccinelli et al. 2011
	299.0559	Alnusin; Pratensein; 2'-hydroxybiochanin A	1.12		Awale et al. 2008
	301.0712	(3S)-ferreirin	0.04		Awale et al. 2008
	601.3523	Xanthochymol; Guttiferone E; Oblongifolin A	1.02		Piccinelli et al. 2011
	255.0660	Liquiritigenin	1.04		Frozza et al. 2013
SH7	269.0827	Medicarpin	4.89		Frozza et al. 2013
	283.0618	Biochanin A	4.06		Frozza et al. 2013
	301.0727	(3S)-ferreirin	4.93		Awale et al. 2008
SH8	267.0662	Formononetin	1.74		Frozza et al. 2013

Table 5. Quantification of markers compounds (μg.mL^{-1}) in red propolis by HPLC-UV.

	EHP1 extract	**SC1**	**SC2**	**SH7**	**SH8**
Liquiritigenin	17.08 ± 0.15	ND	1.25 ± 0.12	ND	ND
Formononetin	34.05 ± 0.22	128.13 ± 0.54	1.57 ± 0.01	1.51 ± 0.01	5.38 ± 0.10
Biochanin A	3.15 ± 0.03	ND	12.38 ± 0.03	ND	ND

Results expressed as mean ± SD of three independent experiments performed in triplicate; ND: not detected.

Table 6. Chemical compounds identified in the crude extract (EHP1) of red propolis from Alagoas by GC-MS.

Sample	R.T. (min)	Compound	Area (%)	Reference
EHP1 extract	18.637	Anethole	2.25	Trusheva et al. 2006
	23.620	Methyleugenol	4.96	Righi et al. 2011
	29.677	Elemicin	8.75	Trusheva et al. 2006
	31.420	Methoxyeugenol	4.87	Righi et al. 2011
	33.220	Isoelemicin	4.25	Trusheva et al. 2006
	60.621	Medicarpin	6.05	Righi et al. 2011
	67.095	Vestitol	17.71	Righi et al. 2011

* Compounds not detected in propolis subfractions.

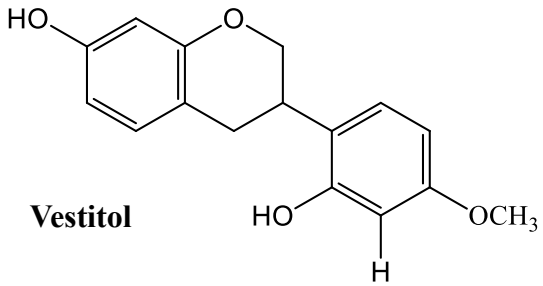
LEGEND TO FIGURES

Figure 1 - Chemical structure of compounds identified in red propolis by High Resolution Mass Spectrometry.

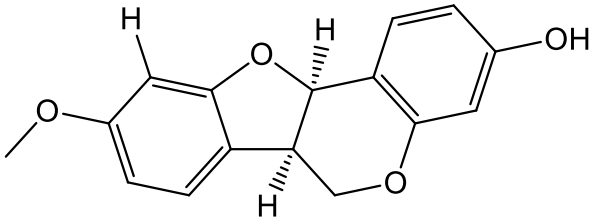
Figure 2 - Full mass spectrum (HRMS) of crude extract (EHP1) obtained of red propolis from Alagoas, in the positive mode.

Figure 3 - Full mass spectrum (HRMS) of SC2 subfraction obtained of red propolis from Alagoas, in negative mode.

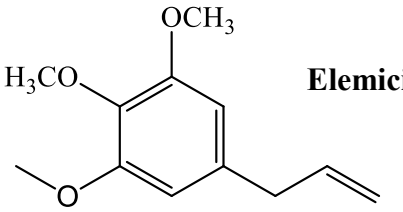
Figure 1



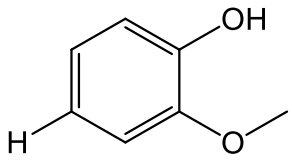
Vestitol



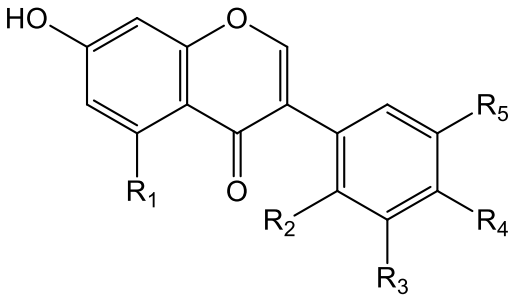
Medicarpin



Elemicin



Guaiacol



	R_1	R_2	R_3	R_4	R_5
Formononetin	H	H	H	OCH_3	H
Biochanin A	OH	H	H	OCH_3	H

Figure 2

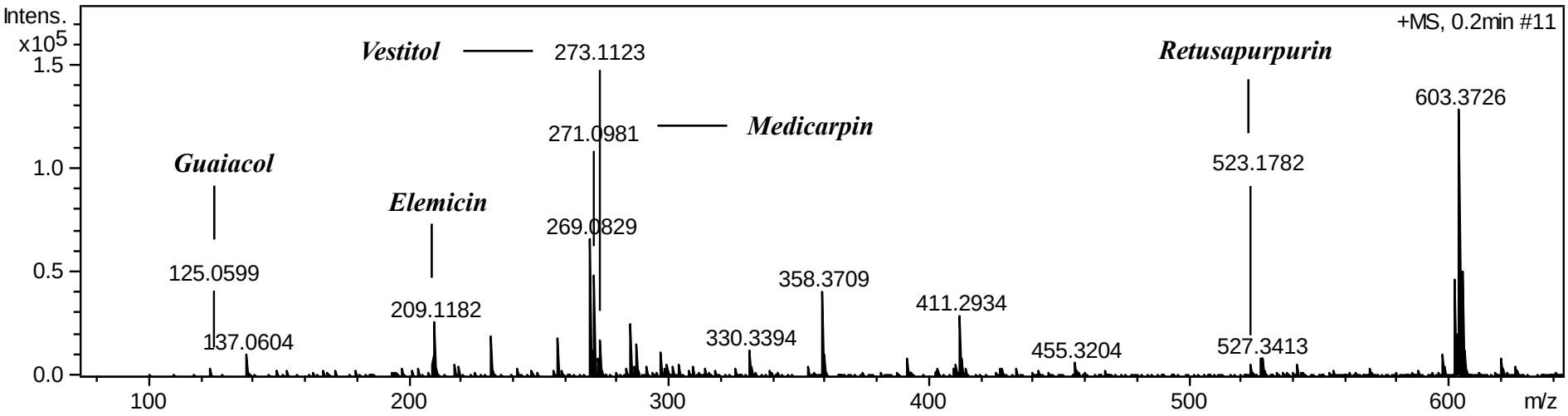
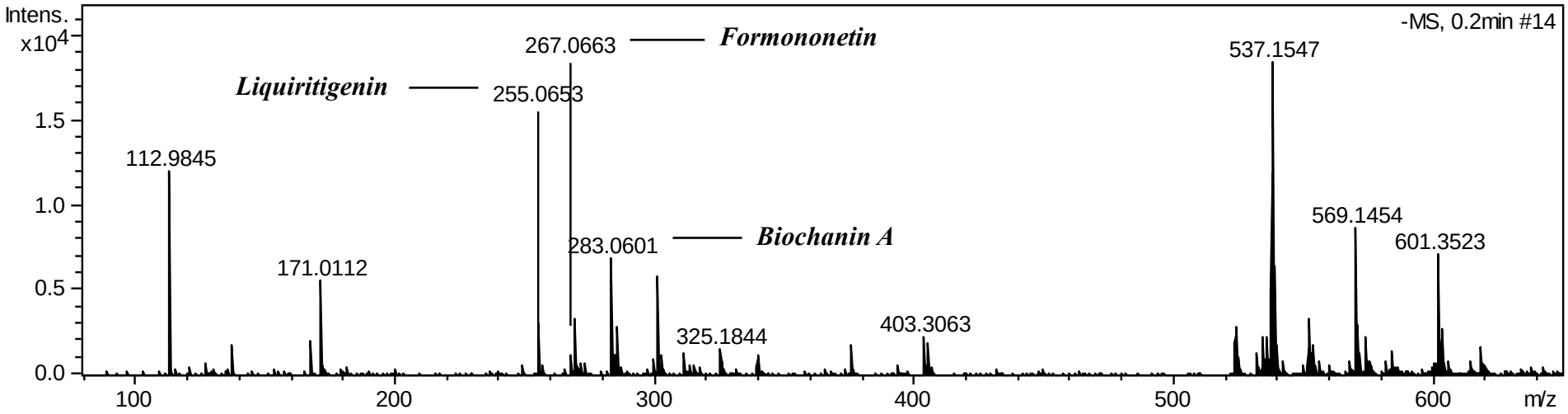

Intens.
x10^5
1.5
1.0
0.5
0.0
+MS, 0.2min #11
Vestitol
273.1123
Medicarpin
271.0981
Guaiacol
125.0599
137.0604
Elemicin
209.1182
269.0829
358.3709
330.3394
411.2934
455.3204
Retusapurpurin
523.1782
527.3413
603.3726
100
200
300
400
500
600
m/z

Figure 3


Intens.
x10^4
-MS, 0.2min #14
267.0663
Formononetin
537.1547
Liquiritigenin
255.0653
112.9845
569.1454
601.3523
283.0601
Biochanin A
171.0112
325.1844
403.3063
1.5
1.0
0.5
0.0
100
200
300
400
500
600
m/z

5. CONCLUSÕES

- O fracionamento bioguiado possibilitou a obtenção de subfrações de própolis vermelha com relevante ação bacteriostática, visto a capacidade de inibir a proliferação dos micro-organismos avaliados.
- As bactérias Gram-positivas (*S. aureus* e *B. subtilis*) foram mais sensíveis que as bactérias Gram-negativas (*E. coli* e *P. aeruginosa*) após exposição ao extrato bruto (EHP1), frações e subfrações da própolis vermelha.
- *B. subtilis* foi mais sensível que *S. aureus*, quando submetido à ação das frações e subfrações.
- *E. coli* apresentou-se mais sensível as frações e *P. aeruginosa* mais sensível as subfrações.
- O extrato bruto (EHP1) apresentou maior atividade contra *S. aureus*.
- As frações que demonstraram melhor atividade frente *S. aureus* foram: FCHex, F4 e F11.
- As frações F4, F11, F12 e F17 foram as mais ativas contra a bactéria *B. subtilis.*
- As frações F12 e F17 foram as mais ativas frente *E. coli*.
- As frações FBut, F6, F13, F14 e F18 apresentaram a maior atividade contra *P. aeruginosa.*
- A subfração SC2 foi a que demonstrou melhor atividade frente todas as bactérias testadas.
- O fracionamento reduziu significativamente a CIM de algumas subfrações quando comparadas as suas frações precursoras, e isso pode estar relacionado ao possível isolamento de compostos com atividade antibacteriana.
- O extrato bruto (EHP1) e as subfrações (SC1, SC2, SH7 e SH8) exibiram efeito

citotóxico frente à linhagem celular não tumoral Vero, evidenciando a importância de se utilizar concentrações inferiores as CIM, a fim de garantir o uso seguro e não citotóxico da própolis vermelha.

- As análises do extrato bruto (EHP1) e das subfrações (SC1, SC2, SH7 e SH8) por EMAR, CLAE-UV e CG-EM identificaram uma série de compostos com atividades biológicas interessantes, em especial a antibacteriana e, dentre eles, estão alguns marcadores químicos essenciais da própolis vermelha, como a Formononetina, Liquiritigenina e Biochanina A.

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7. ANEXOS

ANEXO 1 - Seleção da técnica de extração.

Técnica	Procedimento	Amostra	Solvente	Rendimento (%)
Maceração/ sonicação (Celeghini *et al.*, 2001)	- 1 g/10 mL (1:10) - Ultrassom - 20 min. - Temperatura ambiente - Filtração e evaporação à temperatura ambiente	Própolis Garibaldi	Etanol 70 %	32,74
Maceração com agitação (Frozza *et al.*, 2013)	- 1 g/10 mL (1:10) - Maceração com agitação constante - 24 h (direto) - Temperatura ambiente - Filtração e evaporação à temperatura ambiente	Própolis Garibaldi	Etanol 70 %	39,39
Maceração com agitação (renovação de solvente)	- 50 g/150 mL (1:3) - 1ª extração (mais 3 extrações de 150 mL, utilizando os 50 g iniciais) - Maceração com agitação constante - 6 h cada extração (Total: 24 h) - Temperatura ambiente - Filtração e evaporação em rotaevaporador (35 °C)	Própolis Garibaldi	Etanol 70 %	1ª extração: 36,25 2ª extração: 11,73 3ª extração: 4,55 4ª extração: 1,88 Total: 54,41

- **Maceração com agitação (renovação de solvente):** escolhida como melhor técnica, considerando-se o rendimento total obtido e também devido a menor quantidade de solvente utilizada.

ANEXO 2 - Seleção do solvente de extração.

- Amostra: Própolis da cidade de Garibaldi, RS, Brasil.

- Solventes: água, etanol 20 %, etanol 50 %, etanol 70 %, etanol 80 %, etanol PA, acetato de etila, clorofórmio e hexano.

- Técnica: maceração/sonicação (ultrassom), 20 minutos, temperatura ambiente, evaporação à temperatura ambiente.

Rendimento dos extratos

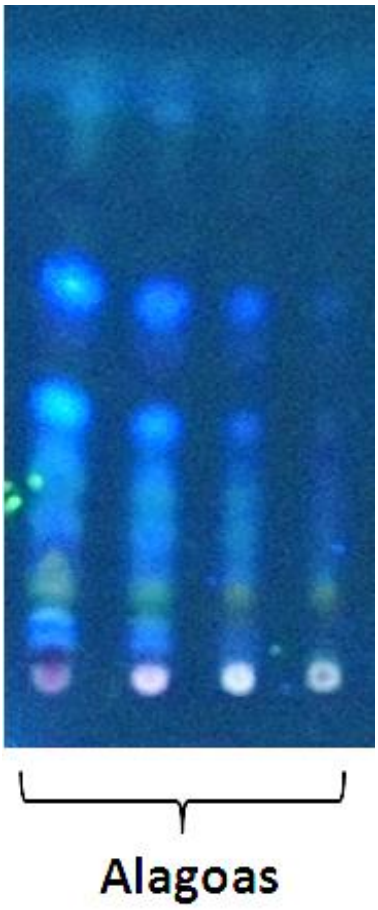
Solvente	Rendimento (%)
Água	5,63
Etanol 20 %	15,12
Etanol 50 %	10,99
Etanol 70 %	32,74
Etanol 80 %	29,05
Etanol PA	27,52
Acetato de etila	45,46
Clorofórmio	47,69
Hexano	13,52

- O solvente Etanol 70 % destaca-se por proporcionar um dos maiores rendimentos e ser menos tóxico (entre aqueles que também apresentaram bons rendimentos). Logo, foi escolhido para a obtenção dos extratos de própolis.

ANEXO 3 - Rendimentos das extrações realizadas com a Própolis Vermelha de Alagoas, Brasil.

Própolis	Extrato (g)	Rendimento (%)
ALAGOAS		
1ª extração	21,47	42,93
2ª extração	6,39	12,78
3ª extração	3,37	6,74
4ª extração	1,43	2,85
Total	**32,66**	**65,30**

ANEXO 4 - Cromatografia em camada delgada (CCD) - extrações realizadas com a Própolis Vermelha de Alagoas, Brasil.



Fase móvel: 3% metanol em clorofórmio
Fase estacionária: Sílica gel 60 F_{254}
Revelador: UV 365nm

- Da esquerda para a direita: 1ª a 4ª extração, com etanol 70 % (renovação de solvente a cada 6 h).

ANEXO 5 - Parâmetros das curvas de calibração (CLAE-UV).

Composto	**Coeficiente (R^2)**	**Níveis de concentração ($\mu g.mL^{-1}$)**	**Equação da reta**
Liquiritigenina	0,9997	1-62	Y = 33,26x + 6,34
Formononetina	0,9998	0,1-250	Y = 39,46x - 34,08
Biochanina A	0,9973	1-31	Y = 46,49x - 56,88

ANEXO 6 - Artigo: Antitumor activity of Brazilian red propolis fractions against Hep-2 cancer cell line.

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

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

Abstract

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

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

1. Introduction

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

Propolis is a natural resinous product made by melliferous bees, used in traditional medicine all over the world. This resin is known to have chemical composition, which presents biological properties such as anti-inflammatory, antifungal, antibacterial, antioxidative and anticancer (Kamiya *et al.*, 2012a). Brazilian red propolis was found at mangrove regions of northeastern Brazilian states, considered the 13th type, which the major botanical source is *Dalbergia ecastophyllum* (Daugsch *et al.*, 2007). The propolis chemical composition has been characterized and varies according to its source (Machado *et al.*, 2016). With the improvement of purification process and instrumental analysis, most compounds can be further qualified and quantified by high-performance liquid chromatography (HPLC) (de Mendonca *et al.*, 2015), gas chromatography–mass spectrometry (GC-MS) (Alencar *et al.*, 2007), liquid chromatography-nana electrospray ionization quadrupole time-of-flight mass spectrometry (LC-nana ESI-Q-TOF-MS) (Sun *et al.*, 2016) and nuclear magnetic resonance (NMR) (Lotti *et al.*, 2010).

However, few studies have identified and characterized chemical composition and activity from red propolis enriched fraction (de Mendonca *et al.*, 2015; Novak *et al.*, 2014), which is considering the first step toward discovery unknown bioactive compounds (Hayacibara *et al.*, 2005).

In this study we identified fraction enriched in biochanin, formononetin and liquiritigenin, which are promising agents against cancer models.

Various studies have shown the anticancer properties of Brazilian red propolis and its compounds, which are effective in reducing cancer cell survival by affecting various growth-promoting and apoptotic pathways (Begnini *et al.*, 2014; de Mendonca *et al.*, 2015). This work aims the understanding the antitumor benefits of red propolis, beyond the identification, and characterization of fractions are mandatory, and mechanism of action need to be determined. Therefore the aim of this present study was to analyze the chemical profile of enriched fractions of hydroalcoholic Brazilian red propolis (HBRP) extract, and to demonstrate its antiproliferative effect on laryngeal cancer cells. In addition, some parameters related to apoptosis as ROS accumulation, mitochondrial membrane potential, will help to elucidate cell signaling involved in propolis mechanism of action.

2. Materials and methods

2.1 Chemicals

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

2.2 Red propolis sample

Red propolis was collected in 2013 in state of Alagoas located in the northeast of

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

2.3 Fractionation

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

2.4 Chemical characterization by ESI – MS/MS and quantification

The dry samples (J and L fractions and HBRP) were dissolved in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid for ESI(+). The solutions were infused individually into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 10μL/min. ESI(+)-MS were acquired using a hybrid high-resolution and high accuracy (5μL/L) microTOF-QII

mass spectrometer (Bruker® Daltonics) under the following conditions: capillary and cone voltages were set to +3500 eV and +40 eV, respectively, with a de-solvation temperature of 100 °C. Diagnostic ions were identified by the comparison of exact *m/z* with compounds determined in previous studies (Table 1). Hystar software (Bruker® Daltonics) was used for data acquisition and processing. The data were collected in the *m/z* range of 70–800 at the speed of two scans per second, providing the resolution of 50.000 (FWHM) at *m/z* 200. No important ions were observed below *m/z* 150 or above *m/z* 800.

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

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

2.5 Cell culture

Hep-2 (human laryngeal epidermoid carcinoma cell) was obtained by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM supplemented with 10% (w/v) heat-inactivated FBS and 1% (w/v) penicillin–streptomycin.

The cultures were maintained in a humidified atmosphere at 37 ºC, 5% CO_2 and 95% air. The study was performed as cells reached 70–80% confluence.

2.6 Cytotoxic assay

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

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

2.7 Morphological examination of cancer cells

Cells were seeded into 24-well plates containing coverslips. After 24 h incubation, the cells were treated with red propolis hydroalcoholic extract and fractions at 50 and 75 µg/mL for 24 h. The negative control group was treated with EtOH–H_2O 2.5% (v/v) instead

of extract for the same period. Then they were stained with Giemsa (Accustain®– Sigma–Aldrich), according to standard Giemsa procedure established by the supplier. Morphological changes of the cells were observed and photographed under a light microscope at 200 x (Olympus®).

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

2.8 Flow Cytometry

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

2.8.1 Annexin-V/PI Assay

The procedure was conducted according to methodology provided in Annexin V-FITC Apoptosis Detection Kit (catalog number APOAF, Sigma, USA). All adhering and

floating cells were harvested and washed twice with PBS before being transferred into sterile centrifuge tube. The cell pellet was then suspended in 100 µL of binding buffer (10 mM HEPES/NaOH pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$). This cell suspension was transferred to a microtube, to which 5 µL of Annexin V-FITC conjugate and 10 µL of propidium iodide were added. The cells were incubated in the dark for 15 minutes at room temperature. The fluorescence of the cells was determined by flow cytometry using FL1 (488/533) and FL3 (488/670) filters.

2.8.2 Mitochondrial Membrane Potential Analysis

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

2.8.3 ROS Analysis

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

2.8.4 Cell Cycle Analysis

Hep-2 cells were cultured with or without red propolis or vehicle (ethanol 2.5%) for 24 h. Following treatment, cells were washed in PBS, trypsinized and collected for centrifugation (1800 rpm for 7 min) in PBS. Cell pellets were gently resuspended, fixed in ice-cold 70% ethanol and stored overnight at −20 °C. Prior to analysis, cells were again collected by centrifugation to remove ethanol, gently resuspended in PBS and re-pelleted.

Cells were then resuspended and permeabilized in Triton X-100 in PBS and incubated with RNAse A and propidium iodide in the dark for 30 min at room temperature. All cell samples were immediately analyzed by flow cytometry for DNA content.

2.9 DNA cleavage assay

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

2.10 Statistical analysis

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

3. Results and Discussion

3.1 Chemical characterization and quantification

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

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

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

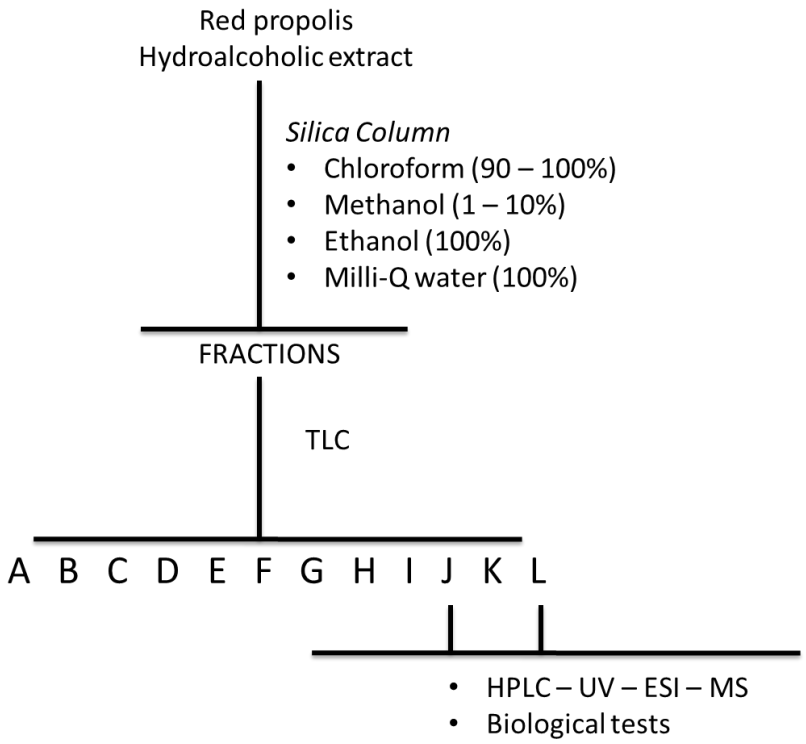


Figure 1. Workflow of fractionation by solvents and column chromatography. 12 fractions (A – L) were collected out of 201 samples, which were grouped according to chemical similarities.

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

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

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

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

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

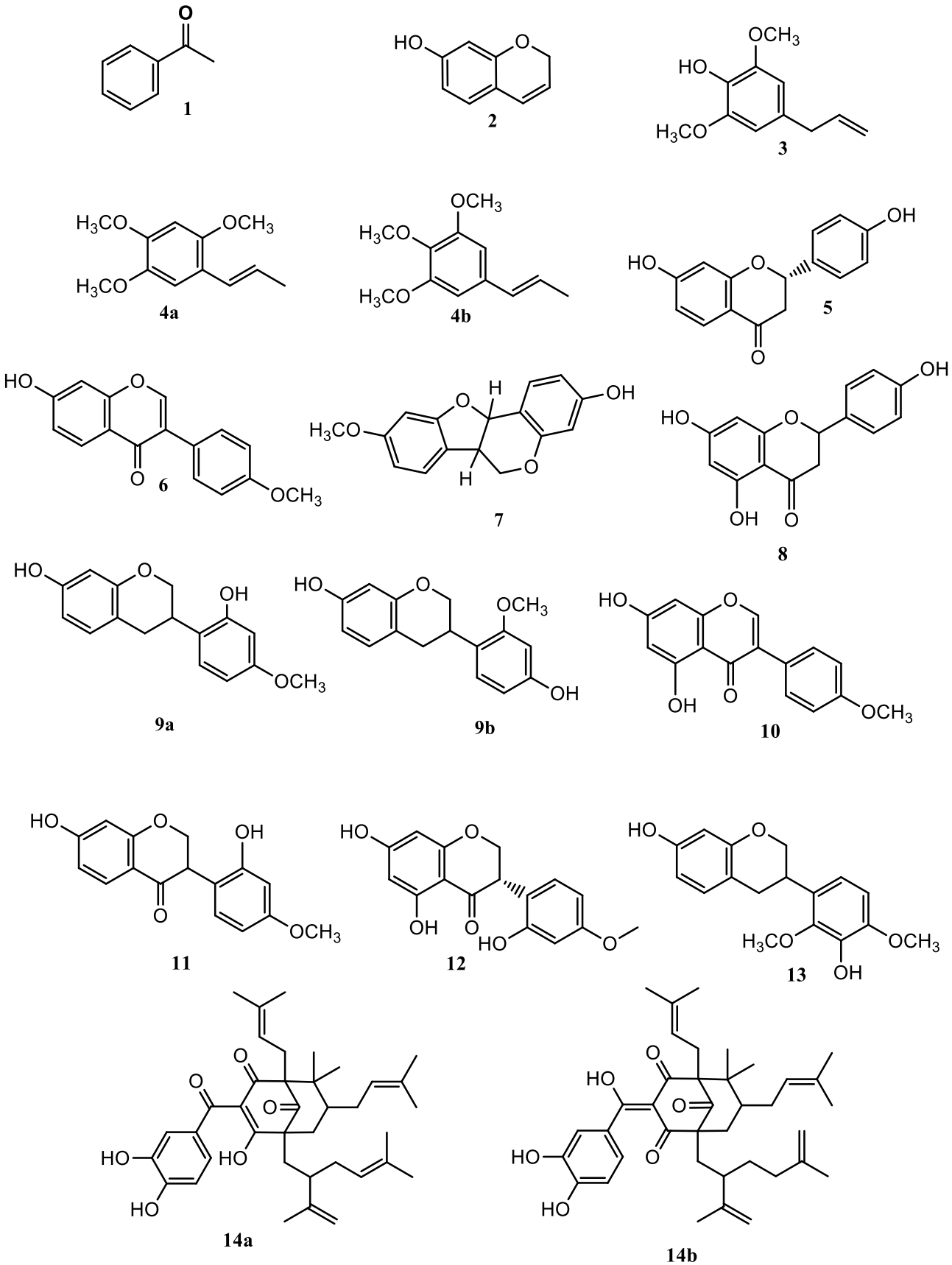


Figure 2. Structures of compounds 1-14 detected in red propolis sample (HBRP) and fractions.

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

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

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

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

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

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

n.d.; not detected

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

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

n.d.; not determinated

3.2 Viability and morphological changes

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

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

culture that received J and L fraction (50 µg/mL) treatment (Figure 4 B and D). The cells that received higher concentrations (75 µg/mL) (Figure 4 C and E) exhibited decreased growth, loss of volume, cytoplasmic shrinkage, nuclear condensation and deformation to a round appearance indicating several biological changes upon extract cytotoxic activity. To confirm the condensation and fragmentation of nuclear DNA was performed a more specific assay, DAPI. In the control group, the nuclei were roundish and homogeneously stained with DAPI (Figure 5A). In contrast, the nuclei in the treated Hep-2 cells were irregularly shaped, small, detached, presenting condensed chromatin and had apoptotic bodies that are typical of the early and late stages of apoptosis (Figure 5B). The condensation and fragmentation of nuclear DNA were not eminent in the HBRP once the concentrations used were below the IC50 for 24 hours of treatment.

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

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

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

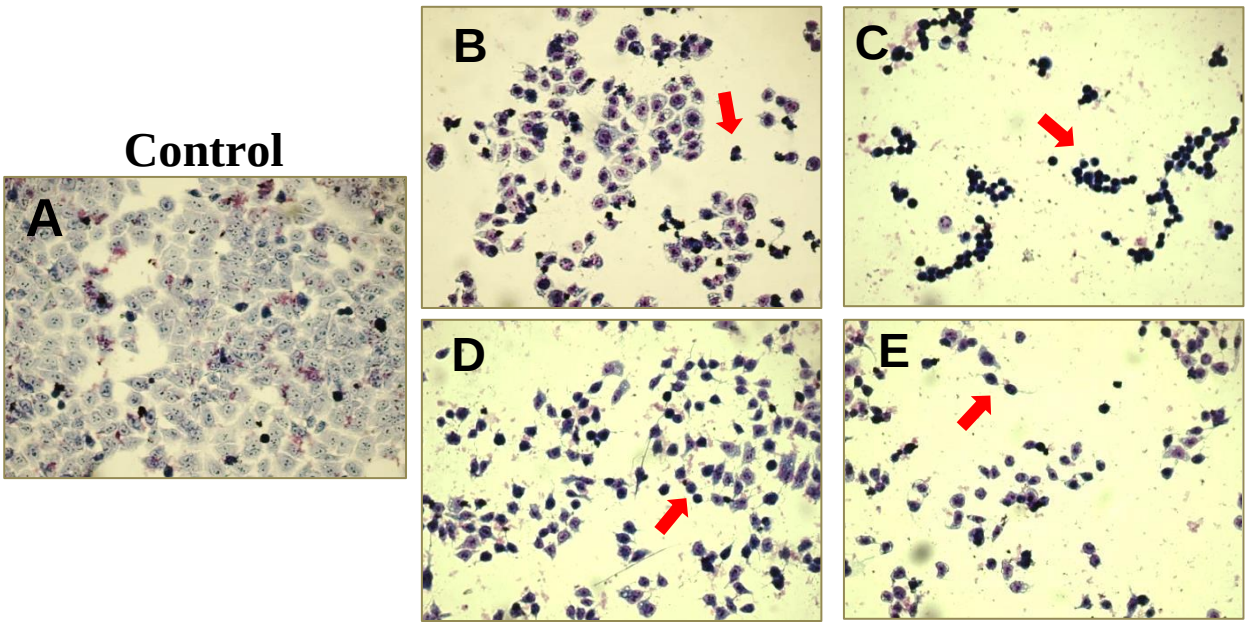


Figure 4. Morphological structure of Hep-2 cancer cells through Giemsa staining after 24 h of treatment. (A) control, (B) J fraction (50 µg/mL), (C) J fraction (75 µg/mL), (D) L fraction (50 µg/mL) and (E) L fraction (75 µg/mL). Arrows indicate nuclear condensation.

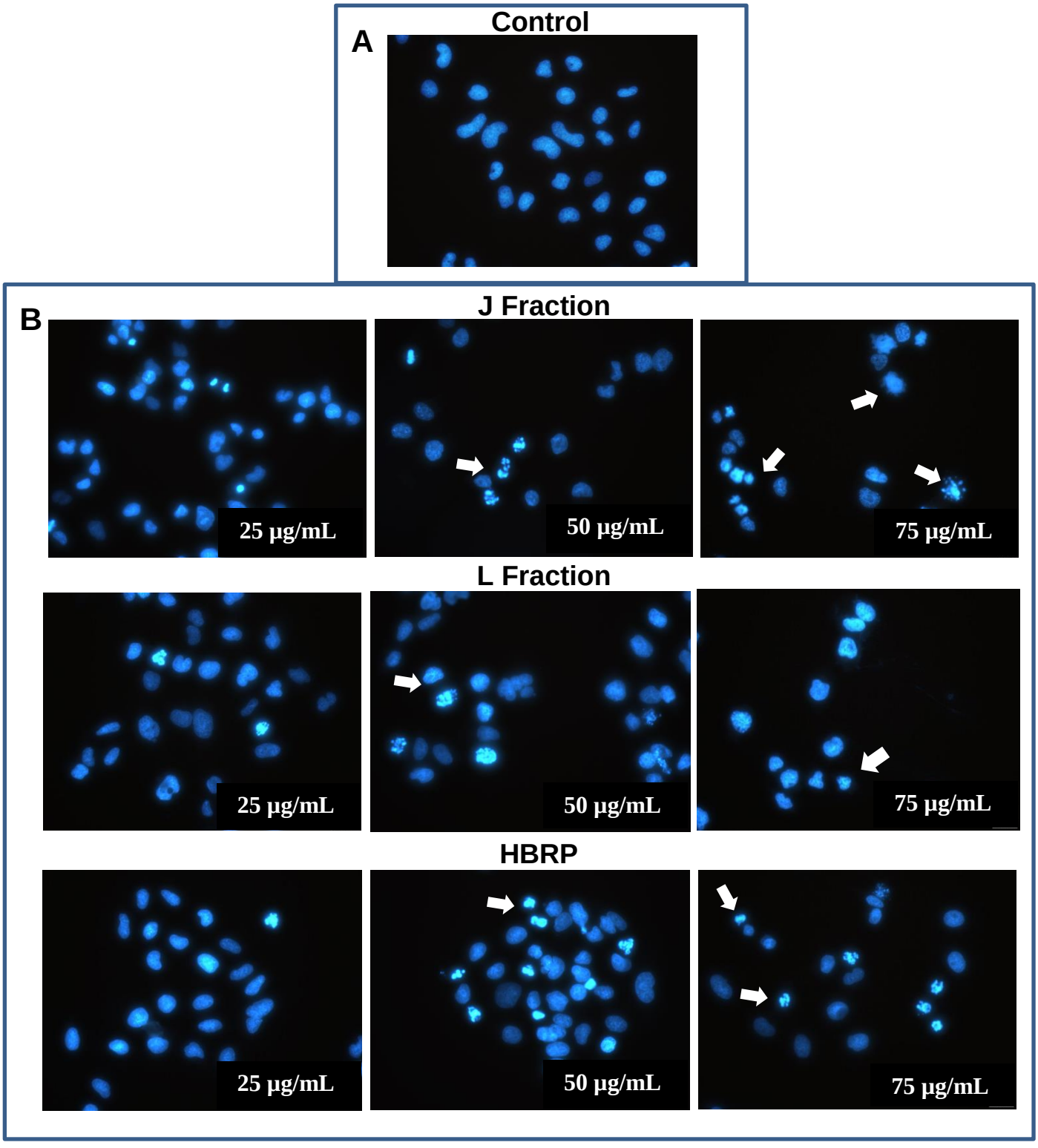


Figure 5. Nuclear morphological changes in Hep-2 cells after treatment with red propolis samples for 24 hours followed by DAPI staining. (A) Control cells treated with media with 2.5% ethanol; (B) Treated groups with 25, 50 and 75 µg/mL of J and L

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

3.3 Flow Cytometry

3.3.1 Annexin/PI

Cells were exposed to different concentrations of red propolis fractions revealed that the percentages of late apoptosis cells were gradually increased (Figure 6). According to our previous studies with samples from Sergipe (Frozza *et al.*, 2014), red propolis extract induced apoptosis in Hep-2 cancer cells in a dose-dependent manner and the signaling to late apoptosis/necrosis increased in a significant way in the cells treated with higher concentrations comparing with control group. Begnini *et al*,. (2014) also presented that red propolis extract induced in 5637 cells a higher percentage of late apoptosis at 100 μg/mL concentration compared to the control. Note that most cells exposed to enriched fractions presented a late stage of apoptosis phenotype compared to the crude extract (HBRP).

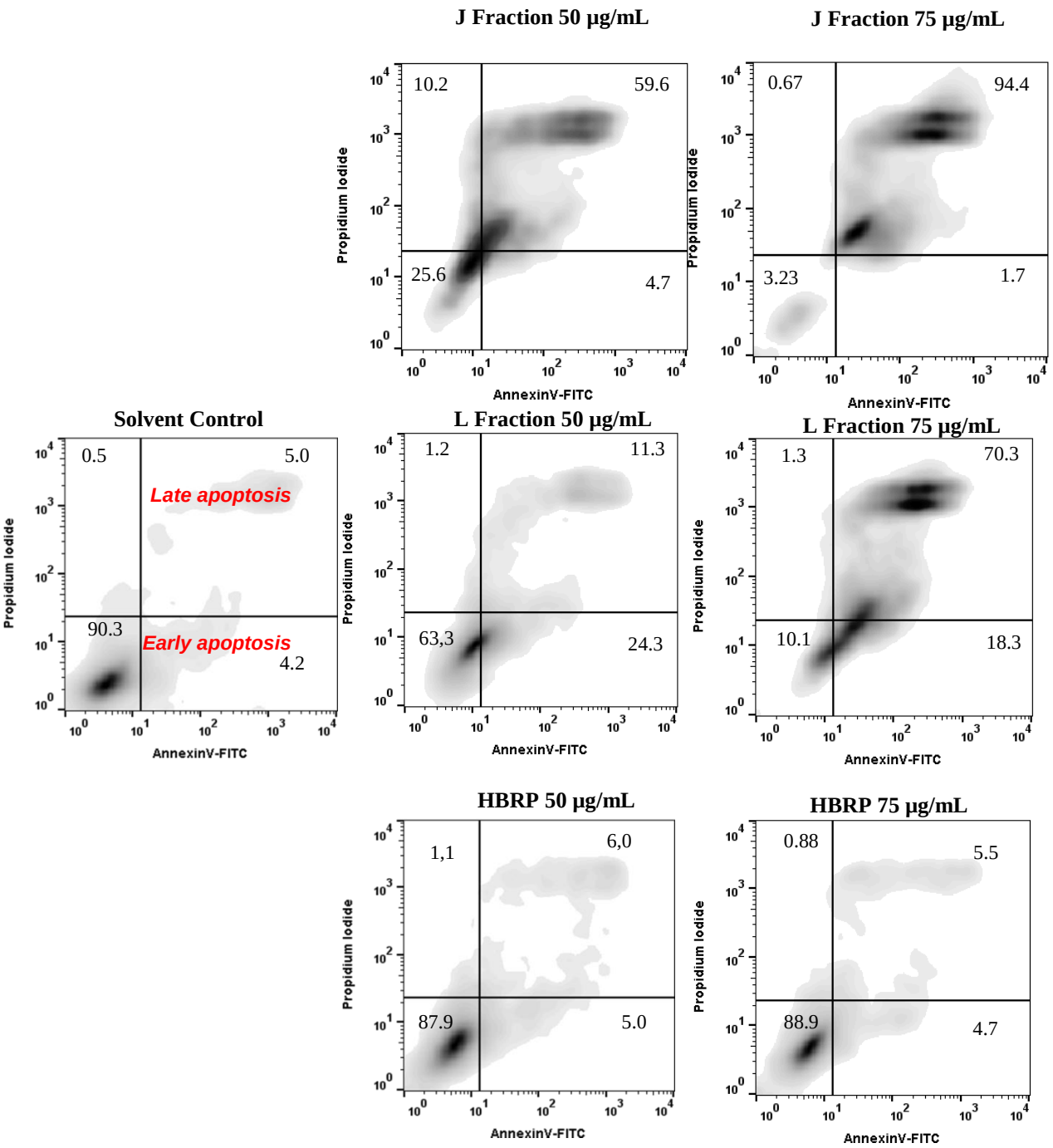


Figure 6. Representative data after 24 h red propolis treatment incubation in Hep-2 cell line. The percentage of Annexin V-positive and PI-negative cells in early stages of apoptosis (bottom right) and Annexin V-positive and PI positive cells that were dead or in late-stages of apoptosis (top right),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

3.3.2 Mitochondrial Membrane Potential and ROS measurement

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

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

Mitochondrial Membrane Potential

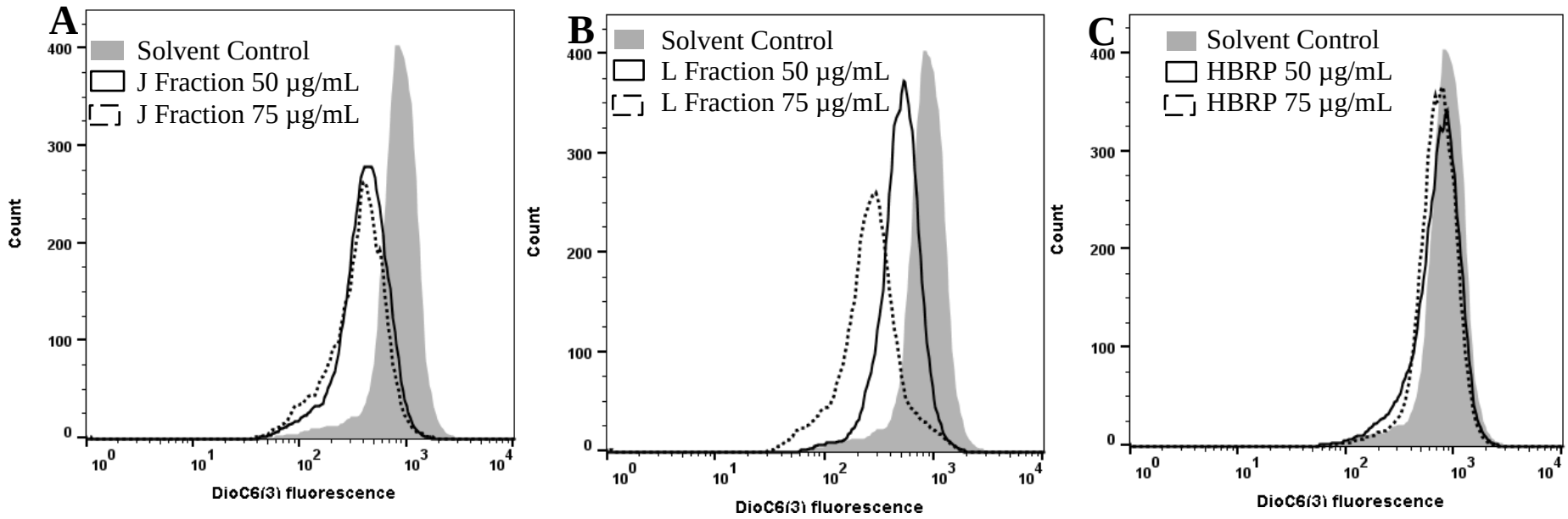


Figure 7. Effects of red propolis samples in MMP determined by fluorescence intensity. A, B – Red propolis fractions (J and L) and C – Crude extract (HBRP).

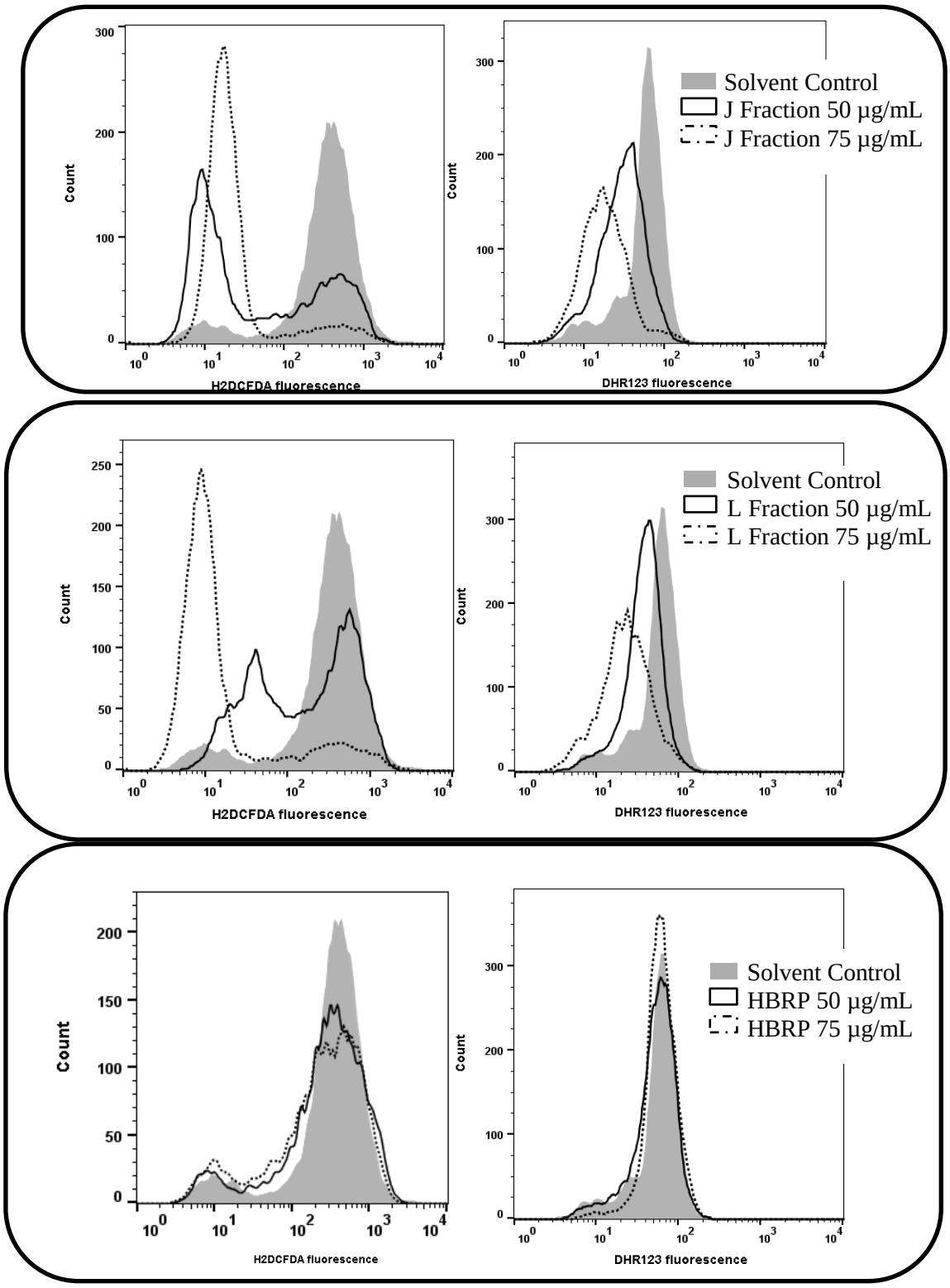
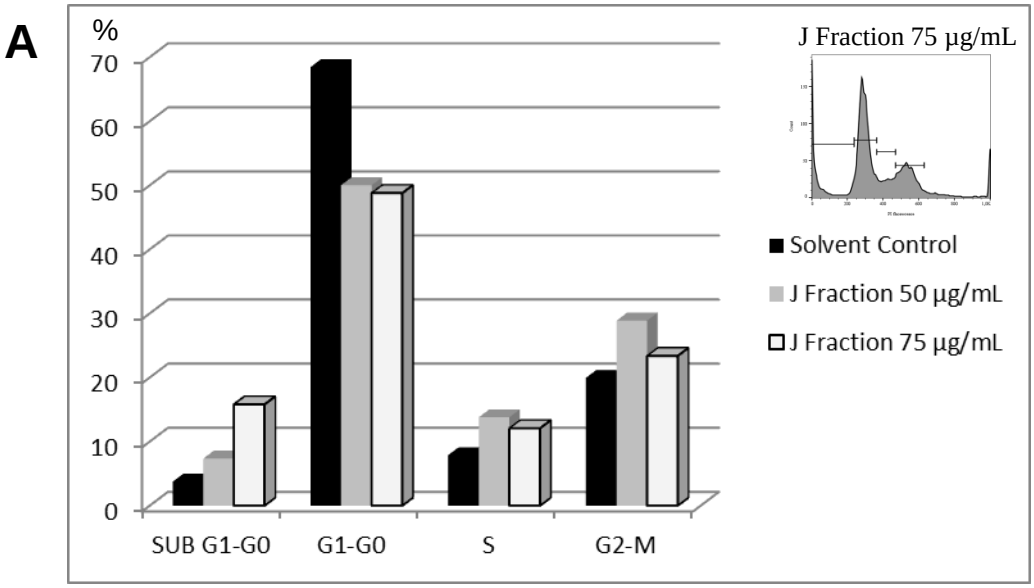


Figure 8. Effects of red propolis samples on intracellular ROS which were determined by fluorescence intensity. A, B – red propolis fractions (J and L) and C – crude extract (HBRP).

3.3.3 Cell Cycle Analysis

To determine whether the cytotoxic effect of the samples on Hep-2 cells was associated with an induction of cell cycle arrest, the distribution of cells in various phases of the cycle was analyzed using flow cytometry. The treated groups showed an accumulation of cells in SUB G1-G0, S and G2-M phase (Figure 9). This increase in SUB G1-G0 phase can be considered as reflecting the induction of cell death. It has been generally accepted that the increase of cells having SUB G1-G0 DNA content is a marker of apoptotic cell death (Mojžišová et al 2016) (Mojzisova *et al.*, 2016). In cell treatments a gradual decrease in the G1-G0 phase was observed. Novak *et al.,* (2014) demonstrated that treated B16F10 cells with a fraction (IV) of Brazilian red propolis for 48 h revealed a significant increase of DNA fragmented (SUB G1-G0 phase) compared to control group.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G1-G0
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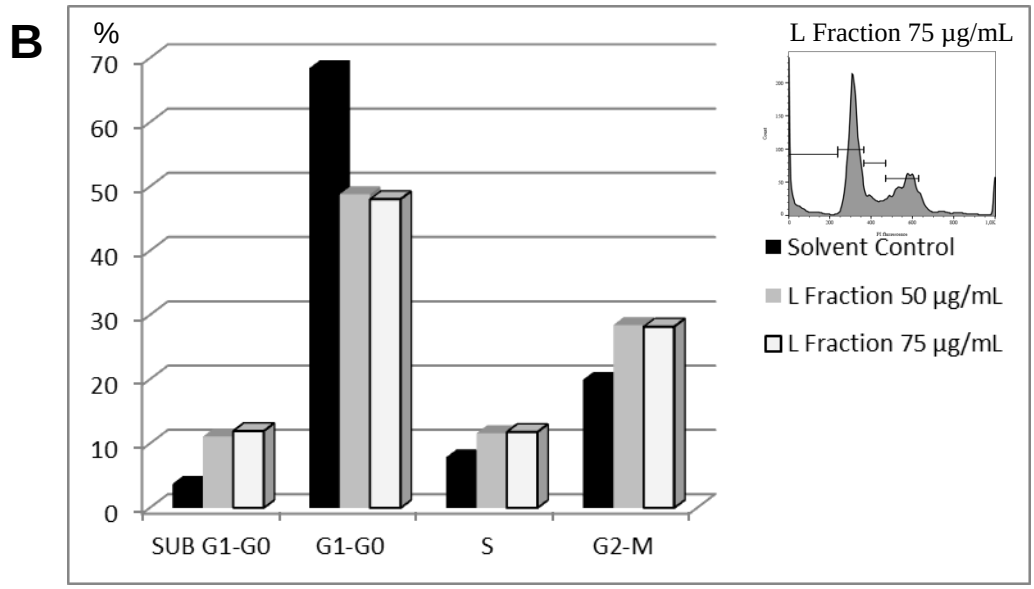
J Fraction 75 µg/mL
Solvent Control
J Fraction 50 µg/mL
J Fraction 75 µg/mL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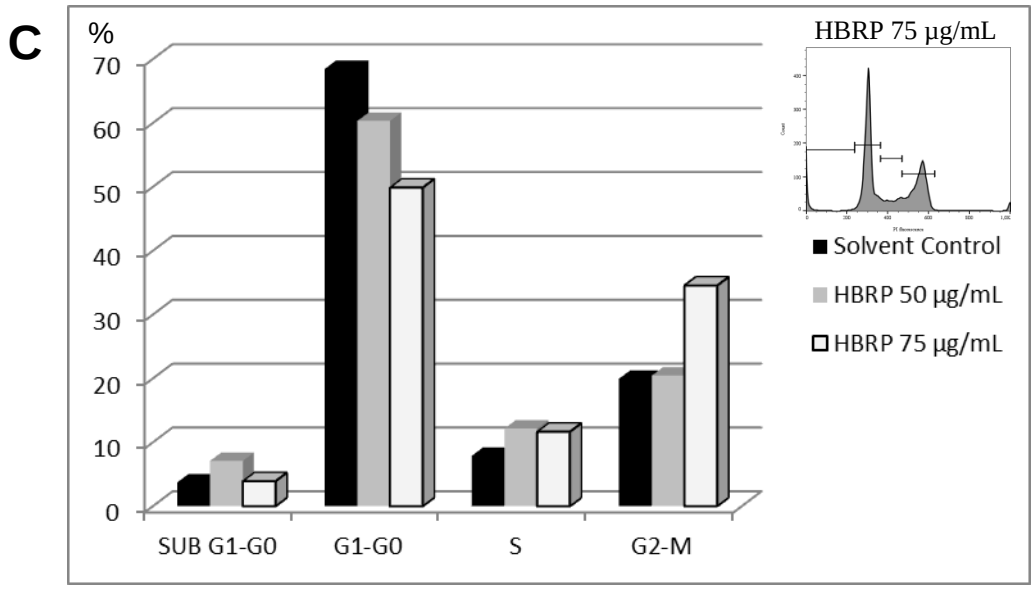
L Fraction 75 µg/mL
Solvent Control
L Fraction 50 µg/mL
L Fraction 75 µg/mL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G2-M
HBRP 75 µg/mL
Solvent Control
HBRP 50 µg/mL
HBRP 75 µg/mL

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

3.1 DNA cleavage assay

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

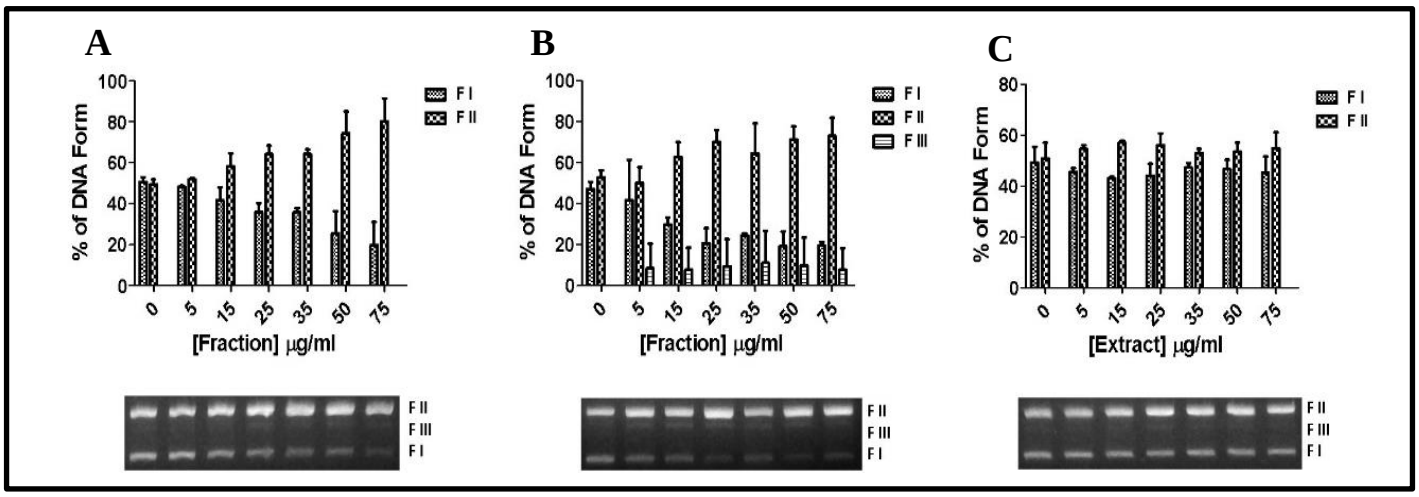

Figure 10. Graphics and photos showing the cleavage activity of J fraction (A), L fraction (B) and of crude extract (C) on plasmid DNA. It can be observed that the activity is higher in the fractions than in the crude extract and the fraction L present the higher activity of all.

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

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

4. Conclusion

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

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

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