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MARCOS DA SILVEIRA REGUEIRA NETO

ANÁLISE COMPARATIVA DOS PERFIS FITOQUÍMICO E MICROBIOLÓGICO DA PRÓPOLIS VERMELHA BRASILEIRA E DA RESINA DE *Dalbergia ecastaphyllum*

Recife
2018

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Biociências, Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de doutor em Ciências Biológicas.

Área de concentração: Biotecnologia

Orientador: Prof. Dr. Valdir de Queiroz Balbino
Coorientador: Prof. Dr. Henrique Douglas Melo Coutinho

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Aprovada em: 28/02/2018

BANCA EXAMINADORA

_____

Prof. Dr. Valdir de Queiroz Balbino (Dep. de Genética/UFPE)

_____

Prof. Dra. Cláudia Sampaio de Andrade Lima (Dep. de Biofísica/UFPE)

_____

Prof. Dr. Antônio Fernando Morais de Oliveira (Dep. de Botânica/UFPE)

_____

Prof. Dra Neide Santos (Dep. de Genética/UFPE)

_____

Prof. Dr. Marcos Antônio de Morais Júnior (Dep. de Genética/UFPE)

A minha mãe, Roseane,
fonte inesgotável de
carinho e ensinamentos,
com amor dedico.

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RESUMO

No Brasil, a própolis é classificada em 13 tipos de acordo com a sua coloração e características fitoquímicas. A própolis vermelha pode ser encontrada em alguns estados do Nordeste. Em sua maioria é composta por flavonoides os quais são atribuidas muitas atividades biológicas. O objetivo deste estudo é comprovar a fonte botânica de resina para a produção de própolis vermelha e a analisar o perfil químico da resina e da própolis, comparar os resultados obtidos e realizar testes de atividade antibacteriana, antileishmania, antitripanossoma e citotóxica. A análise no HPLC da própolis vermelha e da resina permitiu identificar nove compostos: apigenina, ácido cafeico, ácido clorogênico, ácido elégico, luteolina, ácido p-coumárico, quercetina, rutina e vitexina. Uma variação sazonal foi observada no que diz respeito à concentração dos compostos para a própolis vermelha. Os extratos hidroetanólicos da própolis e da resina de *D. ecastaphyllum* apresentaram atividade contra as linhagens bacterianas de *Pseudomonas aeruginosa*, *Escherichia coli* e *Staphylococcus areus*. Duas amostra de própolis vermelha e a amostra de resina apresentaram efeito sinergico quando combinadas com as drogas antibacterianas. Os extratos de propolis vermelha de PE mostraram varação sazonal contra *E. coli* e *S. Aureus,* entretanto não foi observada variação contra *P. Aeruginosa*. Todas as amostras de propolis vermelha e resina apresentaram atividade contra as espécies de *Leishmania* e *T. cruzi* incluídas nesse estudo. De acordo com os nossos resultados, todas as amostras apresentatam atividade antibacteriana e a utilização da própolis vermelha coletada no periodo seco combinada com drogas antimicrobianas pode possivelmente trazer melhores resultados contra infecções multirresistentes. Quando comparada a atividade da própolis vermelha com a resina, observamos uma maior eficiência dos extratos de própolis em relação à resina.

Palavras-chave: Bactéria. *Dalbergia ecastaphyllum*. *Leishmania*. Própolis vermelha. Resina. *Trypanosoma*

ABSTRACT

In Brazil, the propolis is classified in 13 types according to its color and phytochemical characteristics. The 13th type, the red colored propolis, is found in some states of the Northeast of Brazil. It is composed in its majority by flavonoids and many biological activities have been reported. The aim of this study is to verify the botanical source for the production of red propolis and analyses the propolis and resin chemical profile, compare the obtained results and perform antibacterial, antileishmanial, antitrypnosomal and cytotoxic activity tests. The HPLC analysis of the Brazilian red propolis allowed us to identify nine compounds: apigenin, caffeic acid, chlorogenic acid, ellagic acid, luteolin, p-coumaric acid, quercetin, rutin and vitexin. Seasonal variation as observed according to concentrations of the compounds. The hydroethanolic extracts of propolis and the resin of *D. ecastaphyllum* showed bioactivity against the bacterial strains of *Pseudomonas aeruginosa*, *Escherichia coli* e *Staphylococcus areus*. Two red propolis samples and the resin sample presented synergistic effect when combined with standard drugs. The red propolis extracts from Pernambuco showed seasonal variation against *E. coli* and *S. aureus*, however it was not observed any variation against *P. aeruginosa*. All red propolis and resin samples showed activity against the *Leishmania* species and *Trypanosoma cruz*i parasites. According to our results, all samples presented antibacterial activity and the use of red propolis collected in the dry season combined to antibacterial drugs might bring better results in the treatment of multiresistant infections. When the activity of red propolis was compared to the plant resin, we observed a higher efficiency of red propolis extracts over the resin.

Key-words: Bacteria. *Dalbergia ecastaphyllum*. *Leishmania*. Red propolis. Resina. *Trypanosoma.*

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LISTA DE ABREVIATURAS E SIGLAS

A	Antibióticos
AE.	Antiepimastigota
AP.	Antitripomastigota
BHI	*Brain-Heart Infusion*
C.	Indicador de citotoxicidade
CL.	*Cutaneous Leishmaniasis*
CLSI	*Clinical and Laboratory Standards Institute*
CPRG	Ensaio do Clorofenol vermelho-β-D-galactopiranosídeo
DER	*Dalbergia ecastaphyllum* resin
DMSO	Dimetilsulfóxido
DNA	Ácido Desoxiribonucleico
EC ATCC	*Escherichia coli* American Type Culture Collection
EC-06	*Escherichia coli* 06
FBS	*Fetal Bovine Serum*
HPLC DAD.	*High Performance Liquid Chromatography Diode-Array Detection*
IC_{50}	*Half Maximal Inhibitory Concentration*
LacZ	Gene β-galactosidase de *E. coli*
LC	Leishmaniose Cutânea
LM	Leishmaniose Mucocutânea
LOD	*Limit of Detection*
LOQ	*Limit of Quantification*
LV	Leishmaniose Visceral
MIC	*Minimal Inhibitory Concentration*
ML	*Mucocutaneous Leishmaniasis*

MLC	*Minimal Lethal Concentration*
PA03	*Pseudomonas aeruginosa* 03
PA24	*Pseudomonas aeruginosa* 24
RP	*Red Propolis*
RP-AL	*Red Propolis collected in Alagoas*
RP-PED	*Red Propolis from Pernambuco Collected During the Dry Season*
RP-PER	*Red propolis from Pernambuco Collected During the Rainy Season*
SA ATCC	*Staphylococcus aureus American Type Culture Collection*
SA 10	*Staphylococcus aureus* 10
SD	*Standard Deviation*
SNP	*Single Nucleotide Polimorphism*
VL	*Visceral Leishmaniasis*

SUMÁRIO

1 INTRODUÇÃO

Própolis é um produto obtido de uma substância resinosa que é coletada de exsudatos de brotos e árvores, botões florais e pólen de diversas espécies de plantas pelas abelhas do mel (*Apis mellifera*). Essa resina é mastigada, enzimas salivares são adicionadas e o material parcialmente digerido é misturado à cera de abelha e usada na colmeia. As abelhas usam a própolis para reparar os favos de mel, para fechar pequenas frestas, suavizar as paredes internas, embalsamar as carcaças de invasores que as abelhas mataram dentro da colmeia, mas que elas não puderam transportar para fora, bem como proteger a colmeia da invasão de micro-organismos.

Os materiais disponíveis para as abelhas para a produção da própolis são substâncias ativamente secretadas pelas árvores assim como substâncias exsudadas pelas feridas das plantas: materiais lipofílicos nas folhas e brotos de folha, resinas, mucilagem ou goma. A partir da composição da fonte é possível determinar de qual planta (origem botânica) foi produzida a própolis. No Brasil existem ao menos quatro fontes distintas para a produção de própolis: *Baccharis dracunculifolia* (Asteraceae), mais conhecida como alecrim-do-campo, *Dalbergia ecastaphyllum*, popularmento conhecida como rabo-de-bugio (Fabaceae), *Hyptis divaricata* (Lamiaceae) e *Populus alba* (Salicaceae).

A própolis brasileira foi classificada quanto à composição química, origem geográfica e planta fonte em 12 grupos: seis no Sudeste (amarelo, marrom, marrom escuro, marrom esverdeado e verde) e seis no Nordeste (marrom avermelhado, marrom esverdeado, marrom escuro, amarelo, amarelo escuro). Recentemente foi descoberto a própolis vermelha em colmeias localizadas ao longo do litoral e margens de rio do Nordeste, mas precisamente em Alagoas. Na própolis vermelha já foram identificados: flavonoides (isoflavonoides, neoflavonoides, pterocarpanos, isoflavanonol, isoflavanonas, chalconas, isoflavanas, isoflavonas, flavanonas e favonol) além de componentes pertencentes a outros grupos químicos como, benzofenonas preniladas, terpenos e taninos. Porém, a maioria desses estudos estão concentrados na análise da própolis vermelha de Alagoas.

A própolis tem sido usada pela humanidade como produto medicinal desde antes de cristo. Usos como: antisséptico, antioxidante, anti-inflamatório, adesivo, protetor de madeira foram alguns dos usos reportados pelos homens na antiguidade.

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Os egípcios faziam amplo uso da própolis nas múmias como produto antiputrefativo e na idade média era usado como antisséptico e cicatrizante, e os Incas ainda usavam própolis como antipirético. Diante de tantas possibilidades de uso, o interesse global nas pesquisas com própolis é grande e apresentam duas justificativas: a primeira delas é que a própolis apresenta diversas propriedades biológicas e alto valor agregado, onde no Brasil o valor de 30 mL do extrato de própolis vermleha esta em torno de 50 reais.

Com os avanços tecnológicos foi possível aprimorar técnicas para análise do perfil químico dessas substâncias assim como das resinas das quais elas são oriundas. Já foram encontradas mais de 300 substâncias químicas com atividades biológicas, como por exemplo: atividades antitumorais, anti-HIV, antimicrobiana, antifúngica, hepatoprotetora, antiviral, anestésico local, antimofo, além de aplicações na dentítisca. Com a descoberta recente de um novo tipo de própolis, a vermelha, alguns estudos para saber qual a origem botânica, perfil químico e atividades biológicas dos seus componentes foram realizados. Porém, a maioria dos trabalhos focam apenas na própolis vermelha oriunda da resina de *Dalbergia ecastaphyllum*, originaria de Alagoas. Porém, amostras de própolis de outras localidades do Nordeste brasileiro, assim como a avaliação das atividades da resina vegetal de *D. ecastaphyllum* estão subrepresentadas nos estudos científios. Levando em conta o mencionado anteriormente e destacando a importância de se estudar própolis de diferentes regiões por conta da influência dos fatores edafoclimáticos, no presente trabalho realizamos uma avaliação da atividade antiacteriana, antileishmania, antitrypanosoma e cytotoxica de amostras de própolis vermelha de Pernambuco, coletadas em duas estações diferentes (chuvosa e seca), da própolis vermelha de Alagoas e da resina vegetal de *Dalbergia ecastaphyllum*. Além disso, também realizamos uma análise fitoquímica das amostras previamente citadas.

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1.1 OBJETIVOS

1.1.1 Objetivo geral

Analisar o perfil químico da própolis vermelha e da resina, comparar os resultados obtidos e realizar testes de atividade antimicrobiana, antileishmania, antitripanossma e citotóxica.

1.1.2 Objetivos específicos

a) Realizar uma análise comparativa do perfil químico dos extratos de amostras coletadas periodicamente de própolis vermelha de diferentes colônias de abelhas do mel em períodos de chuva e estiagem no estado de Pernambuco;
b) Analisar o perfil químico dos extratos das amostras de resinas coletadas das plantas visitadas pelas abelhas;
c) Confrontar os dados gerados dos perfis químicos das resinas e das amostras de própolis;
d) Comparar os resultados das atividades antimicrobianas, antileishmania e antitripanossoma das amostras de própolis vermelha coletadas no periodo chuvoso e seco com a da resina de *D. ecastaphyllum*;
e) Comparar os resultados obtidos com as amostras da própolis vermelha de Pernambuco e da resina de *D. ecastaphyllum* com os obtidos da análise da própolis vermelha de Alagoas.

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2 REVISÃO BIBLIOGRÁFICA

2.1 HISTÓRIA DEMOGRÁFICA DAS ABELHAS E SEUS PRODUTOS

2.1.1 Origem das abelhas

Abelhas são essencialmente vespas que em um certo momento de sua história evolutiva, abandonaram a predação em favor de cuidar de um ninho, provendo-o com néctar e pólen. Supõe-se que as abelhas tenham evoluído de uma vespa ancestral, provavelmente da família Sphicidae, com partes bucais capazes de ingerir néctar, onde começaram coletando pólen para alimentar suas crias ao invés de se alimentar de presas (WINSTON, 1987).

Análises filogenéticas realizadas com as 10 espécies de abelhas do gênero *Apis* reconhecidas até então dividem este gênero em três grupos distintos: abelhas que nidificam em cavidades (*A. mellifera*, *A. cerana*, *A. koschevnikovi* e *A. nulensis*); abelhas gigantes (*A. dorsata*, *A. laboriosa*, *A. binghami* e *A. nigrocincta*); e as abelhas anãs (*A. florea* e *A. adreniformis*). Com exceção de *A. mellifera*, todas as outras espécies estão restritas ao continente asiático (ARIAS e SHEPPARD, 2005).

As primeiras abelhas podem ter surgido no interior do paleocontinente Gondwana, que também foi o provável ponto de origem das angiospermas (WINSTON, 1987). Porém, a região geográfica de origem das abelhas *A. mellifera* é um tema que ainda hoje é muito discutido entre os cientistas. A distribuição nativa destas abelhas compreende África, Europa e Oeste da Ásia (WALLBERG et al., 2014). Os primeiros trabalhos, baseados em análises morfológicas, sugeriam um centro de origem de *A. mellifera* no Meio Leste ou Nordeste da África. Essa hipótese foi corroborada por análises de DNA mitocondrial e de polimorfismos de nucleotídeo único (SNPs, acrônimo de *Single Nucleotide Polymorphism*). Entretanto, outros trabalhos não dão suporte a esta hipótese, afirmando que os dados obtidos não colocam as linhagens africanas como ancestrais. Sendo assim, levantou-se uma hipótese alternativa de um centro de origem asiático e justificam isso com o fato de que todas as outras espécies do gênero *Apis* são encontradas no continente asiático (HAN et al., 2012; WALLBERG et al., 2014).

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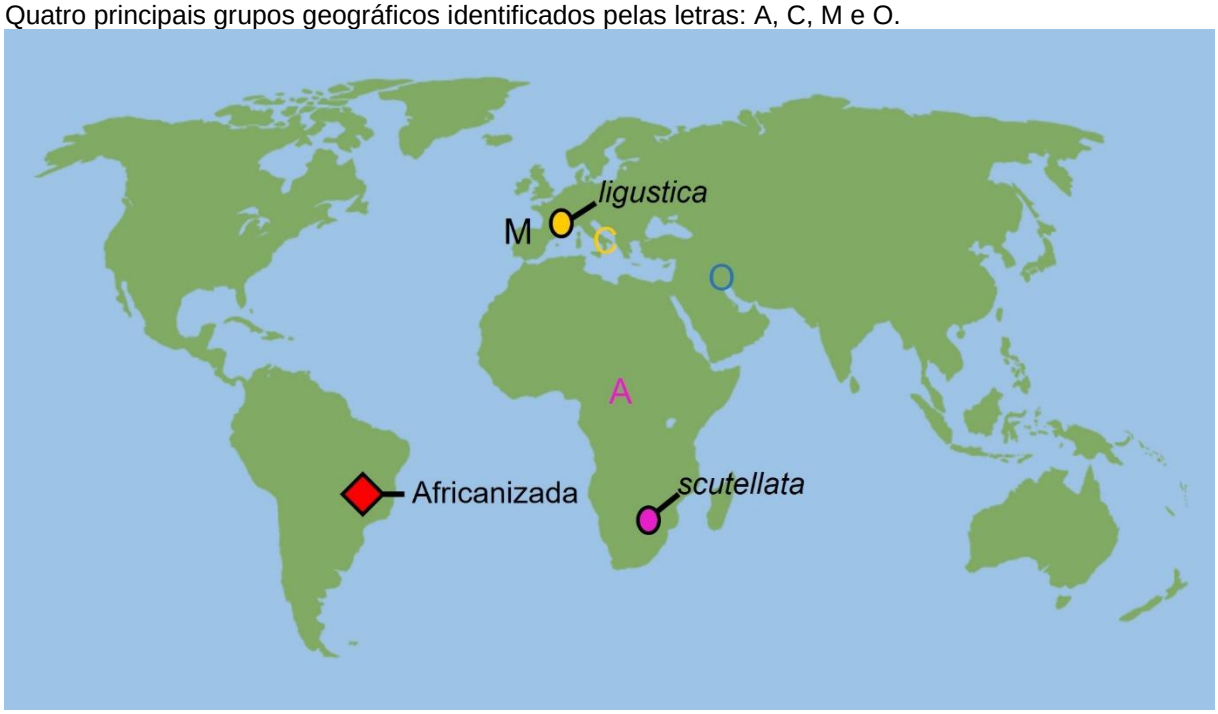
Foram descritas, até então, ao menos 29 subespécies de *A. mellifera* que hoje estão divididas em quatro grandes grupos com base em morfometria e análises genéticas: grupo A, que inclui as subespécies africanas; grupo M, inclui subespécies do Oeste e Norte europeus; grupo C, inclui subespécies do Leste europeu; e o grupo O, que inclui as subespécies da Turquia e do Oriente Médio (Figura 1) (RUTTNER, 1988; ENGEL, 1999; SHEPPARD et al., 2003).

Figura 1 – Distribuição geográfica das duas subespécies de *A. mellifera*, *A. mellifera ligustica* e *A. mellifera scutellata*, que foram introduzidas no Brasil, mais tarde gerando as abelhas Africanizadas. Quatro principais grupos geográficos identificados pelas letras: A, C, M e O.



Fonte: O Autor (2018). Adaptado de WALLBERG et al. (2014).

2.1.2 Introdução das abelhas no Brasil

As primeiras abelhas introduzidas no Brasil foram trazidas do continente europeu pelos imigrantes que trouxeram consigo alguns enxames. Isso aconteceu na primeira metade do século XIX, quando o Imperador D. Pedro II autorizou a importação de abelhas *Apis mellifera mellifera* para o Estado do Rio de Janeiro. Uma outra subespécie de abelha, a *A. mellifera ligustica* (mais conhecida como abelha italiana) foi trazida da Alemanha para o Rio Grande do Sul no final do século XIX (WIESE, 2005).

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Mais uma subespécie de abelha do mel, *A. mellifera scutellata*, foi introduzida em 1956 pelo pesquisador Dr. Warwick Estevan Kerr. Essa subespécie foi coletada na região de Pretória na África do Sul e foram levadas para o Estado de São Paulo. Por conta de uma manipulação descuidada 26 enxames fugiram das colmeias e este episódio deu início a um processo de cruzamentos naturais com as abelhas europeias existentes, resultando na formação de um híbrido denominado de abelha africanizada. Os enxames híbridos rapidamente se dispersaram em direção ao norte, atingindo a América do Norte em menos de 40 anos. Supõe-se que a vantagem das abelhas africanizadas sobre as abelhas europeias deva-se a três fatores primordiais: fatores genéticos, comportamentais e ambientais (WINSTON, 1992; RUBINK et al., 1996; WIESE, 2005).

2.1.3 As abelhas e seus produtos

Além do papel ecológico e econômico que as abelhas desempenham, elas são conhecidas pela sua importância na produção de produtos naturais. Dentre eles, os mais conhecidos são: o mel e a própolis; outros produtos também são muito explorados em pesquisa e pela indústria farmacêutica e de suplementos alimentares, tais como: geleia real, cera e pólen (PYRZYNSKA e BIESAGA, 2009; PREMRATANACHAI e CHANCHAO, 2014; BILIKOVA et al., 2015; VUJIC E POLAK, 2015).

O mel é uma mistura de componentes de plantas e da abelha, sendo um produto alimentício produzido pelas abelhas melíferas a partir do néctar das plantas ou de excreções de insetos sugadores de plantas (BILIKOVA et al., 2015). A geleia real é uma substância secretada por abelhas jovens (nutrizes) com 4 a 12 dias de vida, através de glândulas secretoras [(hipofaringeanas] (KATAYAMA et al., 2008). A cera de abelha é uma substância secretada pelas abelhas operárias entre 14 e 18 dias de vida. A cera é produzida por quatro pares de glândulas cerígenas localizadas na parte inferior do abdômen. A principal função da cera de abelha é a construção de favos (WIESE, 2005). O pólen de abelha é uma mistura de pólens de diferentes espécies de plantas. As abelhas transportam o pólen na pata traseira, numa dobra chamada corbícula, sendo aglutinado com a ajuda de néctar e de enzimas secretadas pelas glândulas salivares das abelhas. Este componente é usado pelas abelhas

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principalmente para o preparo do alimento e da geleia real (DENISOW e DENISOW-PIETRZYK, 2016). E por fim, a própolis, que é uma substância resinosa que é obtida pelas abelhas da partir de exsudatos de plantas e é usada para reparos da colmeia e proteção contra agentes externos (GHISALBERTI, 1979; MARCUCCI, 1995).

2.2 PRÓPOLIS

2.2.1 O que é a própolis

Própolis é o nome genérico dado a um produto obtido de uma substância resinosa que é coletada de exsudatos (resinas, muscilagem, goma ou materiais lipofílicos) de brotos e árvores, botões florais, folhas, feridas de plantas e pólen de diversas espécies pelas abelhas do mel (*Apis mellifera*) (CRANE et al., 1988). As abelhas fazem uso das mandíbulas, com as quais raspam o produto secretado pelas plantas e o tornam maleável, manipulando-o com ajuda das patas, até fixar o material nas corbículas, transportando-o até a colmeia. Essa resina é mastigada, enzimas salivares são adicionadas e o material parcialmente digerido é misturado à cera de abelha e usado na colmeia (GHISALBERTI, 1979; MARCUCCI, 1995).

A própolis é um produto que contém uma mistura complexa composta aproximadamente por 50% de resina e bálsamo, 30% de cera, 10% de óleos essenciais e aromáticos, 5% de pólen e 5% de impurezas (THOMSON, 1990). Além dos fatores já citados que modificam a composição química da própolis, a composição também pode variar em decorrência de fatores como sazonalidade, iluminação, altitude, tipo de coletor, disponibilidade de comida e atividade desenvolvida durante a exploração da própolis (BANKOVA et al., 1998; TORETI et al., 2013).

As abelhas usam a própolis ou “cola de abelha”, como também é comumente conhecida, para reparar os favos de mel, para fechar pequenas frestas, suavizar as paredes internas, embalsamar as carcaças de invasores que as abelhas mataram dentro da colmeia, mas que elas não puderam transportar para fora, bem como proteger a colmeia da invasão de microrganismos (GHISALBERTI, 1979; BURDOCK, 1998).

Pelo homem, a própolis tem sido usada como remédio popular desde 300 a.C. para vários propósitos, a saber: antisséptico; antioxidante; anti-inflamatório; ou como

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adesivo para selar quebras, revestir madeira e outras superfícies. Há registros bíblicos no livro de Gênesis do seu uso; os egípcios usavam a própolis como substância antiputrefativa em suas múmias; na Idade Média foi amplamente usado como antisséptico e cicatrizante; e os Incas o utilizavam como antipirético (TORETI et al., 2013). Inúmeras atividades biológicas foram atribuídas às substâncias presentes nesta mistura, como atividades: antioxidante, anti-inflamatória, melhorias do coração e função circulatória, antilipidemica, antihiperglicêmica, citotóxica, anticâncer (BANKOVA, 2005).

2.2.2 Tipos de própolis

Os diferentes tipos de própolis estão particularmente ligados à flora existente em cada região do planeta. Os principais fatores analisados para diferenciar os tipos de própolis são: características organolépticas; composição química; e fonte botânica. Na Europa, América do Norte, oeste da Ásia e Nova Zelândia, as principais fontes de resina para a produção da própolis são os brotos de choupo (*Populus* spp.). Na Rússia foi observado que a principal fonte vegetal utilizada pelas abelhas é a *Betula verrucosa,* mais conhecida como bétula ou vidoeiro (BURDOCK et al., 1998; BANKOVA et al., 1992). Na América do Sul, mais precisamente na Venezuela e em Cuba, a fonte mais utilizada pelas abelhas do mel para a produção de própolis são espécies do gênero *Clusia* (TOMÁS-BARBERÁN et al., 1993; CUESTA-RUBIO et al, 2002).

No Brasil são conhecidos pelo menos 13 tipos de própolis (Tabela 1) e ao menos quatro espécies vegetais que participam na produção da própolis: *Baccharis dracunculifolia* (Asteracea), mais conhecida como alecrim-do-campo; *Dalbergia ecastaphyllum* (Fabaceae), conhecida no Nordeste do Brasil como rabo de bugio; *Hyptis divaricata* (Lamiaceae); e *Populus alba* (Salicaceae) (PARK et al., 2002a; PARK et al., 2002b; DAUGSCH et al., 2008). Fatores como localização geográfica, origem botânica, composição química e cor foram importantes para encaixar as própolis brasileiras nesses grupos.

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Tabela 1 – Relação dos 13 tipos de própolis encontrados no Brasil.

Extratos Etanólicos da Própolis		
Grupos	**Cor**	**Origem da própolis**
Grupo 1 (RS)	Amarelo	Sul
Grupo 2 (RS)	Marrom	Sul
Grupo 3 (PR)	Marrom escuro	Sul
Grupo 4 (PR)	Marrom	Sul
Grupo 5 (PR)	Marrom esverdeado	Sul
Grupo 6 (BA)	Marrom avermelhado	Nordeste
Grupo 7 (BA)	Marrom esverdeado	Nordeste
Grupo 8 (PE)	Marrom escuro	Nordeste
Grupo 9 (PE)	Amarelo	Nordeste
Grupo 10 (CE)	Amarelo escuro	Nordeste
Grupo 11 (PI)	Amarelo	Nordeste
Grupo 12 (SP)	Verde ou marrom esverdeado	Sudeste
Grupo 13 (AL)	Vermelho	Nordeste

Fonte: O Autor (2018). Adaptado de TORETI et al. (2013)

O perfil químico das amostras de própolis oriundas de regiões tropicais tem sido bastante estudado, especialmente as brasileiras. Os flavonoides, em muitos casos, estão presentes em grande quantidade, embora sejam de origens botânicas diferentes (PARK et al., 2002b; BANKOVA, 2005). Em amostras brasileiras foram encontrados kaempferida, 5,6,7-trihidroxi-3,4'-dihidroxiflavona, aromadendreno-4' metil éter, 3,5,7 – trihidroxi-6,4'-dimetoxiflavona (BANKOVA et al., 2000). Outra nova classe de fenólicos achados em amostras de própolis brasileira são os ácidos p-cumáricos prenilados e seus resíduos com prenil cíclico e acetofenonas (ambos são metabólitos secundários típicos de *B. dracunculifolia*). Artepilina C, um diprenil derivado do ácido p-cumárico, é um componente marcante da própolis verde brasileira (BANSKOTA et al., 1998; TAZAWA et al., 1998; FERNANDES-SILVA et al., 2013). Outros compostos fenólicos foram isolados da própolis brasileira: derivados do ácido cafeoilquinico (típico da própolis Brasileira); c-guaiacilglicerol; e o radical livre limpador, ácido 3-[4-

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hidroxi-3-(3-oxobut-1-enil)-fenilacrílico (TATEFUJI et al., 1996; FERNANDES-SILVA et al., 2013). Di e triterpenos também são importantes classes constituintes da própolis brasileira. Alguns triterpenos alcoólicos estão presentes em própolis do Brasil e Egito como: beta-amirina, triterpenos alcoólicos do tipo amirina e cicloartenol (MARCUCCI et al., 1998). Em baixas concentrações encontra-se componentes voláteis, açúcares e minerais (BANKOVA et al., 2000).

2.3 A PRÓPOLIS VERMELHA

2.3.1 Distribuição geográfica

A própolis vermelha (Figura 2) produzida pela *Apis mellifera* pode ser encontrada atualmente em seis países espalhados em três continentes. No continente africano, a própolis vermelha é produzida na Nigéria. Na Ásia, é encontrada na Arábia Saudita e na China. No continente americano, a própolis vermelha pode ser encontrada no México, em Cuba, na Venezuela e no Brasil (THRUSHEVA et al., 2004; IZUTA et al., 2009; LOTTI et al., 2010; PICCINELLI et al., 2011; OMAR et al.,2016; SADDIQ et al., 2016).

Figura 2 – Própolis vermelha. Quadro retirado de colmeia em apiário localizado em Tamandaré – PE.

Fonte: O Autor (2014).

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No Brasil, a própolis vermelha é encontrada no litoral do Nordeste em colmeias localizadas em regiões de mangue, nos estados da Bahia, Sergipe, Alagoas, Pernambuco e Paraíba. A descoberta da própolis vermelha no Brasil ainda é recente, em torno de uma década desde o seu primeiro relato em publicação científica. THRUSHEVA et al. (2006) publicaram o primeiro trabalho que analisou os constituintes bioativos da própolis vermelha de Alagoas. Nos anos seguintes foram feitos outros estudos com a própolis vermelha proveniente de outros estados (DAUGSCH et al., 2008; FROZZA et al., 2013; REGUEIRA et al., 2017). Porém, apesar de ser encontrada em vários estados da região Nordeste, a própolis vermelha de Alagoas ainda permanece sendo a bem mais estudada. Mais recentemente, ISHIDA et al. (2011) e LÓPEZ et al. (2014) relataram a existência da própolis de cor avermelhada também no Norte do Brasil, nos estados de Roraima e da Amazônia; através da comparação da composição química da própolis vermelha destes estados com a encontrada em outras localidades, os autores afirmam ser um tipo novo de própolis por não ser idêntica a própolis vermelha cubana e nem idêntica à própolis vermelha brasileira.

2.3.2 Origem botânica

A origem botânica da própolis vermelha pode variar nos diferentes países onde ela é encontrada, em decorrência da diversidade florística de cada região (LÓPEZ et al., 2014). Para se investigar de qual fonte botânica as abelhas do mel retiram as substâncias para produção da própolis, três metodologias podem ser empregadas: i. análise comparativa da composição química através dos perfis cromatográficos da própolis e das possíveis plantas fontes; ii. análise palinológica; iii. identificação da fonte botânica da própolis vermelha a metodologia do *DNA barcoding*, que consiste basicamente da extração de DNA da própolis, realização de uma PCR utilizando *primers* para sequências da possível planta fonte e sequenciamento (DAUGSCH et al., 2008; BARTH E LUZ, 2009; JAIN et al., 2014).

As plantas do gênero *Clusia* e *Dalbergia* estão entre as principais espécies procuradas pelas abelhas para a produção da própolis vermelha em diferentes países (TOMÁS-BÁRBERAN et al., 1993; PICCINELLI et al., 2011; LOPEZ et al., 2014). No continente americano, as plantas do gênero *Clusia*, i.e. *C. scrobiculata*, *C. minor* e *C.*

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major, são as fontes botânicas preferenciais para produção da própolis vermelha pelas abelhas na Venezuela, que visitam a flor e coletam a resina secretada na base dos estames (TOMÁS-BÁRBERAN et al., 1993; TRUSHEVA et al., 2006). CUESTA-RUBIO et al. (2004), através de análise cromatográfica, identificaram componentes químicos da resina floral de C*lusia rosea* na própolis vermelha de Cuba. Entretanto, foi relatado a presença, também, de componentes químicos oriundos da resina secretada pela planta *Dalbergia ecastphyllum* em amostras de própolis vermelha cubana (PICCINELLI et al., 2011; LOPEZ et al., 2014). Plantas do gênero *Dalbergia* foram relatadas, também, como fontes botânicas da própolis vermelha da Nigéria através da análise comparativa com os compostos químicos encontrados em *D. ecastaphyllum*, o que se sustenta por conta do número de espécies de *Dalbergia* que são encontradas no Oeste da África (SAHA et al., 2013; OMAR et al., 2016).

No Nordeste brasileiro, a própolis vermelha ocorre ao longo do litoral e está presente nos estados de Alagoas, Bahia, Paraíba, Pernambuco e Sergipe (ALENCAR et al., 2007; DAUGSCH et al., 2008; FROZA et al 2013; LÓPEZ et al., 2014). DAUGSCH et al. (2008) através da observação do comportamento das abelhas e da análise comparativa entre a própolis vermelha e a resina da planta a qual elas estavam visitando, concluíram que *D. ecastaphyllum* é a principal fonte botânica da própolis vermelha nos estados do nordeste do Brasil. LÓPEZ et al. (2014) através de análises cromatográficas dividiu a própolis vermelha em dois grupos: um grupo formado por amostras dos estados de Alagoas e Paraíba, o qual eles identificaram traços químicos fortes de plantas da família Clusiaceae; e um outro grupo formado por amostras de própolis de Cuba e do estado da Paraíba, que apresentaram componentes químicos majoritários de *D. ecastaphyllum*. A hipótese de que componentes químicos da própolis vermelha do Nordeste do Brasil podem ser oriundos de duas fontes botânicas também foi levantada previamente por PICCINELLI et al. (2011). As própolis de cor avermelhada encontradas nos estados de Roraima e da Amazônia, tem como possíveis fontes botânicas plantas da família Clusiaceae, por conta disso, se parecendo mais com a própolis venezuelana e cubana do que com a própolis vermelha encontrada no Nordeste do Brasil (ISHIDA et al., 2011; LÓPEZ et al., 2014).

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2.3.3 Componentes químicos da própolis vermelha

Em geral, a composição química da própolis depende de alguns fatores: altitude, flora ao redor da colmeia, iluminação, sazonalidade e tipo de abelha coletora (SILICI E KUTLUCA et al., 2005; THRUSHEVA et al., 2006; TEIXEIRA et al., 2008; ISLA et al., 2012). A própolis vermelha brasileira tem como componentes químicos majoritários flavonoides (isoflavanonol, chalconas, flavanonas e flavonol) e isoflavonoides (pterocarpanos, isoflavanonas, isoflavanas e isoflavonas) (Figura 3), além de componentes pertencentes a outros grupos químicos [*e.g.* benzofenonas preniladas, terpenos e taninos] (SILVA et al., 2007; DAUGSCH et al., 2008; AWALE et al., 2008; RIGHI et al., 2010; PICCINELLI et al., 2011; LÓPEZ et al., 2014; MAYWORM et al., 2014) Os isoflavonoides são compostos marcadores para a própolis vermelha brasileira e quatro deles se destacam: biochanina A, formonetina, pinocembrina (LÓPEZ et al., 2014) e medicarpina (THRUSHEVA et al., 2006).

Inúmeros trabalhos foram realizados para testar várias atividades biológicas (*e.g.* antioxidante, antimicrobiana, antitumoral, anti-inflamatória, citotóxica), da própolis vermelha brasileira e os compostos químicos encontrados nas amostras testadas têm participação essencial na bioatividade apresentada pela própolis vermelha (ALENCAR et al., 2007; LI et al., 2008; RIGHI et al., 2010; BUENO-SILVA et al., 2013). As isoflavonas encontradas nas amostras de própolis vermelha do Nordeste do Brasil estão associadas às atividades antibacteriana, antifúngica, anti-inflamatória e citotóxica (SILVA et al., 2007; BUENO-SILVA et al., 2013; LÓPEZ et al., 2015). Isoflavonóides têm uma distribuição muito restrita no reino vegetal e ocorre quase que exclusivamente em leguminosas (PICCINELLI et al., 2005). Outros grupos químicos como flavanonas, chalconas e pterocarpanas, encontradas na própolis vermelha, possuem atividade citotóxica (LI et al., 2008).

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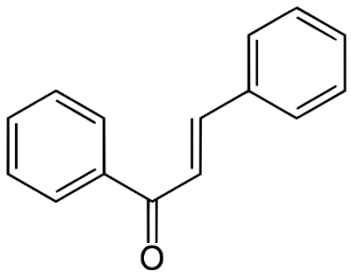
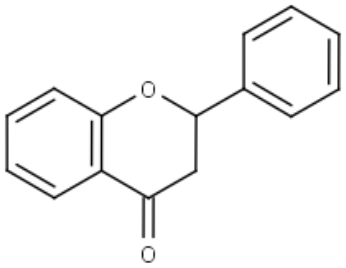
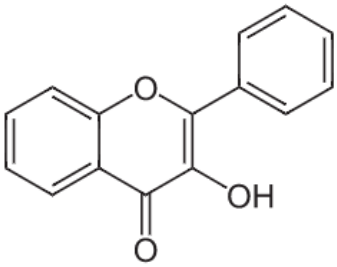
Figura 3 – Núcleos fundamentais das famílias de flavonóides e isoflavonóides encontrados na própolis vermelha.



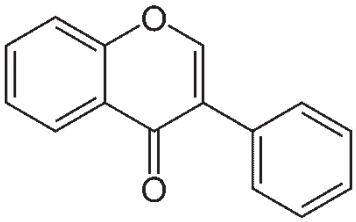
Chalcona



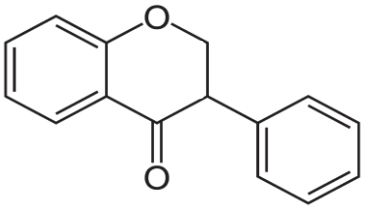
Flavanona



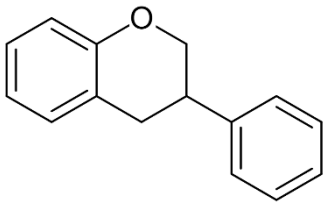
Flavonol



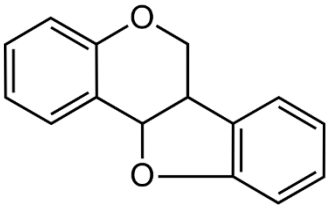
Isoflavona



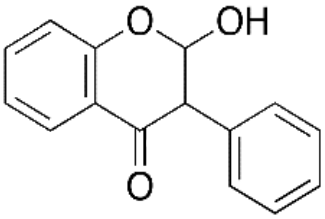
Isoflavanona



Isoflavana



Pterocarpano



Isoflavanonol

Fonte: O Autor (2018).

2.3.4 Atividade antibacteriana da própolis vermelha

A atividade antibacteriana da própolis vermelha brasileira já foi avaliada contra bactérias Gram-negativas – *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebisiella pneumoniae*, *Escherichia coli*, *Proteus mirablis*, *P. morganii*, *Neisseria meningitides*, *Haemophilus influenzae* e *Enterobacter aerogenes* (RIGHI et al., 2010; LÓPEZ et al., 2015) e bactérias Gram-positivas –*Bacillus subtilis*, *Enterococcus faecalis*, *Estreptococcus pyogens*, *Staphylococcus aureus* e *S. epidermidis* (ALENCAR et al., 2007; CABRAL et al., 2009; RIGHI et al., 2010; LOPEZ et al., 2015). Entretanto, a maioria dos estudos foi conduzida com em amostras de própolis vermelha provenientes dos estados de Alagoas, Paraíba e Sergipe.

A própolis vermelha possui atividade antibacteriana por conta da presença de flavonoides em grandes quantidades (DAUGSCH et al., 2008). A atividade antibacteriana dos flavonoides ainda continua sendo bastante estudada e esses compostos podem exercer essa atividade de três modos: matando diretamente a bactéria; através da ativação sinérgica de antibióticos; e através da atenuação da patogenicidade da bactéria (CUSHNIE et al., 2011).

2.3.5 Efeito modulador da atividade antibiótica pela própolis vermelha

Uma questão sempre preocupante com relação ao tratamento de infecções bacterianas e fúngicas é o aparecimento de cepas e linhagens resistentes aos antibióticos comercias (WOJTYCZKA et al., 2013; PIPPI et al., 2015). Microrganismos resistentes aos antibióticos são uma importante questão de saúde pública, uma vez que eles podem facilmente se disseminar no ambiente. Por conta disto, vem-se tentando encontrar uma forma alternativa de tratar infecções microbianas resistentes em seres humanos. A associação de agentes antimicrobianos sintéticos e produtos naturais tem sido estudada e algumas combinações tiveram efeito sinérgico inibindo o crescimento bacteriano (MATIAS et al 2010; HASSAN et al 2016).

A própolis têm sido combinada com alguns tipos de drogas: anticâncer (MARKIEWWICZ-ZUKOWSKA et al., 2013; ORSOLIC et al., 2013); antibacteriana (WOJTYCZKA et al., 2013); antifúngica (PIPPI et al., 2015) e em alguns estudos foi observado efeito sinérgico (ORSI et al., 2012; MARKIEWWICZ-ZUKOWSKA et al.,

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2013; ORSOLIC et al., 2013; HASSAN et al., 2016). A associação da própolis vermelha brasileira com agentes antifúngicos apresentou efeito sinérgico contra *Candida* sp. (PIPPI et al., 2015), entretanto estudos combinando a própolis vermelha e agentes antibacterianos permanecem escassos.

Alguns mecanismos de ação têm sido propostos para a atuação sinérgica de classes de flavonoides presentes na própolis com antibióticos comerciais. Danos causados à membrana citoplasmática (por conta da redução da fluidez da membrana), inibição da síntese de ácidos nucleicos (causada pela inibição da topoisomerase), inibição da energia de metabolismo (causada pela inibição da NADH-citocromo c redutase), inativação da bomba de efluxo e a inibição da formação de biofilme são alguns dos mecanimos de atividade antimicrobiana associados aos flavonoides (CUSHINIE et al., 2011).

2.3.6 Efeito da sazonalidade sobre a própolis

Os fatores ambientais (*e.g.* temperatura, luz e umidade) podem influenciar na biossíntese dos metabólitos nas plantas (TEIXEIRA et al., 2008). No Brasil, diferentemente do que é observado em países com clima temperado, as abelhas coletam material vegetal durante todo o ano, então algumas variações sazonais são possíveis (BANKOVA et al., 1998). Dessa forma, a sazonalidade é um importante fator que pode, também, determinar variações na composição química da própolis. Essas variações são importantes para a aplicação prática dos usos da própolis, por exemplo saber em que época coletar a própolis com maior concentração de compostos bioativos (BANKOVA et al., 1998). BANKOVA et al. (1998) também observaram a presença de ácidos diterpênicos em amostras de própolis coletadas no verão, entretanto observou uma dminuição destes mesmos compostos no outono. Porém, não foi observada uma significante variação da atividade antibacteriana da própolis coletada em difrentes estações (SFORCIN et al., 2000; JORGE et al., 2008; TEIXEIRA et a., 2008).

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2.3.7 Atividade anti-leishmania e antitripanossoma da própolis

Leishmaniose é um grupo de doenças tropicais negligenciadas que podem ser causadas por mais de 20 espécies de protozoarios do gênero *Leishmania* (WHO, 2017a). A doença pode se apresentar através de diferentes formas clínicas, como a cutânea (LC), mucocutânea (LM) e visceral (LV) (WHO, 2017a). A LC é a forma mais comum da doença e é caracterizada por úlceras geralmente localizadas na face, nos braços e pernas, e o número de úlceras pode chegar a 200. Na LM as lesões podem levar à destruição parcial ou total das membranas mucosas do nariz, da boca e das cavidades da garganta. A LV, também conhecida como Calazar, é caracterizada por ataques irregulares de febre, perda de peso, hepato e splenomegalia (WHO, 2017a). A LC pode ser causada primariamente pelas *Leishmania (Leishmania) major*, *L. (Leishmania) amazonensis*, *L. (Leishmania) mexicana*, *L. (Viannia) braziliensis* e outras espécies do subgênero *Viannia*. A maioria dos casos de LM são ocasionados por *L. (Viannia) braziliensis* e acontece em aproximadamente 5% dos indivíduos que já estão com LC. Por fim, a LV pode ser causada pelas *L. (Leishmania) infantum* e *L. (Leishmania) donovani* (AYRES et al., 2007; VALDIVIA et al 2015).

Em ambos os subgêneros *Leishmania* e *Viannia*, os parasitas estão presentes como amastigotas intracelulares no interior de fagolisossomos de fagocitos em hospedeiros invertebrados e nas formas promastigotas nos vetores insetos. A leishmaniose pode ser transmitida pela picada de fêmeas infectadas de flebotomíneos que injetam as formas promastigotas no hospedeiro vertebrado durante sua alimentação. Quando as promastigotas atingem a ferida causada pela alimentação, elas são fagocitadas por macrófagos e outros tipos de células fagocíticas. Uma vez dentro das células, os parasitas passam para a forma amastigota, onde se multiplicam por bipartição e infectam outras células. Nos mosquitos, as formas amastigotas se transformam em promastigotas, se desenvolvem no instestino e migram para a probóscide (Figura 4) (CONCEIÇÃO-SILVA E ALVES, 2014).

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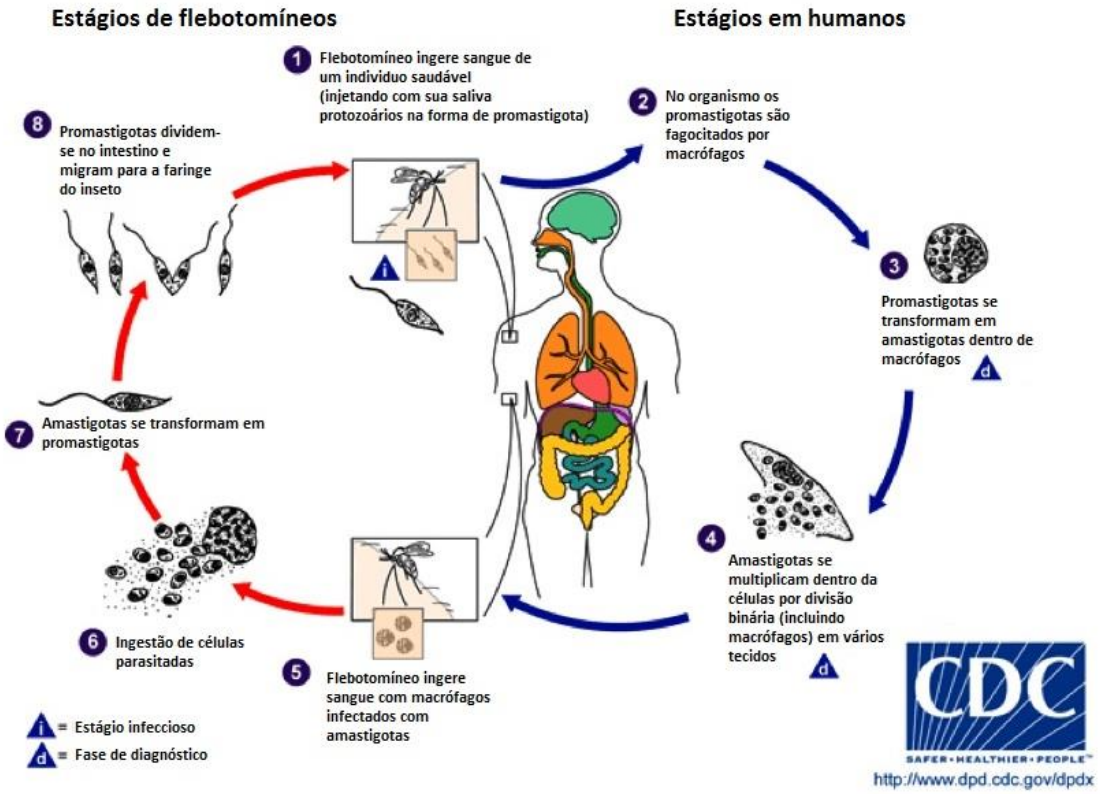
Figura 4 – Ciclo biologico da *Leishmania* spp.



Fonte: CDC, Atlanta, USA (2018).

A doença de Chagas, também conhecida como Tripanossomíase Americana, é uma importante questão de saúde pública na América Latina, sendo causada pelo parasita *Trypanosama cruzi*. Na América Latina, *T. cruzi* é transmitido na maioria dos casos pelo contato com as fezes ou urina de insetos triatomíneos mais conhecidos como barbeiros. A infecção ocorre no momento em que o barbeiro pica uma área de pele exposta e o inseto em seguida defeca próximo à picada (CHATELEIN, 2017; WHO, 2017b).

A Doença de Chagas se apresenta em duas fases: a primeira, fase aguda, pode durar cerca de até dois meses após a infecção. Durante esta fase, há um grande número de parasitas na forma tripomastigota. Nessa fase os sintomas são ausentes ou são fracos. As vezes pode aparecer lesao no local da picada e inchaço das palpebras. Durante a segunda fase, crônica, o parasita se aloca principalmente nas células – sobe a forma amastigota - do coração e de músculos do sistema digestivo (Figura 5). Anos depois da infecção, pode acontecer morte súbita falha do coração causada pela destruição das células e do sistema nervoso (WHO, 2017b).

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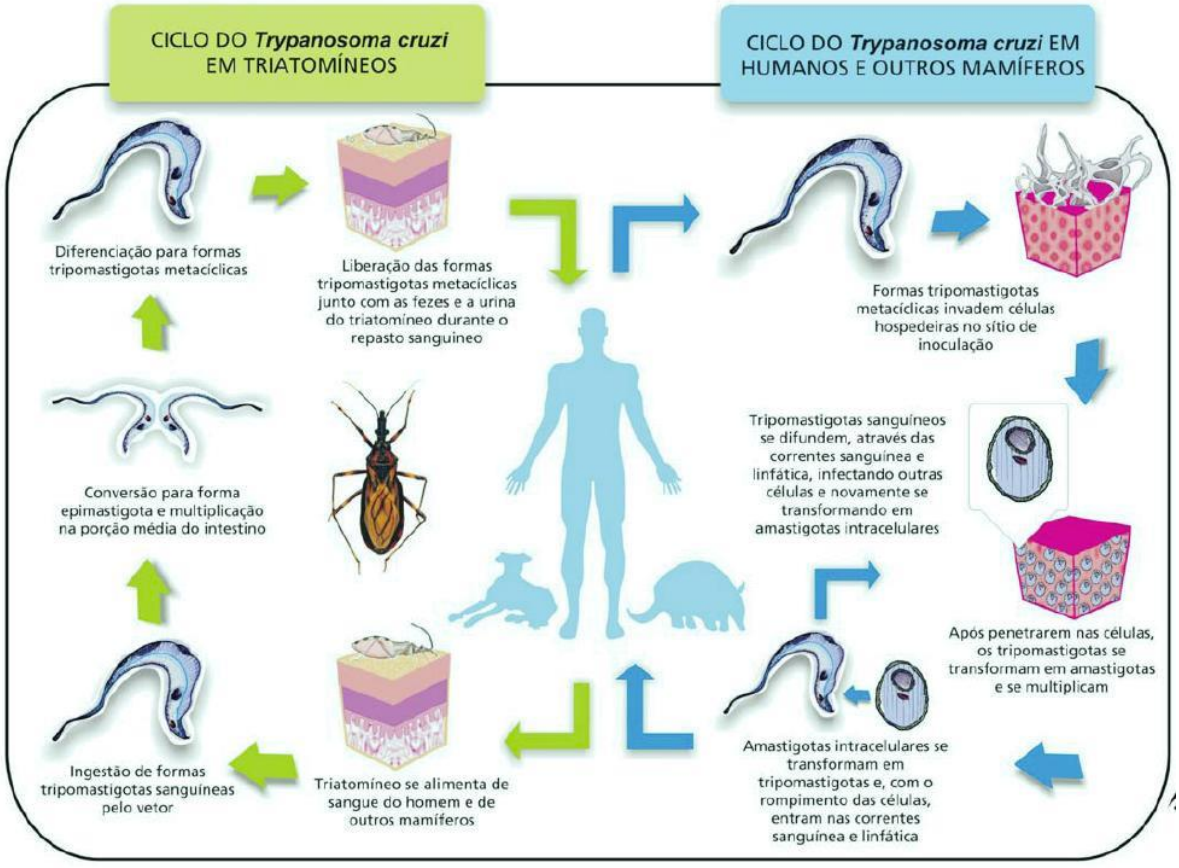
Figura 5 – Ciclo biológico do parasita *Trypanosoma cruzi*.



Ciclo de transmissão do *Trypanosoma cruzi* (simplificado). Infográfico: Venício Ribeiro, ICICT/Fiocruz.

Fonte: http://portaldoprofessor.mec.gov.br/fichaTecnicaAula.html?aula=58597 (2017)

Para o tratamento de pacientes infectados com *Leishmania* spp. ou *Trypanosoma cruzi*, é feito o uso de fármacos de primeira linha. Em pacientes com leishmaniose, antimoniato de meglumina (Glucantime®, Sanofi aventis) é uma droga de primeira linha usada no combate ao protozoário, enquanto que anfotericna B e pentamidina são usadas as vezes como fármacos de segunda ou terceira linha. As drogas previamente citadas são usadas no tratamento de LC e LM (AYRES et al, 2007; HASHGUCHI et al., 2017). A Miltefosina (Paladin, Canadá) é um fármaco de uso oral usado no tratamento de LV (HASHIGUCHI et al. 2017). Atualmente, o tratamento da Doença de Chagas é feito com a utilização de duas drogas nitro-heterocíclicas: benzonidazol (Abarax/ELEA) e nifurtimox (LAMPIT/Bayer). Porém, essas drogas apenas são efetivas na fase aguda da infecção (CHATELEIN, 2017).

Os tratamentos disponíveis atualmente para essas doenças têm se mostrado complicados e dolorosos. Os pacientes com LC geralmente abandonam o tratamento

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com Glucantime® por causa das múltiplas injeções dolorosas da droga, por morarem longe dos centros e dos efeitos colaterais à droga (HASHIGUCHI et al., 2017). No caso dos fármacos utilizados para o tratamento da Doença de Chagas, o uso deles é limitado devido à baixa disponibilidade e efeitos colaterais, tais como: dermatite alérgica; coceiras; e manisfestações gastrointestinais (CHATELEIN et al., 2017). Esses problemas são agravados pelo aparecimento de resistência a essas drogas em áreas endêmicas.

Muitos estudos estão tentando provar a eficácia e incorporar o uso de produtos naturais no tratamento da leishmaniose e da doença de chagas. Com o passar do tempo vários trabalhos têm mostrado que os produtos naturais podem ser uma boa estratégia na busca de compostos bioativos que podem otimizar a atividade biológica dos fármacos e minimizar efeitos colaterais. Uma grande variedade de alcalóides, esteróides, terpenos, cumarinas e flavonoides, tanto de origem vegetal e animal, vêm sendo testados contra diferentes espécies de *Leishmania* e *Trypanosoma*. (CHEUKA et al., 2017)

Nos últimos 20 anos inúmeros estudos sobre as atividades in vitro da própolis contra espécies de *Leishmania spp.* e *Trypanosoma spp.* tem sido realizados. Própolis de diversas origens (*e.g.* América do Sul, Europa e África) e tipos (*e.g.* verde e vermelha) já demonstraram possuir atividades contra espécies de *Leishmania* (*L. amazonensis*, *L. braziliensis*, *L. infantum* e *L. major*) e *Trypanosoma* (*T. cruzi*, *T. brucei* e *T. evansi*) (HIGASHI E CASTRO, 1994; AYRES et al., 2007; MACHADO et al., 2007; OMAR et al., 2016). Em um estudo comparando os efeitos das própolis verde e vermelha contra as formas amastigotas e promastigotas de Leishmania, AYRES et al. (2007) puderam observar que a própolis vermelha foi a mais ativa contra *Leishmania* e menos citotóxica contra os macrófagos e ainda estimulou sua produção. Da própolis vermelha nigeriana foram isolados 10 compostos e foi observada, dentre eles, de moderada a alta atividade contra *Trypanosoma brucei* (OMAR et al., 2016). Os efeitos causados pelo tratamento de formas tripomastigotas de *Trypanosoma* vão desde alterações mitocondriais até danos a membrana plasmática (MENNA-BARRETO et al., 2009).

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2.4 RESINA DE *Dalbergia ecastaphyllum*

2.4.1 *Dalbergia ecastaphyllum*

O gênero pantropical *Dalbergia* L.f. (Fabaceaea: Papilionoideae) consiste de arbustos, trepadeiras, e árvores com cerca de 250 espécies no total, e a Indochina representa um dos seus centros de diversficação com aproximadamente 30 a 45 espécies (HARTVIG et al., 2015). Esse gênero está distribuido nas Américas do Norte, Central e do Sul e oeste da África (TROPICOS, 2018). O gênero é caracterizado por folhas alternas, imparipinada; estípulas geralmente pequenas e caducas. A inflorescência pode ser racemosa ou paniculada. As flores são pequenas e geralmente numerosas. Os frutos são oblongos ou oblongo-elipticos, samaróide, reniforme, orbicular ou suborbicular, membranoso, subcoriaceo ou coriáceo (CARVALHO, 1997).

Dalbergia ecastaphyllum (L.) Taub. (Figura 6) é conhecida popularmente como rabo-de-bugio, bugi, marmelo e marmeleiro-da-praia. Essa espécie ocorre nas Américas, desde a costa sul da Flórida até o sul do Brasil, além da costa oeste africana. *D. ecastaphyllum* está principalmente associada a estuarios, beira de rios, ou manguezais. Menos frequentemente a espécie cresce em vegetação de restinga em solos arenosos como arbustos robustos ou árvores pequenas. *D. ecastaphyllum* está presente atualmente em 18 estados brasileiros (CARVALHO, 1997; SILVA E SANTOS, 2009). Essa espécie vem se tornando popular por ser a principal fonte botânica de resina para a produção da própolis vermelha (ALENCAR et al., 2007).

Figura 6 – Foto de um exemplar *de Dalbegia ecastaphyllum*.

Fonte: Anita Estival (2010), Flora Digital, UFRGS.

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2.4.2 Produção da resina vegetal

Resina vegetal é definida como uma mistura composta por terpenoides e/ou compostos fenólicos secundários solúveis em lipidios que são (i) geralmente secretados em estruturas especializadas localizadas internamente ou na superfície das plantas e (ii) de potencial significância em interações ecológicas. Há dois tipos de secreção resinosa extracelular: endógena, o material se acumula em estruturas internas (glandulas ou ductos) e normalmente esse material é exudado quando planta sofre injuria; exógena, vários tipos de celulas secretórias epidermais descarregam o material para fora do órgão (LANGENHEIM, 2003).

Diferentes organelas estão envolvidas na produção de resinas, como os plastídeos (leucoplastos) e o reticulo endoplasmático liso (REL). Em geral, a síntese de monoterpenos e diterpenos começa nos plastideos e terminam no REL. Flavonóides e fenilpropanoides são sintetisados no REL e acumulados em células secretórias colunares. O transporte dos compontes da resina pode ser feito por vesículas que são envelopadas pela membrana plasmática antes de serem despejadas no espaço intercelular (LANGENHEIM, 2003).

Em alguns casos, a resina é exudada após algum tipo de injúria. No caso de *D. ecastaphyllum*, insetos são responsáveis pela exudação da resina através da produção de furos na casca dos galhos. Essa resina que é um mecanismo de defesa da planta contra invasão de micro-organismos é usada pelas abelhas melíferas para a produção de própolis (NAIR, 2007).

2.4.3 Componentes químicos e atividades biológicas da resina de *Dalbergia ecastaphyllum*

No que se trata do estudo da composição da própolis vermelha brasileira, a resina vegetal a qual as abelhas usam para a produção da própolis (Figura 7) tem sido tratada desde a descoberta da propolis vermelha como coadjuvante. Com o objetivo de identificar a origem botânica da propolis vermelha foi possivel identificar na resina de *D. ecastaphyllum* a presença de flavonóides, e.g. quercetina, crisina, rutina, daidzeina, formonetina, biochanina A, pinocembrina, luteolina, e ácidos fenólicos, como ácido ferúlico (ALENCAR et al., 2007; DAUGSCH et al., 2008; SILVA et al.,

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2007; LÓPEZ et al., 2014). BUENO-SILVA et al. (2017) analisaram a atividade antibacteriana da resina de *D. ecastaphyllum* e observaram que a mesma possui atividade antibacteriana contra bacterias Gram-positivas.

Figura 7 – Exudação da resina vegetal em galho da planta *Dalbergia ecastaphyllum*.

Fonte: O Autor (2015).

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3 RESULTADOS

Os resultados estão apresentados em três artigos.

3.1 ARTIGO 1 - SEASONAL VARIATION OF BRAZILIAN RED PROPOLIS: ANTIBACTERIAL ACTIVITY, SYNERGISTIC EFFECT AND PHYTOCHEMICAL SCREENING

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Seasonal variation of Brazilian red propolis: Antibacterial activity, synergistic effect and phytochemical screening

M.S. Regueira Neto [a, *], Saulo Relison Tintino [b], Ana Raquel Pereira da Silva [b], Maria do Socorro Costa [b], Aline Augusti Boligon [c], Edinardo F.F. Matias [d], Valdir de Queiroz Balbino [a], Irwin R.A. Menezes [b], Henrique Douglas Melo Coutinho [b]

[a] Departamento de Genética, Centro de Ciências Biológicas, UFPE, Brazil
[b] Departamento de Química Biológica, Centro de Ciências Biológicas e Saúde, Universidade Regional do Cariri, Brazil
[c] Department of Industrial Pharmacy, Federal University of Santa Maria, Santa Maria, Brazil
[d] Departamento de Biomedicina, Centro Unversitário Leão Sampaio, Brazil

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ABSTRACT

The aim of this study was to investigate the effect of the dry and rainy season on the antibacterial activity and chemical composition of the Brazilian red propolis. The samples were collected in rainy (RP-PER) and dry (RP-PED) seasons and analyzed by HPLC-DAD. The extracts were tested alone and in association with antibiotics against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The HPLC analysis identified luteolin and quercetin as the main compounds. Seasonal variation was observed according to concentrations of the compounds. The MIC values against *E. coli* ranged from 128 μg/mL to 512 μg/mL (EC 06 and EC ATCC). The red propolis showed MIC values of 512 μg/mL against both strains of *P. aeruginosa* used in our study (PA03 and PA24) and against strains of Gram-positive bacteria *S. aureus* the MICs ranged from 64 μg/mL to ≥1024 μg/mL (SA10). A synergistic effect was observed when we combined the RP-PED with gentamicin against all the strains tested. When we combined the RP-PED with Imipenem, we only observed synergistic effect against *P. aeruginosa*. According to our synergistic activity results, the utilization of red propolis collected in the drier periods can be used as an adjuvant against multiresistant bacterial infections.

1. Introduction

Propolis is a resinous mixture produced by honeybees for various purposes. It is used to avoid intruders by protecting the entrance of the hive and sealing the holes; to prevent contamination inside the hive by bacteria, viruses or parasites because of its antiseptic effect; as well as to cover intruders who died inside the hive in order to avoid their decomposition (Salatino et al., 2005; Righi et al., 2010). Many plant constituents are found in propolis composition e.g. plant resins, pollen and bud excretions. Additionally, it is also found bee products as beewax and bees secretion, so it has been considered an organotherapy product (Daugsch et al., 2007; Mendonça et al., 2015; Anvisa, 2005). After collecting the plant parts, the resin is chewed, then salivary enzymes are added and the partially digested material is mixed with beeswax (Burdock, 1998).

In Brazil, we have 13 groups of propolis based on physiochemical characteristics (Park et al., 2000; Daugsch et al., 2007). The characteristic of each different type of propolis is dependent of some factors as e.g. plant source and edaphoclimatic conditions (Silva et al., 2015). The most recent type of propolis discovered in northeast Brazil has a reddish color and is located in beehives along the river shores (Daugsch et al., 2007). The Brazilian red propolis is found in the states of Alagoas, Paraíba, Pernambuco, Sergipe, Bahia and Roraima (Daugsch et al., 2007; López et al., 2014). However, red propolis can also be found in other countries as Cuba, China, Mexico and Venezuela (Trusheva et al., 2004; Daugsch et al., 2007; Izuta et al., 2009; Lotti et al., 2010; Piccinelli et al., 2011). One species has been suggested to be the botanical source for the Brazilian red propolis: *Dalbergia ecastophyllum*. However, in other countries, other botanical sources are associated to red propolis as *Clusia major*, *C. minor* and *C. scrobiculata* in Venezuela and *C. rosea* in Cuba

* Corresponding author.
E-mail address: mareg1988@gmail.com (M.S. Regueira).

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(Tomas-Barberan et al., 1993; Cuesta-Rubio et al., 2002; Trusheva et al., 2004, 2006).

Previous studies have reported many biological activities for the Brazilian red propolis like antioxidant (Alencar et al., 2007; Righi et al., 2010; Frozza et al., 2013), antimicrobial (Bispo-Junior et al., 2012; Cabral et al., 2009; Righi et al., 2010), anticancer (Awale et al., 2008; Li et al., 2008), anti-inflammatory (Bueno-Silva et al., 2013; Cavendish et al., 2015), cytotoxicity (Alencar et al., 2007), repair of wounds (Batista et al., 2012) and antinociceptive (Cavendish et al., 2015). These properties are attributed to the phenolic compounds, which are present in high amount in Brazilian red propolis, but the substances involved in those biological activities are still being researched. Some studies showed that isoflavones found in red propolis have a participation in many activities as e.g. antimicrobial, antifungal, anticancer and antioxidant (Alencar et al., 2007; Freires et al., 2016). Flavanones, isoflavones, chalcones and pterocarpans have displayed important activity against cancer cell lines (Li et al., 2008). Neovestitol and vestitol isolated from Brazilian red propolis inhibited neutrophil migration in mouse thus showing an anti-inflammatory activity (Bueno-Silva et al., 2013).

In the last decade, many researchers focused their efforts on the discovery of chemicals that participates in the composition of the Brazilian Red Propolis. Among the red propolis samples from the states of Alagoas, Paraíba and Sergipe, it has been found flavones, isoflavones, flavonols, chalcones, aurones, pterocarpans and xanthones (Awale et al., 2008; Li et al., 2008; López et al., 2014; Mendonça et al., 2015). However, some chemical compounds are more frequently found in those samples and between them, there are formonetin, biochanin A, quercetin, pinocembrin, daidzein, medicarpin, homopterocarpin and liquiritigenin (Alencar et al., 2007; Cabral et al., 2009; Zulueta et al., 2009; Righi et al., 2010; Piccinelli et al., 2011; Oldoni et al., 2011; Frozza et al., 2013; Cavendish et al., 2015; Lopez et al., 2015). These major compounds are also present in the exudate of *Dalbergia ecastophyllum*, the mean source for the production of red propolis in the Northeast of Brazil. On the other hand, López et al. (2014) showed that probably a second plant species participates as one of the mean source of resins for the red propolis in Brazil.

The red propolis of some Northeastern Brazilian states (e.g. Alagoas, Paraíba and Sergipe) have been tested for antibacterial activity against Gram-negative bacteria – *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebisiella pneumoniae*, *Escherichia coli*, *Proteus mirablis*, *P. morganii*, *Neisseria meningitides*, *Haemophilus influenzae* and *Enterobacter aerogenes* – (Righi et al., 2010; Lopez et al., 2015) and Gram-positive bacteria – *Bacillus subtilis*, *Enterococcus faecalis*, *Estreptococcus pyogens*, *Staphylococcus aureus* and *S. epidermidis* (Alencar et al., 2007; Cabral et al., 2009; Righi et al., 2010; Lopez et al., 2015). Trusheva et al. (2006) attributed the antibacterial activity found in the Brazilian red propolis to the flavonoids, especially isoflavonoids and prenilated benzophenones. However, studies about the antimicrobial activity of the red propolis from other Brazilian states remains very scarce.

Resistance of microorganism to commercial antimicrobials drugs has been reported in the literature, so scientists all over the world are gathering efforts to find an alternative way to treat resistant microbial infections in humans diseases (Mandal et al., 2010; Eumkeb et al., 2012). The association between synthetic antimicrobial agents and natural substances have been studied and some combinations have had synergistic effect to inhibit bacterial growing (Matias et al., 2010; Hassan et al., 2016). Propolis has been combined with some kind of drugs, e.g. anticancer (Guo et al., 2015), antibacterial (Wojtyczka et al., 2013), antifungal (Pippi et al., 2015) and in some studies, it was observed a synergistic effect (Fernandes et al., 2005; Wojtyczka et al., 2013). The association of Brazilian red propolis with antifungals showed synergistic effect against *Candida* sp, however studies with red propolis and antimicrobials agents remains scarce (Pippi et al., 2015).

The major effect that can affect the chemical composition of propolis is the geographical location. Every place on earth has its own particularities concerning to the diversity of plants, climate, soil, and these factors together strongly influences the chemical composition of propolis (Teixeira et al., 2008; Sampaio et al., 2016). However, seasonality is also an important factor that can determine the propolis composition due to the influence of phenological factors in biosynthesis of plant metabolites (Teixeira et al., 2008). Seasonal variation of chemical compounds from propolis were previously described for Brazilian propolis, but was not observed a significant variation for antibacterial activity (Sforcin et al., 2000; Jorge et al., 2008; Teixeira et al., 2008).

The Northeast of Brazil has two seasons (drier and rainy) that are very distinct between them and one of these differences comprises the amount of rain during the year (APAC, 2016). Accordingly, these seasonal variations, as mentioned above, can influence the biosynthesis of plant metabolites and consequently affect the resin that is secreted by plants, which is used by the honeybees to produce propolis (Teixeira et al., 2008). Therefore, we aimed to investigate the effect of the dry and rainy season on the antibacterial activity and chemical composition of the Brazilian red propolis to bring the possibility whether a better antibacterial activity could be assessed for the red propolis alone or in association with standard drugs.

2. Material and methods

2.1. Propolis sampling and processing

Red propolis produced by Africanized *Apis mellifera* was collected in apiaries located in Tamandaré, state of Pernambuco. Those samples were collected in two moments: rainy season (between April and august of 2014) and dry season (between December/2015 and March/2016). Those periods are historically known by have the higher and the lowest precipitation in the state of Pernambuco (APAC, 2016), respectively. Raw red propolis from Pernambuco Rainy (RP-PER) and Dry (RP-PED) season were stored at −20 °C and then the propolis samples were dissolved in hydroethanolic solution (ethanol 54%) for 72 h. Afterward, the extract was filtered and concentrated using a rotary vacuum evaporator (model Q-344B-Quimis, Brazil). The concentrated solution was frozen and then lyophilized to obtain a fine powder of the red propolis hydroethanolic extract. For the microbiological assays, the dry extract of red propolis was solubilized in dimethyl sulfoxide (DMSO) and afterward, the DMSO-red propolis solution was diluted in sterile water to reach the concentration of 1024 μg/mL.

2.2. Chemical, apparatus and general procedures for HPLC-DAD

All chemical were of analytical grade. Acetonitrile, methanol, phosphoric acid, chlorogenic acid, caffeic acid, *p*-coumaric acid and ellagic acid were purchased from Merck (Darmstadt, Germany). Quercetin, apigenin, vitexin, rutin and luteolin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). The standards vitexin and rutin were used only for RP-PER samples and the p-coumaric acid for the RP-PED samples. High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

2.3. HPLC-DAD analysis

2.3.1. Red propolis rainy season

Chromatographic analyses were carried out under gradient conditions using Phenomenex C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% formic acid and methanol. Methanol concentration was gradually increased as follows: 0–5 min, 10%; 5–7 min, 20%; 7–31 min, 31%; 32–44 min, 40%; 44–50 min, 100%; and 55–60 min, 3%; following the method described by Boligon et al. (2015) with slight modifications. Red *propolis* hydroethanolic extract and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use, the extract was analyzed at a concentration of 25 mg/mL. The flow rate was 0.6 mL/min and the injection volume was 40 μl. The sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030–0.200 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 325 for chlorogenic acid, caffeic acid and ellagic acid; and 367 nm for rutin, vitexin, quercetin, apigenin and luteolin. Chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–500 nm). All chromatography operations were carried out at ambient temperature and in triplicate. Calibration curve for chlorogenic acid: $Y = 13519x + 1267.4$ ($r = 0.9999$); caffeic acid: $Y = 11964x + 1173.5$ ($r = 0.9998$); ellagic acid: $Y = 11845x + 1309.6$ ($r = 0.9995$); rutin: $Y = 12573x + 1258.9$ ($r = 0.9999$); quercetin: $Y = 13508x + 1267.2$ ($r = 0.9997$); vitexin: $Y = 12549x + 1182.3$ ($r = 0.9994$); luteolin: $Y = 12651x + 1376.4$ ($r = 0.9998$) and apigenin: $Y = 11987x + 1293.6$ ($r = 0.9999$).

2.3.2. Red propolis dry season

RP-PED hydroethanolic extract at a concentration of 15 mg/mL was injected by means of a model SIL-20A Shimadzu Auto sampler. Separations were carried out using Phenomenex C_{18} column (4.6 mm × 250 mm × 5 μm particle size). The mobile phase was water with 1% phosphoric acid (v/v) (solvent A) and HPLC grade acetonitrile (solvent B) at a flow rate of 0.6 mL/min and injection volume 40 μL. The composition gradient was: 5% solvent B reaching 15% at 10 min; 30% solvent B at 25 min, 65% solvent B at 40 min and 98% solvent B at 45 min, followed by 50 min at isocratic elution until 60 min. At 80 min the gradient reached the initial conditions again, following the method described by Carvalho et al. (2016) with slight modifications. The sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the acetonitrile: water (1:1, v/v) at a concentration range of 0.030–0.500 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 254 nm for ellagic acid; 327 nm for caffeic acid, *p*-coumaric acid and chlorogenic acids; and 366 for quercetin, apigenin and luteolin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–600 nm). Calibration curve for chlorogenic acid: $Y = 12457x + 1045.9$ ($r = 0.9996$); caffeic acid: $Y = 13509x + 1173.2$ ($r = 0.9998$); apigenin: $Y = 10865x + 1473.7$ ($r = 0.9999$); ellagic acid: $Y = 12758x + 1064.8$ ($r = 0.9993$); quercetin: $Y = 11470x + 1357.8$ ($r = 0.9997$); *p*-coumaric acid: $Y = 13541x + 1269.5$ ($r = 0.9999$) and luteolin: $Y = 10582x + 1346.9$ ($r = 0.9997$). All chromatography operations were carried out at ambient temperature and in triplicate.

2.4. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al. (2015). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.5. Bacterial strains

The strains used were the clinical isolates of *Escherichia coli* 06 (EC06), *Pseudomonas aeruginosa* 03 (PA03), *P. aeruginosa* 24 (PA24) and *Staphylococcus aureus* 10 (SA10) and standard strains of *E. coli* ATCC 25922 (ECATCC) and *S. aureus* ATCC 6538 (SAATCC). The bacterial strains were maintained and activated, prior to the assay, overnight at 37 °C on Nutrient Agar (Difco Laboratories).

2.6. Drugs

Gentamicin and Imipenem were obtained from Sigma Chemical Co. All drugs were dissolved in sterile water.

2.7. Minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of Red Propolis from Pernambuco Rainy and Dry season were determined in a microdilution assay using 100 μL of each suspended bacterial strain inoculum in saline solution which corresponds to 0.5 of McFarland scale, followed by the addition of 900 μL brain heart infusion (BHI) in 1,5 mL microtubes. The BHI-inoculum solutions were transferred to a 96-well microtiter plates and serial dilutions of each red propolis extract was performed with concentrations ranging from 512 to 8 μg/mL (1:1). The plates were incubated for 24 h at 37 °C and the bacterial growth was assessed by the use of resazurin. The MIC was defined according to CLSI (2008) in which is the lowest concentration where no growth can be observed. The antibacterial assays were performed in triplicates and results were expressed as an average of replicates.

2.8. Combined effect of red propolis with standard antibiotics

We combined the RE-PER and the RE-PED with Imipinem and Gentamicin to assess whether the red propolis can decrease the MIC of standard antibiotics. The increase of the antibiotic activity of the standard antibiotics was tested using a sub-inhibitory concentration of the extracts of red propolis (MIC/8). For the control, 150 μL of each bacterial strain in saline solution (0.5 in McFarland scale), were added to a 1,5 mL micro tubes together with 1350 μL of BHI broth. For the tests, 150 μL of each bacterial strain in saline solution (0.5 in McFarland scale), were added together with the red propolis extract from the rainy and dry season (MIC/8) and completed with BHI until reach 1,5 mL. The previous solutions were then transferred to a 96-well microtiter plate and serial dilutions (1:1) were performed with 100 μL of the antimicrobial drugs. The plates were incubated at 37 °C for 24 h and the bacterial growth was assessed with resazurin. The plates were incubated at 37 °C for 24 h, and bacterial growth was assessed by the use of resazurin. MIC was defined with the concentrations of antibiotics between 2500 and 1,22 μg/mL. The MIC of controls were assessed using the antibiotics alone.

2.9. Statistical analysis

2.9.1. Microbiological results

The assays were performed in triplicates and results were

expressed as average of replicates. The results are expressed as the geometric mean. Two-Way ANOVA was applied as Statistical hypothesis analysis followed by Bonferroni post hoc using GraphPad Prism 6.0 software (2008).

2.9.2. HPLC-DAD

Differences between groups of HPLC were assessed by an analysis of variance model and Tukey's test. The level of significance for the analyses was set to $p < 0.05$. These analyses were performed by using the free software R version 3.1.1. (R Core Team, 2014).

3. Results

3.1. Chemical characterization of red propolis

3.1.1. Rainy season

HPLC fingerprinting of RP-PER hydroethanolic extract revealed the presence of two major peaks (4 and 7) identified as vitexin and luteolin and the others were identified as chlorogenic acid, caffeic acid, ellagic acid, rutin, quercetin and apigenin (Fig. 1 and Table 1).

3.1.2. Dry season

The HPLC profile of RP-PED is shown in Fig. 2. Two major peaks (5 and 6) stands out and they were identified as quercetin and luteolin. The sample contains other minor compounds in addition to the other two: chlorogenic acid, caffeic acid, ellagic acid, *p*-coumaric acid and apigenin (Fig. 2 and Table 1).

4. Microbiological assays

4.1. Minimal inhibitory concentration

The red propolis showed MIC values (Table 2) ranging from 128 μg/mL to 512 μg/mL against *E. coli* strains and from 64 μg/mL to ≥1024 μg/mL against *S. aureus* strains. However, for the *P. aeruginosa* strains the MIC values were all the same, 512 μg/mL.

4.2. Combined effect of red propolis with standard antibiotics

The Brazilian red propolis, which was collected in the dry season, has demonstrated to improve the imipenem and gentamicin activity against strains of *P. aeruginosa* and *S. aureus*. However, there was no synergistic effect between the red propolis collected in the rainy season and the imipenem and gentamicin against the same strains (Fig. 3). The RP-PER did not influenced the effect of gentamicin against the PA03, SA10 and SAATCC, except against the PA24 strain, which there was an antagonist effect. When the RP-PER was combined with imipenem we also observed no effect against all of the strains used in this study. On the other hand, the RP-PED improved the gentamicin activity against all bacterial strains. A synergistic effect was also observed against *P. aeruginosa* (PA03 and PA24) when we combined the RP-PED with imipenem. The RP-PED did not improve the imipenem activity against *S. aureus* strains (SA10 and SAATCC).

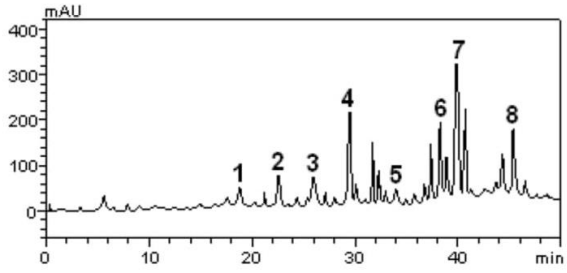

Fig. 1. Chemical profile of the RP-PER hydroethanolic extract. Chlorogenic acid (peak 1), caffeic acid (peak 2), ellagic acid (peak 3), vitexin (peak 4), rutin (peak 5), quercetin (peak 6), luteolin (peak 7) and apigenin (peak 8).

5. Discussion

5.1. Chemical characterization of red propolis

Honey Bees that produces red propolis has been already reported in three continents: Africa (Nigeria), Asia (Saudi Arabia) and America (Brazil, Cuba, Mexico and Venezuela) (Trusheva et al., 2004; Piccinelli et al., 2005, 2011; Lotti et al., 2010; Lopez et al., 2015; Omar et al., 2016; Saddiq and Mohamed, 2016). The red propolis found at those locations has its own particularities regarding to chemical composition. Those specific characteristics are associated with the geographical location where the red propolis is produced. The edaphoclimatics conditions and the botanical source are important factors, which can affect the chemical composition of propolis (Silva et al., 2015).

The HPLC analysis of the Brazilian red propolis sampled from the state of Pernambuco allowed us to identify nine compounds: apigenin, caffeic acid, chlorogenic acid, ellagic acid, luteolin, p-coumaric acid, quercetin, rutin and vitexin. The compounds apigenin, chlorogenic acid, ellagic acid and vitexin were never described before for red propolis. Among them we have flavonoids, acid hidroxycinnamic and derivatives of acid hidroxycinnamic. Those bioactive compounds are most known by have some biological activities, e.g. neuroprotective, anti-cancer, anti-inflammatory, antioxidante, antimicrobial and anti-leishmania (Ural et al., 2015; Bak et al., 2016; Fonseca-Silva et al., 2015; Kasala et al., 2016; Tsang et al., 2016; Pei et al., 2016). Flavonoids represents more than 60% of the composition of the Brazilian red propolis and many chemical classes have been found, e.g. flavones, flavanones, isoflavones, isoflavonoids, chalcones, aurones, flavonols and neoflavonoids (Silva et al., 2007; Daugsch et al., 2007; Alencar et al., 2007; Li et al., 2008; Cabral et al., 2009; Zulueta et al., 2009; Righi et al., 2010; Piccinelli et al., 2011; Oldoni et al., 2011; Frozza et al., 2013; Mendonça et al., 2015; Cavendish et al., 2015; Lopez et al., 2015).

The compounds p-coumaric acid, rutin, quercetin, Luteolin and caffeic acid were previously reported only in Brazilian red propolis samples from Alagoas, Brazil (Silva et al., 2007; Daugsch et al., 2007; Cabral et al., 2009; Mendonça et al., 2015). However, a variety of chemical compounds have been found in red propolis samples from the Northeast of Brazil (Freires et al., 2016). Most of the compounds identified in red propolis were from the State of Alagoas (Alencar et al., 2007; Cabral et al., 2009; Mendonça et al., 2015), followed by the states of Segipe (Pinheiro et al., 2014) and Paraiba (Lopez et al., 2015). The red propolis from the states of Pernambuco and Bahia are placed as less studied in number of publications.

5.2. Seasonal effect on chemical compounds of Brazilian red propolis

This is the first report of seasonal variation of the concentrations of chemicals compounds in red propolis. When we compared the concentration of the compounds found in both propolis used in this study, we observed that the concentrations of quercetin, caffeic acid and ellagic acid were almost two times higher in RP-PED than in RP-PER. However, we observed an increase of luteolin and apigenin in the RP-PER compared to RP-PED. The concentration of

Table 1
Components of red *propolis* hydroethanolic extract.

Compounds	RP-PER	LOD	LOQ	RP-PED	LOD	LOQ
	mg/g	μg/mL	μg/mL	mg/g	μg/mL	μg/mL
Chlorogenic acid	0.73 ± 0.01 a	0.011	0.037	0.97 ± 0.03 a	0.015	0.049
Caffeic acid	1.32 ± 0.03 b	0.009	0.029	2.15 ± 0.01 b	0.008	0.026
Ellagic acid	1.28 ± 0.01 b	0.013	0.042	2.09 ± 0.03 b	0.026	0.085
Vitexin	3.69 ± 0.01 c	0.028	0.093			
Rutin	0.45 ± 0.02 d	0.031	0.102			
p-Coumaric acid				0.18 ± 0.04 c	0.013	0.043
Quercetin	3.19 ± 0.03 e	0.015	0.051	6.34 ± 0.01 d	0.024	0.079
Luteolin	7.51 ± 0.01 f	0.026	0.085	4.11 ± 0.02 e	0.016	0.052
Apigenin	2.97 ± 0.04 e	0.017	0.058	1.95 ± 0.05 b	0.011	0.034

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$.

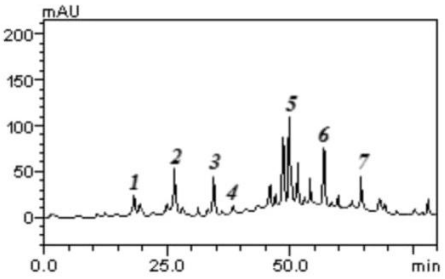

Fig. 2. Chemical profile of RP-PED hydroethanolic extract. Chlorogenic acid (peak 1), caffeic acid (peak 2), ellagic acid (peak 3), *p*-coumaric acid (peak 4), quercetin (peak 5), luteolin (peak 6) and apigenin (peak 7).

Table 2
Values of MIC of the Brazilian red propolis from rainy and dry season against bacterial strains.

Bacterial strains	MIC (μg/mL)	
	RP-PER	RP-PED
EC06	128	512
ECATCC	512	161,3
PA03	512	512
PA24	512	512
SA10	64	101,6
SAATCC	≥1024	512

chlorogenic acid were slightly higher in the RP-PED. A previous study with Argentinian propolis showed that the extracts of propolis collected in the summer and spring had a higher content of phenolic and flavonoid contents than the samples collected in winter and autumn (Isla et al., 2012). It was also observed that concentrations of phenolic compounds from Brazilian propolis showed seasonal variations. Other studies reported that in samples of alecrim propolis an inversely proportional amount of triterpenoids and phenolic was observed (Teixeira et al., 2008). Similar phenomenon was observed by Bankova et al. (1998), where diterpenes started appearing in summer and reached a maximum in autumn.

That variation found in chemical compounds of red propolis is probably due to a seasonal pressure suffered by the botanical font. We believe that because the mean element in the production of red propolis is a resin secreted by *Dalbergia ecastophyllum* trees and the production and accumulation of secondary metabolites of plants are influenced by seasonal variations. In a study about the effect of the environment on the secondary metabolic profile of *Tithonia diversifolia*, Sampaio et al. (2016) observed that samples collected during the rainiest period were rich in primary metabolites, meanwhile the leaf and stem samples collected during the drier periods were rich in secondary metabolites. The mentioned study could also explain why the red propolis samples collected in the drier periods had a higher concentration in four of the six compounds compared with the rainiest period.

5.3. Antimicrobial activity

Previous studies have shown that Brazilian red propolis has a large content of phenolic compounds, mostly flavonoids and these phenolic compounds found in red propolis are associated to antibacterial activity (Cabral et al., 2009; Freires et al., 2016). The antibacterial activity of the hydroethanolic extract of Brazilian red propolis from the state of Pernambuco were tested against strains of *E. coli*, *P. aeruginosa* and *S. aureus*. The MIC values of *E. coli* ranged from 128 μg/mL to 512 μg/mL (EC 06) and from 161 μg/mL to 512 μg/mL (EC ATCC). These results are similar to those found by Righi et al. (2010), Bispo-Junior et al. (2012) and Lopez et al. (2015) when they evaluated the MIC of red propolis from the states of Alagoas, Sergipe and Paraíba. The red propolis samples showed MIC values of 512 μg/mL against both strains of *P. aeruginosa* used in our study (PA03 and PA24). Red propolis samples from the states of Alagoas, Paraíba and Sergipe showed MIC values above 200 μg/mL (Lopez et al., 2015). Red propolis samples from Alagoas analyzed by Righi et al. (2010) presented MIC 256 μg/mL. Red propolis MIC values against strains of Gram-positive bacteria *S. aureus* ranged from 64 μg/mL to 101,6 μg/mL (SA10) and from 512 μg/mL to ≥1024 μg/Ml (SAATCC). Similar results against *S. aureus* were reported for red propolis from Alagoas, Paraíba and Sergipe (Alencar et al., 2007; Cabral et al., 2009; Oldoni et al., 2011; Bispo-Junior et al., 2012; Bueno-Silva et al., 2013; Lopez et al., 2015). In a study with plant extract, Kuete (2010) showed that an extract has significant antibacterial activity if MIC is 100 μg/mL, moderate if its MIC is between 100 and 625 μg/mL and low when MIC is above 625 μg/mL. Based on this, we can deduce that both red propolis hydroethanolic extract has antibacterial effect against the bacterial strains used in this study. As we observed in our results, the red propolis extract was more effective against Gram-positive bacteria than the Gram-negative bacteria. This was also observed in previous studies when the ethanolic extract of red propolis was tested against *S. aureus* (Alencar et al., 2007; Cabral et al., 2009; Oldoni et al., 2011; Bispo-Junior et al., 2012; Bueno-Silva et al., 2013; Lopez et al., 2015). The mechanisms of antibacterial activity, as mentioned above, involves the flavonoids present in the propolis. In the literature, some reviews summarized these mechanisms, they are: (1) inhibition of nucleic acids synthesis (DNA and RNA); (2) inhibitory mechanism on DNA gyrase; (3) inhibition of cytoplasmic membrane function (damage to the cytoplasmic membrane caused by a reduction in the fluidity); (4) Inhibition of energy metabolism (due to the damage to

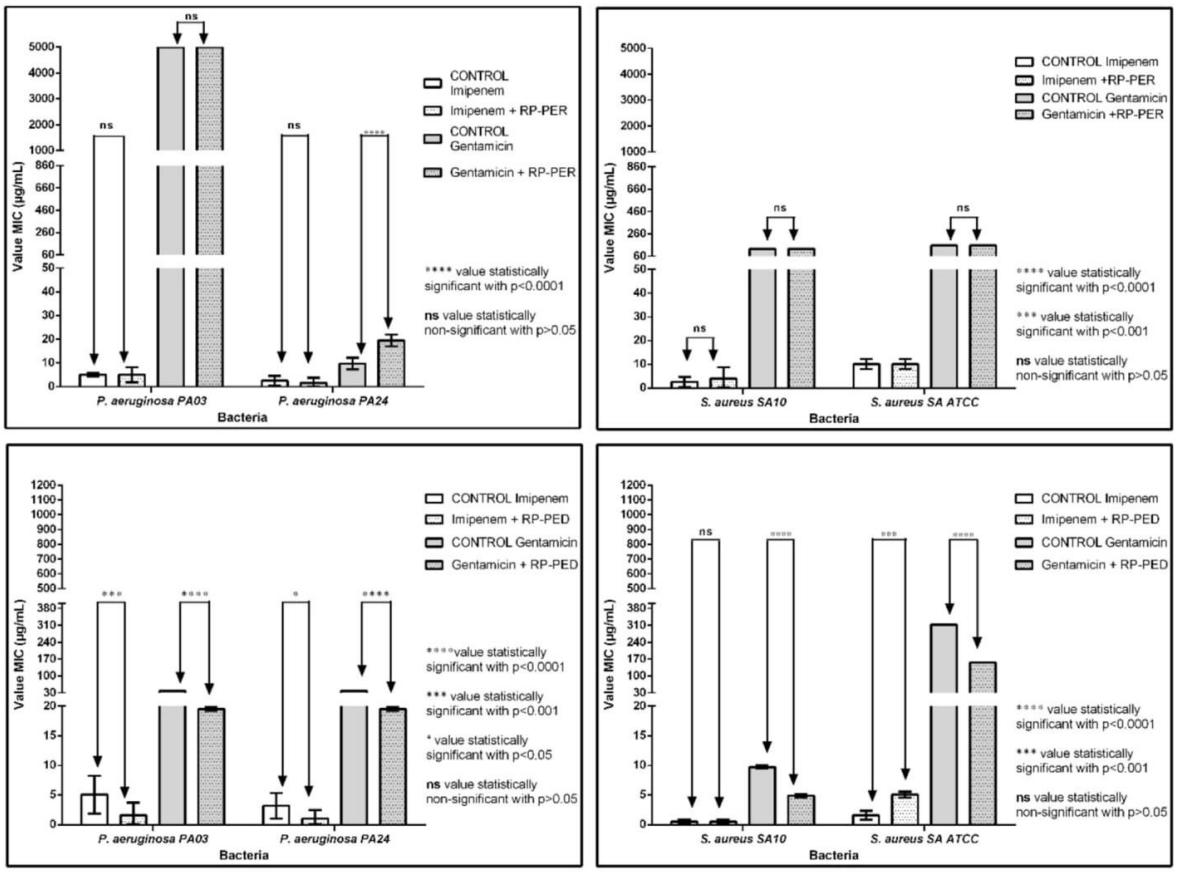

Fig. 3. Combined effect of red propolis and antibacterial drugs. The histograms show the results of the antibiotic activity of the extract of red propolis from rainy and dry season combined with standard drugs, against strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

the cytoplasmic membrane the exchange of nutrients and metabolites is disturbed, inhibiting the energy supply); (5) Inhibition of attachment and biofilm formation (Xie et al., 2015; Freires et al., 2016).

5.4. Seasonal variation of minimal inhibitory concentration

In our study, we compared the antibacterial activity of samples of red propolis from rainy and dry seasons, then we could observe different MIC values between them. Then we observed that the hydroethanolic extract of RP-PER had the lowest MIC against the multiresistant strain of *Staphilococcus aureus* (SA10) that inhibited the bacterial growing with 64 µg/mL. However, the MIC of RP-PED against same strain had an increase (101,6 µg/mL). The same happened when we tested the red propolis samples against the a multiresistant strain of *Escherichia coli*. The RP-PER showed the lowest MIC (128 µg/mL) when compared to the MIC of RP-PED (512 µg/mL). The hydroethanolic extract of RP-PED showed a lower MIC in comparison to the RP-PER extract against the ECATCC and SAATCC strains. The red propolis samples from rainy and drying seasons had the same MIC for both *Pseudomonas aeruginosa* strains (512 µg/mL). In other studies, seasonal variation of the antibacterial activity of propolis against *S. aureus* and *E. coli* were observed, but no significant differences was observed along the year (Sforcin et al., 2000; Jorge et al., 2008). However, the Argentinian propolis sample collected in the summer and autumn showed higher antibacterial activity than the other samples (Isla et al., 2012).

5.5. Seasonal variation of combined effect of red propolis with standard antibiotics

Gentamicin and Imipenem are effective antibiotics against Gram-positive and Gram-negative bacteria, e.g. *Escherichia coli*, *Klebisiella* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Selim et al., 2012; Zhang et al., 2014; Saha et al., 2016; Vázquez et al., 2016). However, multidrug resistance it is a major concern all over the world, especially in the hospital environment, and it has significantly increased in recent years (Özçelik et al., 2008; Biedenbach et al., 2016). Previous studies have shown that association of standard drugs with phenolic compounds can enhance the activity of these drugs against a range of diseases (Mun et al., 2013; Amin et al., 2015; Guo et al., 2015). Taking in to account what was mentioned above, we decided to test both of our red propolis samples combined with standard antibiotics against strains of *S. aureus* and *P. aeruginosa*. Our results showed that RP-PER combined with imipenem and gentamicin had no synergistic effect against the strains of *S. aureus* and *P. aeruginosa*. On the other hand, a synergistic effect was observed when we combined the RP-PED

with gentamicin against all the strains tested. When we combined the RP-PED with Imipenem, we only observed synergistic effect against *P. aeruginosa* strains. However, The RP-PED-imipenem association was not effective against *S. aureus* strains. A synergistic effect of red propolis was also observed against *Candida* spp. when the propolis was combined with fluconazole (Pippi et al., 2015).

Other types of propolis also showed synergistic activity against microorganisms. Synergistic effect between propolis and gentamicin against *S. aureus* was also observed by the Kirby and Bauer and E-test methods (Fernandes-Júnior et al., 2005). Wojtyczka et al. (2013) tested the ethanolic extract of propolis associated with 10 different antimicrobial drugs against clinical isolates of *S. aureus*. The study revealed synergism between the ethanolic extract of propolis and eight antimicrobial drugs against all tested strains of *S. aureus* (Wojtyczka et al., 2013). In a study with Bulgarian and Brazilian propolis the authors also observed a synergism with antimicrobial drugs against *Salmonella Typhi* (Orsi et al., 2006, 2012). The application of the association of chemical compounds isolated from natural products with standard drugs against bacterial infections could open a new horizon for the application of bee products, as propolis, in the treatment of bacterial infections, which became resistant to standard drugs.

Bacteria cell wall or membrane damage is probably the mean mechanism in which propolis chemical compounds participates. That mechanism makes the entrance inside the cell easier for antibiotics and, consequently, enhance its activity (Orsi et al., 2006, 2012). Some studies have shown that flavonoids, eg. apigenin, quercetin, galangin, pnocembrin, and caffeic acid, benzoic acid, cinnamic acid act synergistically with antibiotics causing functional and structural damages to the outer cytoplasmic membrane (Scazzocchio et al., 2006; Xie et al., 2015). Also, other mechanisms could be involved in the synergism between the flavonoids found in propolis and other drugs, as peptidoglycan synthesis inhibition and inhibition of the activity of β-lactamase enzymes (Eumkeb and Chukrathok, 2013). In addition, it was observed that pinocembrin, a common flavonoid found in the Brazilian red propolis, inhibited the efflux activity, decreasing the MIC of ethidium bromide (EtBr) and rifampicin against mycobacteria (Gröblacher et al., 2012).

Here, we have the first report of synergistic effect of Brazilian red propolis combined with imipenem and gentamicin against *P. aeruginosa* and *S. aureus*. As mentioned above, many studies have been performed with Gram-positive bacteria, however studies with Gram-negative bacteria remains scarce. A previous report made by Taher (2015) showed that a resistant strain of the Gram-negative *Klebsiella pneumoniae* became susceptible when the antibiotic drugs were combined to propolis. In our study, we observed that only propolis collected in the drier period showed synergistic effect with imipenem and gentamicin. What could explain this variation is the fact that the concentrations of compounds are higher in RP-PED than in RP-PER due to the influence of seasonality on resin plants what directly affects the chemical profile of red propolis.

6. Conclusions

The HPLC analysis allowed us to identify four compounds that have never been reported before for the Brazilian red propolis. We could also observe a seasonal variation according to concentrations of the compounds that were identified. We compared the concentration of six compounds in the rainiest and driest periods and we observed that four of them had the highest concentration in the dry season.

The antibacterial tests showed that the red propolis collected in the state of Pernambuco is effective against strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. We observed seasonal variations on MICs against *E. coli* and *S. aureus*, but no seasonal variation was observed against *P. aeruginosa*.

The results obtained with the synergistic analysis showed that the RP-PED had a better performance than the RP-PER when combined with the antibacterial drugs imipenem and gentamicin. The RP-PER had no synergistic effect against all the used in this study. However, when we combined the RP-ED with gentamicin we obtained synergistic effect against all bacterial strains. On the other hand, when we combined the RP-PED with imipenem, we only observed a synergistic effect against the *P. aeruginosa* strains. These results encourage us to explore the seasonal variants of natural products and look for to that one who has the best results against pathogenic bacteria.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.03.052.

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3.2 ARTIGO 2 - ANÁLISE COMPARATIVA DA ATIVIDADE ANTIBACTERIANA E DO PERFIL FITOQUÍMICO DA PRÓPOLIS VERMELHA BRASILEIRA E DA RESINA DE *Dalbergia ecastaphyllum* (L) TAUB

Marcos da Silveira Regueira-Neto[a], Saulo Relison Tintino[b], Ana Raquel Pereira da Silva[b], Maria do Socorro Costa[b], Aline Augusti Boligon[c], Irwin R.A. Menezes[b], Valdir de Queiroz Balbino[a], Henrique Douglas de Melo Coutinho[b].

[a]Laboratório de Bioinformática e Biologia Evolutiva, Departamento de Genética, Centro de Biociências, UFPE, Recife, PE, Brazil

[b]Laboratório de Microbiologia e Biologia Molecular, Centro de Ciências Biológicas e Saúde, Universidade Regional do Cariri, Crato, CE, Brazil

[c]Phytochemical Research Laboratory, Department of Industrial Pharmacy, Federal University of Santa Maria, Santa Maria, Brazil

Corresponding author:

Marcos da Silveira Regueira-Neto

mareg1988@gmail.com

Departamento de Genética, CB, Universidade Federal de Pernambuco

Pernambuco, Recife, Brazil

Abstract

Red propolis is a resinous substance produced by honey bees using a plant resin that is exuded by *Dalbergia ecastaphyllum* (L) Taub. This type of propolis has been extensively explored in studies that tested the bioactivity against Gram-positive and

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Gram-negative bacteria, however the plant resin that is the source of bioactive compounds is underrepresented in those studies. The aim of this study was to investigate the antibacterial activity of red propolis and resin and their association with standard antibiotics to evaluate possible differences of activity. We also submitted red propolis and the resin to a HPLC analysis to confirm the botanical origin. The hydroethanolic extracts of red propolis and *D. ecastaphyllum* resin were analyzed by HPLC-DAD. The extracts were tested against strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* to obtain MIC. The extracts were also tested against *P. aeruginosa* and *S. aureus* in association with gentamicin and imipenem. The HPLC analysis identified seven compounds with six of them present in both substances. The lowest MIC values obtained in this study were observed against *S. aureus*. In general, MIC values showed to be lower for red propolis against all species tested in comparison to resin. Red propolis and resin showed synergistic effect against all the strains tested when combined with gentamicin. However, when we combined these substances to imipenem we only observed synergistic effect against *P. aeruginosa* strains. Despite the synergistic behavior to be similar for both substances we observed that inhibitory concentrations of drugs were lower when associated with red propolis in comparison to resin. In conclusion, both of the substance tested has antibacterial activity but the results showed that red propolis had a better performance and possibly indicate that honey bees can improve the activity of compounds present in red propolis. When we compared the quantitative values we observed that the inhibitory concentrations of drugs were lower when associated with red propolis.

Keywords: Red propolis; Resin; *Dalbergia ecastaphyllum*; HPLC; Antibacterial activity

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Abbreviations: A, antibiotics; BHI, Brain Heart Infusion; DER, Dalbergia ecastaphyllum resin; DMSO, dimethyl sulfoxide; EC06, *Escherichia coli* 06; ECATCC, *Escherichia coli* ATCC; LOD, limit of detection; LOQ, limit of quantification; MIC, minimal inhibitory concentration; PA03, *Pseudomonas aeruginosa* 03; PA24, *Pseudomonas aeruginosa* 24; RP, red propolis; SA10, *Staphylococcus aureus* 10; SAATCC, *Staphylococcu aureus* ATCC; SD, standard deviation.

1. Introduction

The genus *Dalbergia* (Fabaceae) consists of shrubs, lianas and trees which comprises around 250 species in total (Hartvig et al., 2015). This pantropical genus is distributed in the North, Central and South America and West Africa (zipcodezoo, 2017). Despite *Dalbergia ecastaphyllum* (L) Taub to be a non-mangrove plant, this species grows in association with mangrove in Brazil and its distribution comprises all Brazil's coastal region (Diop et al., 2001).

Many insect's species from the families Cerambicidae (Coleoptera), Curculionidae (Coleoptera), Pyralidae (Lepidoptera), Oecophoridae (Lepidoptera), Cossidae (Lepidoptera), are known for cause a variety of damages to trees causing many losses to the wood industry (Nair, 2007). In response to that insect attack trees have some mechanisms to avoid infestation and the entry of fungi. In tropical leguminous trees that produces resin there are pocket secretory structures. Those resin pockets are filled with resin secreted by resin-producing epithelial cells. The exudation of the resin, in some situations, is caused by injuries made by an agent attempting to enter the tissue. The resin pressure enables the trees to seal off injured areas, thereby inhibiting the entry of microrganisms (Langenheim, 2003).

The plant resin exuded by *D. ecastaphyllum* is used by honey bees *Apis mellifera* in the Northeast of Brazil for the production of red propolis. The red colored resin from *D. ecastaphyllum* is collected, chewed and salivary enzymes are added and then the partially digested material is mixed with bee's wax (Burdock, 1998). That resinous mixture called propolis is used by the bees to avoid intruders in the hive by protecting the entrance and sealing holes. Propolis is used also to prevent contamination inside the hive because of its antiseptic effect (Righi et al., 2010).

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Since the old times propolis has been used by humans around the world in the treatment of a range of diseases (Toreti et al., 2013). Propolis from different origins have specific physiochemical characteristics and in Brazil it was reported until now 13 different types of propolis (Park et al., 2000; Daugsch et al., 2007). The thirteenth type of Brazilian propolis, the red propolis, can be found in the northeast region of Brazil (Daugsch et al., 2007; Lopez et al., 2014). Red propolis samples from several Brazilian states (Alagoas, Paraíba, Pernambuco and Sergipe) were tested against Gram-negative and Gram-positive bacteria (Cabral et al., 2009; Righi et al., 2010; Lopez et al., 2015; Regueira Neto et al., 2017). The antibacterial activity of this type of propolis has been attributed to flavonoids, specially isoflavonoids and prenylated benzophenones (Trusheva e al., 2006).

Antibacterial resistance has become a big problem in public health (Biedenbach et al., 2016). In a study Lautenbach et al. (2010) reported that in the last decade the resistance of *Pseudomonas aeruginosa* to imipenem increased from 13% to 20%. In Addition to that, resistance to aminoglycosides has been observed in Gram-negative bacteria and one of the possible solutions to overcome this problem is the combination of drugs or drugs and natural products to reach a synergistic effect (Gad, Mohamed, and Ashour 2011). Previous studies has combined different types of propolis with antimicrobial and antifungal drugs and the authors observed synergistic effect between them (Pippi et al. 2015; Wojtyczka et al. 2013) . However, there are no reports of synergistic study using the plant resin of *Dalbergia ecastaphyllum*.

Red propolis gets all attention regard to application in medical studies, however the Resin that is the source of bioactive components is mostly explored when it comes to confirm the botanical origins of propolis through chemical characterization (Silva et al.,

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105 2007; Piccinelli et al., 2011; Lopez et al 2014). Antibacterial activity studies remain
106 scarce for the resin of *Dalbergia ecastaphyllum* and previous studies just tested the
107 resin against Gram-positive bacteria (Bueno-Silva et al. 2017).

108 The process in which the resin is submitted when it is chewed by honey bees still
109 unknown, but researchers have pointed out the chewing process and the mixing with
110 bee's saliva as an important step in propolis production (Ghisalberti, 1979; Marcucci,
111 1995). In addition to this, we also do not know how that process can affect the
112 compounds present in propolis. Therefore, the aim of this study was to investigate the
113 antibacterial activity of the resin and red propolis with and without association with
114 standard antibiotics to evaluate possible differences of activity. We also performed a
115 chemical characterization of the resin and red propolis to confirm the botanical origin.

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2. Material and Methods

2.1 Propolis and resin samples

Red propolis (RP) samples were collected in an apiary located at Japaratinga, state of Alagoas and resin (DER) samples were collected from *Dalbergia ecastaphyllum* trees located at Tamandaré, state of Pernambuco. The raw samples of red propolis and resin were stored at -20 °C and then they were dissolved in hydroethanolic solution (ethanol 54%) for 72 h. Afterward, the extract was filtered and concentrated using a rotary vacuum evaporator (model Q-344B-Quimis, Brazil). The concentrated solution was frozen and then lyophilized to obtain a fine powder. For the microbiological assays, the dry extract of RP and DER were solubilized in dimethyl sulfoxide (DMSO) and then DMSO-extract solutions were diluted in sterile water to reach the concentration of 1024 µg/mL.

2.2 Chemical, apparatus and general procedures

All chemicals were of analytical grade. Acetonitrile, phosphoric acid, chlorogenic acid, caffeic acid, *p*-coumaric acid and ellagic acid were purchased from Merck (Darmstadt, Germany). Quercetin, apigenin and luteolin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

2.3 HPLC-DAD analysis

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Propolis and Resin at a concentration of 15 mg/mL was injected by means of a model SIL-20A Shimadzu Auto sampler. Separations were carried out using Phenomenex C_{18} column (4.6 mm x 250 mm x 5 μm particle size). The mobile phase was water with 1% phosphoric acid (v/v) (solvent A) and HPLC grade acetonitrile (solvent B) at a flow rate of 0.6 mL/min and injection volume 40 μL. The composition gradient was: 5% solvent B reaching 15% at 10 min; 30% solvent B at 25 min, 65% solvent B at 40 min and 98% solvent B at 45 min, followed by 50 min at isocratic elution until 60 min. At 80 min the gradient reached the initial conditions again, following the method described by Carvalho et al. (2016) with slight modifications. The sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the acetonitrile: water (1:1, v/v) at a concentration range of 0.030 – 0.500 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 254 nm for ellagic acid; 327 nm for caffeic acid, *p*-coumaric acid and chlorogenic acids; and 366 for quercetin, apigenin and luteolin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 600 nm). Calibration curve for chlorogenic acid: Y = 12457x + 1045.9 (r = 0.9996); caffeic acid: Y = 13509x + 1173.2 (r = 0.9998); apigenin: Y = 10865x + 1473.7 (r = 0.9999); ellagic acid: Y = 12758x + 1064.8 (r = 0.9993); quercetin: Y = 11470x + 1357.8 (r = 0.9997); *p*-coumaric acid: Y = 13541x + 1269.5 (r = 0.9999) and luteolin: Y = 10582x + 1346.9 (r = 0.9997). All chromatography operations were carried out at ambient temperature and in triplicate.

2.4 Limit of detection (LOD) and limit of quantification (LOQ)

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LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al. (2015). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.5 Bacterial strains

The bacterial strains used in this study were the clinical isolates of *Escherichia coli 06* (EC06), *Pseudomonas aeruginosa* 03 (PA03), *P. aeruginosa* 24 (PA24) and *Staphylococcus aureus* 10 (SA10) and standard strains of *E. coli* ATCC 25922 (ECATCC) and *S. aureus* ATCC 6538 (SAATCC). The microorganisms EC06, SA10, PA03 and PA24 were obtained from clinical isolates (Table 1). The bacterial strains were maintained and activated, prior to the assay overnight at 37 °C on Nutrient Agar (Difco Laboratories).

Table 1 - Bacterial source and resistance profile of clinical isolates.

Bacteria	Source	Resistance profile
Escherichia coli 06	Urine culture	Cf, Cef, Ca, Cro
Pseudomonas aeruginosa 03	Urine culture	Cpm,Ctz,Imi,Cip,Ptz,Lev,Mer,Ami
Pseudomonas aeruginosa 24	Nasal secretion	Cpm,Ctz,Imi,Cip,Ptz,Lev,Mer,Ami
Staphylococcus aureus 10	Rectal swab	Ca, Cef, Cf, Oxa, Pen, Amp, Amox, Mox, Cip, Lev, Asb, Amc, Eri, Cla, Azi, Clin

Amp - Ampicillin; Asb - Ampicillin + Sulbactam; Ami - Amikacin; Amox - Amoxicillin; Amc - Amoxicillin + Ac. clavulanic; Azi - Azithromycin; Ca - Cefadroxil; Cf - Cephalotin; Cef - Cephalexin; Cla - Clarithromycin; Cro - Ceftriaxone; Ctz - ceftazidime; Cip - Ciprofloxacin; Cpm=Cfepime; Clin - Clindamycin; Eri – Eritromicin; Oxa - oxacillin; Imi

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– Imipenem; Lev - levofloxacin; Mer - meropenem; Mox - Moxifloxacin; Pen - Penicillin; Ptz - Piperacillin + Tazobactam.

2.6 Drugs

Gentamicin and Imipenem were obtained from Sigma Chemical Co. All drugs were dissolved in sterile water.

2.7 Minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of red propolis and resin were determined in a microdilution assay using 100 µL of each suspended bacterial strain inoculum in saline solution which corresponds to 0.5 of McFarland scale, followed by the addition of 900 µL brain heart infusion (BHI) in 1,5 mL microtubes. The BHI-inoculum solutions were transferred to a 96-well microtiter plates and serial dilutions of each extract were performed with concentrations ranging from 512 to 8 µg/mL (1:1). The plates were submitted to an incubation for 24 h at 37 °C and the bacterial growth was assessed by the use of resazurin. The MIC was defined according to CLSI (2008) in which is the lowest concentration where no growth can be observed. The antibacterial assays were performed in triplicates and results were expressed as an average of replicates.

2.8 Combined effect of red propolis and resin with standard antibiotics

Red propolis and resin hydroethanolic extract were combined with Imipinem and Gentamicin to assess whether these substances can decrease the MIC of standard antibiotics. The increase of the antibiotic activity of the standard drugs was tested using a sub-inhibitory concentration of the extracts of samples included in this study (MIC/8). For the control, 150 µL of each bacterial strain in saline solution (0.5 in McFarland scale), were added to a 1,5 mL micro tubes together with 1350 µL of BHI broth. For

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the tests, 150 µL of each bacterial strain in saline solution (0.5 in McFarland scale), were added together with the red propolis and resin extracts (MIC/8) and completed with BHI until reach 1,5 mL. The previous solutions were then transferred to a 96-well microtiter plate and serial dilutions (1:1) were performed with 100 µL of the antimicrobial drugs. The plates were incubated at 37 °C for 24 h and the bacterial growth was assessed with resazurin. The plates were incubated at 37° C for 24 h, and bacterial growth was assessed by the use of resazurin. MIC was defined with the concentrations of antibiotics between 2500 and 1,22 µg/mL. The MIC of controls were assessed using the antibiotics alone.

2.9 Statistical analysis

2.9.1 Microbiological results

We performed the assays in triplicates and results were expressed as average of replicates. The results are expressed as the geometric mean. Two-Way ANOVA was applied as Statistical hypothesis analysis followed by Bonferroni post hoc using GraphPad Prism 6.0 software (2008).

2.9.2 HPLC-DAD

Differences between groups of HPLC were assessed by an analysis of variance model and Tukey's test. The level of significance for the analyses was set to $p < 0.05$. These analyses were performed by using the free software R version 3.1.1. (R Core Team, 2014).

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3. Results

3.1 Chemical characterization of red propolis and plant resin

3.1.1 Red propolis

The chemical compounds of the red propolis is shown on the HPLC profile in Fig. 1. Two major peaks were observed in the profile (5 and 6) they were identified as quercetin and luteolin, respectively. The other compounds present in the red propolis were chlorogenic acid, caffeic acid, ellagic acid, p-coumaric acid and apigenin.

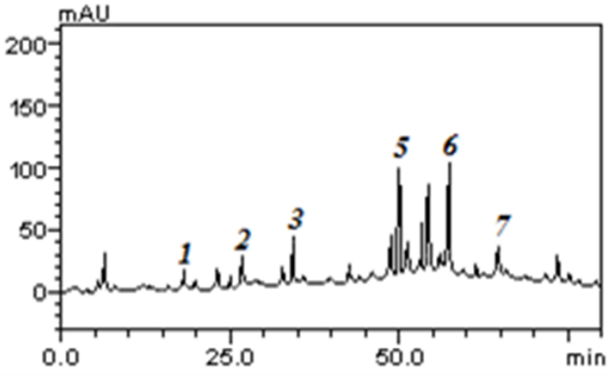

Fig. 1. Representative high performance liquid chromatography profile of red propolis hydroethanolic extract. Chlorogenic acid (peak 1), caffeic acid (peak 2), ellagic acid (peak 3), quercetin (peak 5), luteolin (peak 6) and apigenin (peak 7).

3.1.2 Resin

The HPLC fingerprint of the hydroethanolic extract of the resin of *Dalbergia ecastaphyllum* showed the presence of one major peak (5) identified as quercetin. The others were identified as chlorogenic acid, caffeic acid, ellagic acid, p-coumaric acid, luteolin and apigenin (Fig. 2 and Table 2).

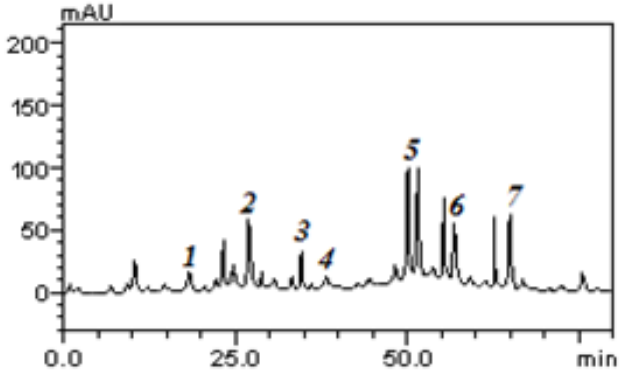

Fig. 2. Representative high performance liquid chromatography profile of resin hydroethanolic extract. Chlorogenic acid (peak 1), caffeic acid (peak 2), ellagic acid (peak 3), *p*-coumaric acid (peak 4), quercetin (peak 5), luteolin (peak 6) and apigenin (peak 7).

Table 2 – Components of the hydroethanolic extract of red propolis and *D. ecastaphyllum* resin.

Compounds	*RP*	*DER*	LOD	LOQ
	mg/g	mg/g	μg/mL	μg/mL
Chlorogenic	0.36 ± 0.01 a	0.86 ± 0.02 a	0.015	0.049
Caffeic acid	0.69 ± 0.03 b	3.97 ± 0.04 b	0.008	0.026
Ellagic acid	1.17 ± 0.02 c	1.15 ± 0.01 c	0.026	0.085
p-Coumaric acid	-	0.23 ± 0.01 d	0.013	0.043
Quercetin	5.73 ± 0.04 d	5.84 ± 0.03 e	0.024	0.079
Luteolin	5.78 ± 0.03 d	2.67 ± 0.01 f	0.016	0.052
Apigenin	0.64 ± 0.01 b	3.94 ± 0.02 b	0.011	0.034

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$

3.2 Microbiological assays

3.2.1 Minimal inhibitory concentration

The MIC values for red propolis (Table 3) ranged from 384 μg/mL to ≥ 1024 μg/mL against *E. coli*. The MIC values observed against strains of *S. aureus* ranged from 53

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259 µg/mL to 512 µg/mL. However, for the *P. aeruginosa* used in this study the MIC values
260 were all the same, 512 µg/mL.

261 The plant resin showed MIC values (Table 3) ranging from 682 µg/mL to ≥ 1024 µg/mL
262 against two strains of *E. coli* and *P. aeruginosa*. MIC against *S. aureus* ranged from
263 85 µg/mL to 1024 µg/mL.

264

265 **Table 3 – MIC values of the red propolis and plant resin against Gram-positive**
266 **and Gram-negative bacterial strains**

Bacterial strains	MIC (µg/mL)	
	Red propolis	Resin
EC06	≥ 1024	≥ 1024
ECATCC	384	682
PA03	512	682
PA24	512	≥ 1024
SA10	53	85.3
SAATCC	512	≥ 1024

267

268 *3.2.2 Combined effect of red propolis and Dalbergia ecastaphyllum resin with standard*
269 *antibiotics*

270 The red propolis and *Dalbergia ecastaphyllum* resin showed similar results when they
271 were associated to antibiotics (Fig. 3). RP and DER showed synergistic activity when
272 combined to imipenem and gentamicin against both strains of *P. aeruginosa* (PA03 e
273 PA24). The RP and DER also showed synergistic effect against *S. aureus* (SA10 and

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SAATCC) when combined to gentamicin, however no effect was observed when the samples were combined to imipenem and tested against SA10 and SAATCC. The values obtained for these tests showed that RP reached lower MICs against all strains used in this study when compared to the values obtained with the DER (Table 4).

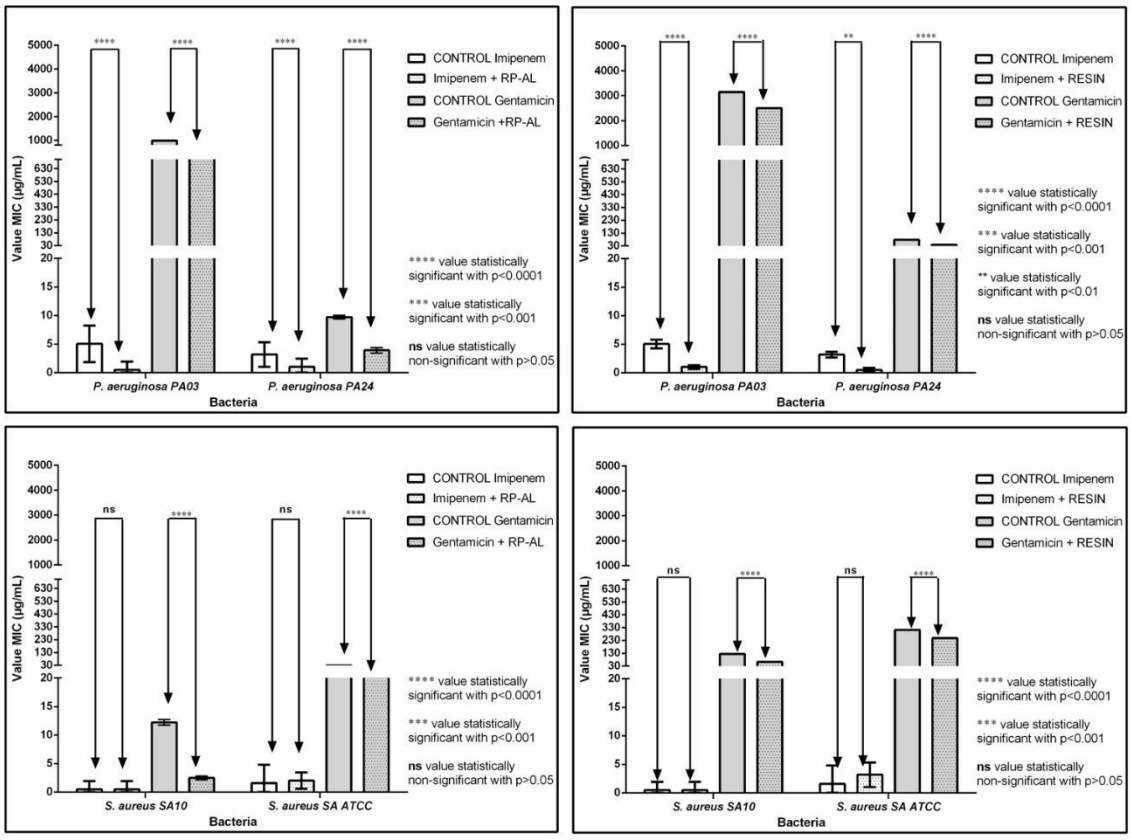

Fig. 3 – Association of red propolis and *D. ecastaphyllum* resin with standard antibiotics. The histograms show the results of the antibiotics activity of RD and DER combined with standard drugs against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Table 4 – Values of the bacterial growth inhibition reached with the association of the RP and DER with standard drugs

	RP x PA03		RP x PA24		RP x SA10		RP x SAATCC	
Antibiotics	RP+A	Control	RP+A	Control	RP+A	Control	RP+A	Control
Imipenem	0.5 µg/mL	5.0 µg/mL	1 µg/mL	3.2 µg/mL	0.5 µg/mL	0.5 µg/mL	2 µg/mL	1.5 µg/mL
Gentamicin	787 µg/mL	992 µg/mL	3.9 µg/mL	9.7 µg/mL	2.5 µg/mL	12 µg/mL	24 µg/mL	30 µg/mL
	DER x PA03		DER x PA24		DER x SA10		DER x SAATCC	
Antibiotics	DER+A	Control	DER+A	Control	DER+A	Control	DER+A	Control
Imipenem	1 µg/mL	5 µg/mL	0.5 µg/mL	3.17 µg/mL	0.5 µg/mL	0.5 µg/mL	3.17 µg/mL	1.58 µg/mL
Gentamicin	2500 µg/mL	3149 µg/mL	39 µg/mL	78 µg/mL	62 µg/mL	124 µg/mL	248 µg/mL	312 µg/mL

Results are expressed as mean ± standard deviations (SD) of three determinations. A= antibiotics; RP= red propolis; DER= *Dalbergia ecastaphyllum* resin; PA03: *Pseudomonas aeruginosa* 03; PA24: *P. aeruginosa* 24; SA10*: Staphylococcus aureus* 10; SAATCC: *S. aureus* ATCC.

4. Discussion

Right after the discovery of red propolis in the Northeastern region of Brazil the first publications started to appear and it was determined by chromatographic methods the botanical origin of the Brazilian red propolis. Silva et al. (2007) compared HPLC profiles of resin samples from 20 species of plant to samples of red propolis and only one plant showed a similar profile to that found for red propolis, and the plant was identified as *Dalbergia ecastaphyllum*. A similar study was also conducted by Dougsch et al. (2007), in addition to HPLC analysis they also observed bees collecting red resin from plants that they later confirmed that was *D. ecastaphyllum*. In these previous studies the chemical profile of red propolis was exactly the same as those *for D. ecastaphyllum* (Dougsch et al., 2007; Silva et al., 2007).

In our study we detected seven compounds: chlorogenic acid, caffeic acid, ellagic acid, quercetin, luteolin and apigenin were found in samples from both red propolis and *D. ecastaphyllum* resin. However, p-Coumaric acid was only detected in samples from DER. This phenolic acid, which is not present in our sample collected in Alagoas, along with it caffeic acid, quercetin and luteolin were previously reported in other samples of red propolis from Alagoas and Pernambuco (Silva et al., 2007; Daugsch et al., 2007; Cabral et al., 2009; Regueira Neto et al., 2017). Chlorogenic acid, ellagic acid and apigenin were also reported in red propolis from Pernambuco (Regueira Neto et al., 2017). Quercetin and luteolin were also found in *D. ecastaphyllum* resin from previous studies using samples from Alagoas (Dougsch et al., 2007; Silva et al., 2007). Neuroprotective, anti-cancer, anti-inflammatory, antioxidante, antimicrobial and anti-leishmania are among the bioactivites reported for the compounds identified in our

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316 study (Ural et al., 2015; Bak et al., 2016; Fonseca-Silva et al., 2015; Kasala et al.,
317 2016; Tsang et al., 2016; Pei et al., 2016).

318 Once the amount of substances per gram of red propolis analyzed in this study was
319 compared with the values we found in a previous study (Regueira Neto et al 2017), we
320 could observe that the amount of chlorogenic acid, caffeic acid, ellagic acid, p-
321 coumaric acid, quercetin, luteolin and apigenin were higher in samples collected in the
322 state of Pernambuco. This result does not surprise us because the red propolis sample
323 used in this study was collected in the rainy season and as we observe in the previous
324 study (Regueira Neto et al., 2017), samples of red propolis collected in the rainy
325 season tend to bear a lower amount of the substances.

326 The hydroethanolic extract of red propolis from the Brazilian states of Alagoas,
327 Pernambuco, Paraíba and Sergipe has been tested against Gram-positive and Gram-
328 negative bacteria (Alencar et al., 2007; Bueno-Silva et al., 2013; Lopez et al., 2015).
329 However, reports about the antibacterial activity of the plant resin produced by
330 *Dalbergia ecastaphyllum* remains scarce. In our study the lowest MIC was observed
331 for RP (53 µg/mL) against SA10 strain followed by the MIC for DER (85 µg/mL) for the
332 same *S. aureus* strain. When we compared the MIC values between RP and DER for
333 all the bacterial strains we observed that the values were lower for RP (Table 2). In the
334 one-year period study analyzing the antibacterial activity of red propolis and *D.*
335 *ecastaphyllum* resin against *Streptococcus mutans*, *S. sobrinus*, *Stapylococcus*
336 *aureus* and *Actinomyces naeslundii* performed by Bueno-Silva et al 2016, the authors
337 also showed that the MIC values for hydroethanolic extract of red propolis were lower
338 than the MIC observed for *D. ecastaphyllum* (collected in March, July and September)
339 resin against *Staphylococcus aureus* and *Actinomyces naeslundii*. However, for the

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most of samples collected in other months any difference was not observed between red propolis and resin (Bueno-Silva et al., 2017).

Drug resistance is a major concern in hospitals over the world (Biedenbach et al., 2016), accordingly many studies have been carried out testing antibacterial drugs association with natural products. The synergistic behavior between antimicrobial drugs and propolis from different types and geographical regions have been performed against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Salmonella* and *Candida* strains and the researches successfully obtained synergistic effect with those association (Orsi et al., 2012; Wojtyczka et al., 2013; Pippi et al., 2015; Regueira Neto et al., 2017). In our present study, when we combined the hydroethanolic extract of red propolis with gentamicin we could observe synergistic effect against all strains used in this study. However, when we combined the same red propolis extract with imipenem we only had synergistic effect against *P. aeruginosa* strains. In a previous work, we found similar results when we combined red propolis collected in the state of Pernambuco during the dry season (Regueira Neto et al., 2017). Here we have the first report of the synergistic behavior between *Dalbergia ecastaphyllum* resin and the antibacterial drugs gentamicin and imipenem. The association between the ethanolic extract of DER and the antibiotic drugs we observed synergistic effect against all strains of *S. aureus* and *P. aeruginosa*. However, when we combined the DER extract with imipenem we only had synergistic effect against *P. aeruginosa* strains. The DER-drugs results are similar to those found for RP-drugs, but when we compared the quantitative values we observed that inhibitory concentrations of drugs were lower when associated with RP (Table 4).

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One thing that caught our attention in these results is the fact that RP had better results than DER. It might be due to modifications that the resin is submitted during propolis production by honey bees. As we mentioned above, after collecting, the resin is chewed and salivary enzymes are added to the mixture. The hypopharyngeal gland of honey bees produces digestive enzymes that possibly play a role in propolis production. Polyphenols act as a substract for various enzymes and are subjected to be metabolized by glucosidase (Vauzour et al., 2012). Some fungi β-glucosidases enzymes are known for hydrolyzing plant fungitoxic glycosides to less toxic or less soluble aglyca (Schmidt et al. 2011).

5 Conclusions

It was possible through HPLC analysis to identify six compounds in common between red propolis and *Dalbergia ecastaphyllum* resin with p-coumaric acid only present in the plant resin. The MIC assay showed that both red propolis and *D. ecastaphyllum* resin are more effective against the Gram-positive bacteria *Staphylococcus aureus*. In addition to this the study also showed that red propolis had lower MICs for all bacteria species included in this study when compared to *D. ecastaphyllum* resin. Red propolis and resin showed similar synergistic behavior when combined with gentamicin and imipenem. However, the quantitative results showed that the MICs values for red propolis were also lower in comparison to *D. ecastaphyllum* resin. These results possibly indicate that honey bees can improve the activity of compounds present in red propolis and open doors for further studies to try to elucidate the mechanisms in which the botanical material is processed by honey bees during propolis production and what is the impact of this step in propolis bioactivity.

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Conflict of Interest

None.

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3.3 ARTIGO 3 - ANTITRYPANOSOMAL, ANTILEISHMANIAL AND CYTOTOXIC ACTIVITIES OF BRAZILIAN RED PROPOLIS AND PLANT RESIN OF *DALBERGIA ECASTOPHYLLUM* (L) TAUB.

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Antitrypanosomal, antileishmanial and cytotoxic activities of Brazilian red propolis and plant resin of *Dalbergia ecastaphyllum* (L) Taub

Marcos da Silveira Regueira-Neto[a,*], Saulo Relison Tintino[b], Miriam Rolón[c], Cathia Coronal[c], Maria C. Vega[c], Valdir de Queiroz Balbino[a], Henrique Douglas de Melo Coutinho[b]

[a] Laboratório de Bioinformática e Biologia Evolutiva, Departamento de Genética, Centro de Biociências, UFPE, Brazil
[b] Laboratório de Microbiologia e Biologia Molecular, Centro de Ciências Biológicas e Saúde, Universidade Regional do Cariri, Brazil
[c] Centro para el Dessarollo de la Investigación Cientifica (CEDIC), Fundacion Moisés Bertoni/Labortórios Díaz Gill, Asunción, Paraguay

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ABSTRACT

The treatment for leishmaniasis and Chagas disease can be hard and painful, such that many patients give up on the treatment. In order to find an alternative path for the treatment of these diseases, researchers are using natural products to fight these parasites. The aim of this study was to evaluate the antiprotozoan and cytotoxic activities of red propolis samples collected from different Brazilian states and seasons whilst searching for possible activity differences. We also compared the red propolis results with the ones obtained for the plant resin extract collected from *Dalbergia ecastaphyllum* trees. The hydroethanolic red propolis extracts from Pernambuco and Alagoas, and the *D. ecastaphyllum* resin were evaluated regarding their antileishmanial, antitrypanosomal and cytotoxic activity. All extracts showed antiprotozoan and cytotoxic activity. RP-PER showed to be more cytotoxic against protozoan parasites and fibroblast cells. All propolis extracts showed a higher cytotoxic activity when compared to resin extracts. The propolis sample collected in Pernambuco during the rainy season killed the parasites with lower concentrations than the sample collected in the dry season. The IC_{50} observed against the parasites could be used without high fibroblast cell damage.

1. Introduction

Leishmaniasis and American Trypanosomiasis are diseases caused by protozoan parasites and are considered a life-threatening illness and a public health problem in Latin America (WHO, 2017, 2018). Leishmaniasis is a group of complex diseases caused by more than 20 *Leishmania* species transmitted by sand flies. The most common form of the disease is the cutaneous leishmaniasis (CL), followed by two other forms of leishmaniasis: mucocutaneous (ML) and visceral (VL) (WHO, 2018). CL and ML can be caused by *Leishmania braziliensis* and VL can be caused by *L. infantum* (Bomfim et al., 2017; Ponte-Sucre et al., 2017). American Trypanosomiasis also known as Chagas disease is a vector-borne disease caused by the protozoan parasite *Trypanosoma cruzi*. Chagas disease is transmitted to humans by the faeces or urine from triatomine bugs known as 'kissing bugs'. Chagas disease is characterised by a skin lesion or a swelling of the eye lids, cardiac disorders and oesophageal or colonic enlargement (WHO, 2017).

Treatments available for these diseases are made using first line drug choices. For the treatment of CL and ML, meglumine antimoniate (Glucantime®, Sanofi aventis) is the first line choice in Ecuador, followed by amphotericin B and pentamidine. For VL treatment the oral drug miltefosine is used (Paladin, Canadá) (Ayres et al., 2007; Hashiguchi et al., 2017). Benzinidazole and nifurtimox are used in the treatment for Chagas Disease. However, those drugs are only effective against the acute phase of the infection (Chatelain, 2017). Despite drug availability, the treatment may be abandoned by patients due to the painfulness of the drug injection, strong adverse reactions, high cost, complicated drug administration, and drug toxicity (Hashiguchi et al., 2017; WHO, 2017). These problems are aggravated by the development of parasitic drug resistance. Researchers facing these problems are trying to find an alternative path for treating these infections. A variety of studies are trying to prove the efficacy of the use of natural plant or animal products for the treatment of leishmaniasis and Chagas disease. Those studies have shown that natural products are a good source of bioactive compounds which might optimise the biological activity of drugs and minimise adverse reactions. A large amount of chemical compounds such as alkaloids, steroids, terpenes, coumarins and flavonoids from plant and animal origins have been tested against different *Leishmania* and *Trypanosoma* species (Cheuka et al., 2016; Oryan, 2015; Pereira et al., 2008).

* Corresponding author. Departamento de Genética, CB, Universidade Federal de Pernambuco, Pernambuco, Recife, Brazil.
E-mail address: mareg1988@gmail.com (M.d.S. Regueira-Neto).

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Propolis is a resinous substance produced by *Apis mellifera* honey bees with the purpose of protecting the hive, sealing holes, preventing contamination of the hive's internal environment by fungi and bacteria and covering intruders who die inside the hive in order to avoid their decomposition (Righi et al., 2011; Salatino et al., 2005). Honey bees use plant materials such as plant resins, pollens and bud excretions to produce propolis where the physiochemical characteristics of the different propolis types are a reflection of the plant species from each region (da Silva et al., 2015; Daugsch et al., 2008). Propolis in Brazil is divided into 13 groups based on their physiochemical characteristics (YongKun et al., 2000; Daugsch et al., 2008). The thirteenth type of Brazilian propolis, red propolis, discovered by 2007 in the Northeast of Brazil, has demonstrated since then a variety of biological activities, e.g. antioxidant (Alencar et al., 2007; Righi et al., 2011), antimicrobial (Bispo Junior et al., 2012; Regueira et al., 2017), anti-cancerous (Awale et al., 2008; Frozza et al., 2017), anti-inflammatory (Bueno-Silva et al., 2013; Cavendish et al. 2015), cytotoxic (Alencar et al., 2007), wound repairing (Batista et al., 2012), antinociceptive (Cavendish et al. 2015) and antiprotozoal (Nascimento et al., 2016; Omar et al., 2016).

In the last 20 years numerous studies on the *in vitro* bioactivity of propolis have been performed against *Leishmania* and *Trypanosoma* species. Propolis from diverse geographical origins, e.g. South America, Europe and Africa, and types, e.g. brown, green and red, have already demonstrated activity against the *Leishmania* species: *L. amazonensis*, *L. braziliensis*, *L. infantum* and *L. major*, and the *Trypanosoma* species: *T. cruzi*, *T. brucei* and *T. evansi* (Higashi and de Castro, 1994; Ayres et al., 2007; Machado, Leon, and De Castro, 2007; Omar et al., 2016). Ayres et al. (2007) compared the effect of green and red propolis against *Leishmania* promastigote and amastigote forms where they observed that the red propolis was the most effective against *Leishmania* and least cytotoxic against macrophages with the red propolis also enhancing its production. From Nigerian red propolis, 10 compounds were isolated and a moderate to high activity was observed against *Trypanosoma brucei* (Omar et al., 2016).

The geographical location where the propolis is produced has an important influence on the propolis' physiochemical characteristics. Different places on different continents and countries have their very own plant species, climate and soil diversity, where these factors strongly affect propolis characteristics (Sampaio et al., 2016; Teixeira et al., 2010). The chemical compounds, antibacterial and antifungal activities of a variety of propolis types were previously explored in seasonality effect studies with some of them showing seasonal influence over propolis biological activities (Bankova et al., 1998; Isla et al., 2012; Mendonça et al., 2015; Regueira et al., 2017). However, no seasonal red propolis effect against protozoan parasites has been reported until now.

For a drug or a natural product to be used as a treatment against microbial infections it is important that they are not cytotoxic to healthy human cells. A variety of propolis types, e.g. brown, green, yellow and red, from various regions in the world were proven to be cytotoxic against a range of cancer cells (Machado et al., 2016.). However, when it comes to the *in vitro* cytotoxic activity evaluation against healthy cells, some studies have shown that propolis from various geographical places and types may in some cases present cytotoxic or protective activities when applied to fibroblast cultures (Gjertsen et al., 2011; Tyszka-Czochara et al., 2014; Zare Jahromi et al., 2014). Those studies are important for the development of new drugs or cosmetics, e.g. sunscreen and anti-aging products (Alshaher et al., 2004; Ebadi and Fazeli, 2017).

The present study aimed to evaluate the leishmanicide, trypanocide and cytotoxic activities of red propolis collected from different Brazilian states and seasons to evaluate any possible activity differences. We also compared the results obtained with the red propolis with the ones obtained with the plant resin extract collected from *Dalbergia ecastaphyllum* trees, recognized as the botanical source of the Brazilian red propolis.

2. Material and methods

2.1. Propolis and resin samples

Red propolis samples were collected in the Brazilian states of Pernambuco in the municipality of Tamandaré (RP-PED and RP-PER) and Alagoas in the city of Japaratinga (RP-AL). The Pernambuco's samples were collected during two seasons: rainy (between April and August) (RP-PER) and dry (between December and March) (RP-PED). Those periods are historically known to have the highest and the lowest precipitation in the state of Pernambuco (APAC, 2016). Plant resin collection from *Dalbergia ecastaphyllum* trees was performed in mangroves near the apiary in the state of Pernambuco. The raw red propolis and resin samples were stored at −20 °C and these were then dissolved in hydroethanolic solution (ethanol 54%) for 72 h. Afterward, the extract was filtered and concentrated using a rotary vacuum evaporator (model Q-344B-Quimis, Brazil). The concentrated solution was frozen and then lyophilised to obtain a fine powder.

2.2. Antiparasitic activity

2.2.1. Cell lines

The Chagas clone *Trypanosoma cruzi* CL-B5, was used as described by Le-Senne et al. (2002). Parasites were stably transfected with the *Escherichia coli* b-galactosidase gene (lacZ). Epimastigote forms were grown at 28 °C in liver infusion tryptose broth (Difco, Detroit, MI) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), penicillin (Ern, S.A., Barcelona, Spain) and streptomycin (Reig Jofre' S.A., Barcelona, Spain), then harvested during the exponential growth phase (Roldos et al., 2008). The duration of the test was of 24 h. *Leishmania Braziliensis* Vianna and *L. infantum* were obtained from the Instituto de Investigaciones en Ciencias de la Salud, Asunción, Paraguay–IICS. Strain maintenance, cultivation form and promastigote form isolations were performed according to Roldos et al. (2008). The *L. braziliensis* Vianna (MHOM/BR/75/M2903) and *L. infantum* (MCAN/ES/92/BCN83) strains were used in the promastigote inhibition assays, where they were grown at 22 °C in Schneider's *Drosophila melanogaster* medium supplemented with 20% FBS. For the cytotoxic assays, the fibroblast cell line NCTC929 was used and grown in Minimal Essential Medium (Sigma, St. Louis, MO). The culture medium was supplemented with heat-inactivated FBS (10%), penicillin G (100 U/mL) and streptomycin (100 mg/mL). Cultures were kept at 37 °C in a humid atmosphere with 5% CO2. The viability of the strains was evaluated according to Roldos et al. (2008), through the use of Resazurin as a colorimetric method.

2.2.2. Chemicals and drugs

Resazurin sodium salt (Sigma-Aldrich, St. Louis, MO) was stored at 4 °C protected from light. Resazurin solution was prepared by adding 1% phosphate buffer, pH 7, filtered and sterilised prior to use. Chlorophenol red-b-D-galactopyranoside (CPRG; Roche, Indianapolis, IN) was dissolved in 0.9% Triton X-100 (pH 7.4). Penicillin G (Ern, S.A., Barcelona, Spain), streptomycin (Reig Jofre' S.A., Barcelona, Spain) and dimethylsulphate were also used. The liver infusion tryptose broth (LIT) (Difco, Detroit, MI) culture medium was used.

2.2.3. In vitro epimastigote susceptibility assay by CPRG assay

The assay was performed according to Vega et al. (2005) using 96-well microplates with cultures which had not reached the stationary phase. Epimastigote forms of the *Trypanosoma cruzi* parasite were seeded (1×10^5/mL) in 200 µL of LIT medium. The plates were then incubated with the red propolis and resin samples (15.62–1000 µg/mL) at 28 °C for 72 h 50 µL of CPRG solution was added to reach a final concentration of 200 mM. The plates were incubated at 37 °C for an additional 6 h and the β-galactosidase activity was then measured at an absorbance of 595 nm. Each experiment was performed three-times and independently, each concentration was tested in triplicate in each

experiment. The efficacy of each compound was estimated by calculating the anti-epimastigote and the result was expressed in percentage inhibition.

2.2.4. In vitro leishmanicidal assay

L. brasiliensis Vianna and *L. infantum* promastigotes were grown to a concentration of 106 cells/mL and then transferred to the test. The red propolis and resin samples were dissolved in Dimethyl sulfoxide (DMSO) used at an atoxic concentration to achieve the concentrations to be tested where the solution was then transferred to microplates. The compound activity was evaluated after 72 h by direct cell counting after serial dilutions and compared with an untreated control.

2.2.5. Cytotoxic assays

NCTC 929 fibroblasts were added to a 96-well microplate at a final concentration of 104 cells/well. The cells were grown at 37 °C in an atmosphere of 5% CO_2. The medium was removed and the samples were added until reaching 200 µL. A 24 h culture was performed. After this incubation, 20 µL of a 2 µM Resazurin solution was added to each well. The plates were incubated for 3 h and the resazurin reduction was measured at 490 and 595 nm and compared to a blank control.

2.2.6. Statistical analysis

The statistical analysis of IC_{50} values are expressed as mean ± S.D., using the software Probit TSK version 1.5 (SPSSInc., Chicago, IL).

3. Results

3.1. Leishmanicidal activity of the red propolis and the D. ecastaphyllum resin extracts

The *in vitro* antileishmanial activity of the hydroethanolic extracts from the Brazilian red propolis from Pernambuco and Alagoas and the plant resin collected from *D. ecastaphyllum* trees were performed against *Leishmania braziliensis* and *L. infantum* promastigote forms. After 72 h of extract incubation with the parasites we observed that the lowest IC_{50} belongs to RP-PER, both against *L. braziliensis* and *L. infantum* (Table 1). The RP-PED had the second lowest IC_{50} against *L. braziliensis*, however its IC_{50} against *L. infantum* was a little higher. The red propolis extract from Alagoas had an IC_{50} similar to those observed for RP-PED. The resin showed one of the highest IC_{50} for both treated species when compared to the propolis extract. All aforementioned results are shown in Table 1. An interesting observation is that the RP-PED extract was the only one which the highest concentration used to treat *Leishmania* could kill 100% of the parasites. These results are followed by the ones observed for the RP-AL and DER against *L. infantum* promastigote forms.

3.2. Trypanocide activity of the red propolis and the D. ecastaphyllum plant resin extracts

In the antitrypanosomal test, the RP-PER extract showed the lowest IC_{50} against *Trypanosoma cruzi* epimastigote forms (Table 2). The RP-PER IC_{50} result is followed by RP-PED, RP-AL and DER. The highest RP-PER concentration tested (1000 µg/mL) killed almost 100% of the parasites. However, the other extracts maintained the epimastigote death percentage around 80% for the highest concentration tested. The RP-PED, RP-AL and DER extracts also showed similar death percentages (around 80%) for the extracts concentrations 1000, 500, 250 and 125 µg/mL.

3.3. Cytotoxic effect of the Brazilian red propolis and the D. ecastaphyllum plant resin extracts

As we observed in the previous experiments, the RP-PER also showed the lowest IC_{50} when compared to the IC_{50} values of the other extracts. The RP-PER 1000, 500, 250 and 125 µg/mL concentration values presented exactly or almost 100% fibroblast cell death. However, the other extracts, at the previously mentioned concentrations, displayed a fibroblast death percentage near 70%. These results can be observed in Table 2.

RP-PER – Red propolis from Pernambuco rainy season; RP-PED – Red propolis from Pernambuco Dry Season; RP-AL – Red propolis from Alagoas; DER- *Dalbergia ecastaphyllum* resin; %AP – Percentual of killed promastigotes forms; ± SD – Standard Deviation; IC_{50Lb} – Concentration in which 50% of *L. braziliensis* parasites are killed; IC_{50Li} – Concentration in which 50% of *L. infantum* parasites are killed.

RP-PER – Red propolis from Pernambuco rainy season; RP-PED – Red propolis from Pernmabuco Dry Season; RP-AL – Red propolis from Alagoas; DER- *Dalbergia ecastaphyllum* resin; %AE – Percentual of killed epimastigotes forms; ± SD – Standard Deviation; %C – Percentual of killed fibroblasts NCTC 929; IC_{50Tc} – Concentration in which 50% of *T. cruzi* parasites are killed; IC_{50F} – Concentration in which 50% of fibroblasts cells are killed.

4. Discussion

4.1. Antileishmanial activity of the red propolis and Dalbergia ecastaphyllum resin

The propolis *in vitro* antileishmanial activity has been reported for propolis samples from different geographical origins, e.g. Ecuador (Cuesta-Rubio et al., 2017), Portugal (Falcão et al., 2014), Cuba (Monzote et al., 2012), Bolivia (Nina et al., 2016), Bulgaria (Machado et al., 2007), Turkey (Duran et al., 2011) and Brazil (Ayres et al., 2007), as well as from a variety of types, e.g. green (Ayres et al., 2007), brown (Monzote et al., 2012), yellow (Monzote et al., 2012) and red (Nascimento et al., 2016). Our study analysed the leishmanicidal activity of three different red propolis samples from Pernambuco and Alagoas against *Leishmania braziliensis* and *L. infantum*. The red propolis sample from Pernambuco RP-PER displayed the lowest IC_{50} against *L. braziliensis* and *L. infantum*. These results show that the red propolis from Pernambuco can be very cytotoxic against *Leishmania* promastigote forms. Ayres et al. (2007) in a study using red propolis samples from Alagoas observed an 84.5% *L. amazonensis* inhibition using a red propolis extract solution of 6 µg/mL. Those results differ from ours results from Alagoas in which we found an IC_{50} of 55.79 µg/mL for *L. braziliensis* and 109.49 µg/mL against *L. infantum*. Similar to our results are those found by Nascimento et al., (2016) where they reported the use of nanoparticles loaded with red propolis from Alagoas against *L. braziliensis* promastigote forms. When we compared the leishmanicidal activity of the three red propolis samples used in our study against both *Leishmania* species we observed that *L. braziliensis* promastigote forms were more susceptible to all red propolis extracts than *L. infantum* parasites. The red propolis samples used in this study were previously analysed by our group to identify the chemical composition of the red propolis samples (Regueira et al., 2017). In this study we identified nine compounds: apigenin, caffeic acid, chlorogenic acid, ellagic acid, luteolin, p-coumaric acid, quercetin, rutin and vitexin. It has been reported in the scientific literature that flavonoids such as quercetin, luteolin and rutin have high leishmanicidal activity (Nascimento et al., 2016). Other studies say that chalcones present in the red propolis can inhibit *Leishmania* parasite growth by affecting the cytoplasmic membrane and the mitochondrial complex (Chen et al., 2001; Torres-Santos et al., 2009).

In this study we report for the first time the antileishmanial activity of the plant resin used by honey bees for red propolis production. We had the chance to compare the biological activity of the red propolis and the plant resin collected from *Dalbergia ecastaphyllum* against *L. braziliensis* and *L. infantum* in this study. According to the results displayed in Table 1 the red propolis samples from Alagoas and Pernambuco showed a better performance compared to the *Dalbergia*

Table 1
Antileismanial activities of the hydroethanolic extracts of red propolis and *Dalbergia ecastaphyllum* plant resin.

Sample	Conc. μg/mL	%AP ± SD (*L. braziliensis*)	Conc. μg/mL	%AP ± SD (*L. infantum*)
RP-PER	1000	100.00 ± 0.21	1000	100.00 ± 0.24
	500	100.00 ± 0.32	500	84.77 ± 0.92
	250	99.12 ± 0.21	250	95.27 ± 0.81
	125	97.17 ± 0,16	125	92.80 ± 0.71
	62.5	85.87 ± 0.29	62.5	87.18 ± 2.05
	31.25	48.20 ± 0.09	31.25	43.51 ± 1.78
	15.62	–	15.62	–
	IC_{50Lb}: 35.22		IC_{50Li}: 37.45	
RP-PED	1000	68.11 ± 3.75	1000	62.41 ± 0.56
	500	66.92 ± 2.80	500	54.17 ± 1.82
	250	65.59 ± 0.55	250	54.56 ± 2.19
	125	64.82 ± 0.32	125	50.31 ± 0.57
	62.5	60.94 ± 0.12	62.5	45.58 ± 0.14
	31.25	57.95 ± 0.45	31.25	0.00 ± 0.55
	15.62	1.51 ± 0.92	15.62	0.00 ± 2.22
	IC_{50Lb}: 42.29		IC_{50Li}: 200.66	
RP-AL	1000	69.43 ± 0.85	1000	97.57 ± 6.23
	500	66.03 ± 4.90	500	79.26 ± 1.86
	250	66.03 ± 0.21	250	67.09 ± 0.85
	125	65.89 ± 0.06	125	62.22 ± 0.10
	62.5	63.75 ± 0.14	62.5	62.24 ± 1.28
	31.25	48.11 ± 0.01	31.25	10.54 ± 2.05
	15.62	19.17 ± 0.64	15.62	0.00 ± 0.03
	IC_{50Lb}: 55.79		IC_{50Li}: 109.49	
DER	1000	79.72 ± 4.08	1000	91.86 ± 1.10
	500	67.10 ± 0.06	500	94.20 ± 0.66
	250	66.08 ± 3.32	250	74.05 ± 1.07
	125	64.32 ± 1.33	125	64.30 ± 0.98
	62.5	62.17 ± 1.46	62.5	0.00 ± 0.91
	31.25	31.33 ± 1.63	31.25	0.00 ± 1.20
	15.62	0.00 ± 4.31	15.62	0.00 ± 0.66
	IC_{50Lb}: 90.46		IC_{50Li}: 134.56	

ecastaphyllum samples. In a study using *D. oliveri* and *D. cultrate* timber, Takahashi et al. (2004) obtained the MLC (minimum lethal concentration) and the MIC (minimal inhibitory concentration) of *Dalbergia* timber extracts against *Leishmania major* promastigote forms. They observed that *D. oliveri* obtained a MLC and MIC of 400 and 200 μg/mL, respectively whilst *D. cultrate* obtained 50 and 25 μg/mL, respectively. A hypothesis that could explain the difference between the red propolis and the plant resin activity against *Leishmania* parasites could be the fact that the plant material composition used in the propolis production process might be enhanced by honey bees during the chewing stage.

4.2. Red propolis and Dalbergia ecastaphyllum resin trypanocide activity

In the last decades a variety of studies investigated the use of natural products for the treatment of Chagas disease. In this study we also evaluated the *in vitro* antitrypanosomal activity of red propolis and the resin exuded by *Dalbergia ecastaphyllum* trees. As we observed with *Leishmania* promastigotes, the RP-PER showed the lowest IC_{50} against *Trypanosoma cruzi* epimastigote forms. This result was followed by the RP-PED, RP-AL and DER extracts. Both of the Pernambuco's red propolis extracts had a lower IC_{50} than Alagoas'. As we also observed against *Leishmania* parasites, the three red propolis extracts had a better effect compared with the plant resin extract. In a study comparing the antitrypanosomal activity of three different types of Brazilian propolis, green, brown and red, against *T. cruzi*, Dantas Silva et al., (2017) observed that Brazilian red propolis samples displayed the highest activity compared to other types of propolis. Also against *T. cruzi*, Salomão et al. (2008) observed that Brazilian red propolis was cytotoxic against trypomastigote forms and they reported that the red propolis extract was the most active among the tested extracts. The Nigerian red propolis, which is very similar to Brazilian propolis, showed to be more effective against *Trypanosoma brucei* as a crude extract than the isolated compounds (Omar et al., 2016). As it is widely reported for a range of red propolis activities, the propolis trypanocidal activity is also due to the presence of flavonoids in their chemical composition. The mean mechanisms by which propolis may kill *Trypanosoma* parasites involves mitochondrial and plasma membrane alterations. In *T. cruzi* epimastigote forms mitochondrial swelling, altered vacuoles and myelin figure formations were observed. Such changes compromise the lipid biosynthesis of *T. cruzi* epimastigotes, consequently affecting their plasma membrane (Dantas et al., 2006; Menna-Barreto et al., 2009).

4.3. Red propolis and D. ecastaphyllum resin cytotoxic activity

Propolis use by humans as medicine has been reported since 300 B.C. (Toreti et al., 2013). The development of techniques over the centuries has made human propolis use to be applied for the most diverse purposes. Currently, numerous biological activities have been attributed to propolis, e.g. antioxidant, anti-inflammatory, antibacterial, anti-fungal, anticancer, wound repair, antinociceptive, and cytotoxic (Alencar et al., 2007; Batista et al., 2012; Lima Cavendish et al., 2015; da Silva Frozza et al., 2013; Machado et al., 2016; Righi et al., 2011). In an attempt to guarantee the safe use of propolis derivatives or even raw propolis, researches are testing propolis from diverse geographical origins to evaluate the cytotoxic potential of those samples (Jacob et al., 2015; Murase et al., 2013). In this study we

Table 2
Trypanocide and cytotoxic activities of the hydroethanolic extracts of red propolis and *Dalbergia ecastaphyllum* plant resin.

Sample	Conc. µg/mL	%AE ± SD (*T. cruzi*)	Conc. µg/mL	%C ± SD (Fibroblast)
RP-PER	1000	99.77 ± 1.63	1000	100.00 ± 0.35
	500	81.95 ± 0.21	500	100.00 ± 0.90
	250	87.78 ± 0.51	250	99.62 ± 0.79
	125	77.83 ± 0.49	125	97.06 ± 0.94
	62.5	76.70 ± 0.57	62.5	90.63 ± 0.26
	31.25	63.74 ± 0.07	31.25	9.30 ± 1.92
	15.62	–	15.62	–
	IC_{50Tc}: < 31		IC_{50F}: 48.09	
RP-PED	1000	80.86 ± 1.20	1000	71.31 ± 0.08
	500	77.30 ± 0.00	500	66.95 ± 0.08
	250	77.15 ± 1.27	250	68.98 ± 0.48
	125	75.19 ± 0.52	125	51.94 ± 0.42
	62.5	68.76 ± 0.98	62.5	33.53 ± 0.42
	31.25	40.56 ± 0.72	31.25	4.63 ± 1.48
	15.62	25.13 ± 0.81	15.62	0.00 ± 6.29
	IC_{50Tc}: 54.86		IC_{50F}: 142.26	
RP-AL	1000	79.79 ± 1.20	1000	63.27 ± 0.35
	500	77.46 ± 0.49	500	62.76 ± 0.78
	250	76.28 ± 0.81	250	60.20 ± 0.68
	125	76.82 ± 0.47	125	55.24 ± 0.14
	62.5	60.54 ± 1.01	62.5	37.87 ± 2.62
	31.25	32.16 ± 0.07	31.25	0.01 ± 2.12
	15.62	5.75 ± 1.98	15.62	1.92 ± 2.97
	IC_{50Tc}: 59.84		IC_{50F}: 127.31	
DER	1000	81.25 ± 0.95	1000	60.21 ± 0.71
	500	80.56 ± 0.49	500	61.58 ± 0.01
	250	77.89 ± 0.42	250	58.35 ± 0.42
	125	74.38 ± 0.49	125	24.64 ± 2.33
	62.5	42.79 ± 0.35	62.5	1.84 ± 2.12
	31.25	20.27 ± 1.63	31.25	0.00 ± 0.92
	15.62	–	15.62	0.00 ± 0.07
	IC_{50Tc}: 88.86		IC_{50F}: 228.02	

analysed the red propolis and the *D. ecastaphyllum* plant resin cytotoxic activity against mouse fibroblasts. The lowest IC_{50} was observed for RP-PER with 48.09 µg/mL while the other extracts obtained IC_{50} values three to five-fold higher. If we take a look at the extract concentrations which killed close to 0% of the fibroblasts, these being 31.25 µg/mL for RP-PER, RP-PED and RP-AL, and 62.5 µg/mL for DER, and compare it with percentage values of *L. braziliensis*, *L. infantum* and *T. cruzi* which were killed at these same concentrations, we observe that in most of cases the extracts killed almost 50% of the *L. braziliensis* and *T. cruzi* parasites. The aforementioned observation shows that the red propolis extracts from Pernambuco, Alagoas and the plant resin can probably be used against *L. braziliensis* and *T. cruzi* parasites without causing significant damage to healthy cells. Previous studies analysed the propolis cytotoxic potential against gingival and pulp fibroblasts cells and the authors observed that propolis solutions were safe whilst also decreasing apoptosis, increasing metabolic activity and proliferation of periodontal ligament cells (Alshaher et al., 2004; Gjertsen et al., 2011; Sonmez et al., 2005). However, it was also observed that green propolis at concentrations of 31.25 µg/mL or more might be cytotoxic to mouse fibroblasts (Funari et al., 2007).

4.4. Seasonal effect on red propolis antiprotozoal and cytotoxic activity

The geographical location where the propolis is produced plays an important role in determining the chemical composition of each type, where the plant, soil, climate and seasonality diversity influence the chemical composition of propolis (Sampaio et al., 2016; Teixeira et al., 2010). In this study we analysed the impact of seasonality on antileishmanial, antitrypanosomal and the cytotoxic activity of the red propolis collected from Pernambuco, Brazil during the dry and rainy seasons. In our study we observed that the RP-PER showed a more efficient antiprotozoan and cytotoxic activity than the RP-PED. The IC_{50} obtained against *Leishmania infantum* and mouse fibroblasts were five-fold and three-fold higher when compared to the results obtained with RP-PER, respectively. Regarding their activity against *L. braziliensis* and *Trypanosoma cruzi* there were slight differences, however the RP-PED still obtained a higher IC_{50} compared to RP-PER. These results demonstrate that the red propolis collected during the rainy season is more efficient against the parasites than the samples collected in the dry season. We observed the opposite when we tested the same samples combined with standard drugs against two bacteria species – *Pseudomonas aeruginosa* and *Staphylococcus aureus* – in a previous study. The red propolis collected in the dry season when combined to drugs had a more efficient synergistic activity than the samples from the rainy season (Regueira et al., 2017). On the other hand, Mendonça et al. (2015) in study on the seasonality of the red propolis from Sergipe, Brazil observed a positive correlation between yield and the pluviosity. Thus we can see here that seasonality influence on propolis bioactivity and how the extracts act against microorganisms is complex. Different red propolis samples collected in specific periods might have an enhanced bioactivity against specific targets, which is the case of the red propolis from Pernambuco, Brazil where the sample collected during the dry season had a better activity against bacteria while the sample collected during the rainy season performed better against protozoan parasites. To properly address this question, other factors which influence propolis activity need to be explored.

5. Conclusions

The evaluation of the *in vitro* cytotoxic activity of red propolis samples from Pernambuco and Alagoas against *Leishmania* and *Trypanosoma* parasites showed that all the extracts possessed high cytotoxic activity against the parasites. However, the RP-PER presented the lowest IC_{50} against all tested species when compared to the others. Between the *Leishmania* species included in this study, *L. braziliensis* was the most susceptible to the action of the extracts. Propolis samples, in general, showed a better performance against the parasites when compared to the *Dalbergia ecastaphyllum* resin extract. According to this, honey bees may possibly modulate the chemical compounds present in the plant resin when they mix the plant material with their saliva and wax during the production of red propolis. To prove this, further studies are necessary. The concentration of extracts needed to kill at least 50% of fibroblasts cells are higher than the concentrations needed to kill the parasites, which makes the red propolis, despite its high toxicity, safe to be used at specifics concentrations. We also observed the effect of seasonality on red propolis samples from Pernambuco, in which the red propolis collected during the rainy season showed to be more effective than the one collected in the dry season.

Conflicts of interest

The authors have no competing interests to declare.

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4 CONCLUSÕES

1. Nove compostos químicos foram identificados nos extratos da própolis vermelha e da resina de *Dalbergia ecastaphyllum*. As diferenças de concentração deles nos extratos hidroetanólicos podem estar associados a fatores geográficos e sazonais;

2. Todos os extratos de própolis vermelha coletados em Pernambuco e Alagoas, incluindo a resina de *D. ecastaphyllum*, apresentaram atividades antibacteriana, antitripanosoma, atileishmania e citotóxica. Essas atividades são atribuidas à presença de flavonóides na composição;

3. A estação em que a própolis é coletada tem uma influência significativa na atividade biológica desses compostos;

4. Em todos os testes realizados, os extratos hidroetanólicos da própolis vermelha apresentaram uma maior eficácia frente aos micro-organismos em relação à resina vegetal e essa diferença pode estar relacionada à potencialização da ação dos compostos da resina durante à produção da própolis;

5. A própolis vermelha pernambucana é similar do ponto de vista de atividade biológica e composição química à própolis vermelha de Alagoas.

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ANEXO A – NORMAS DA REVISTA FOOD AND CHEMICAL TOXICOLOGY

FOOD AND CHEMICAL TOXICOLOGY

AUTHOR INFORMATION PACK

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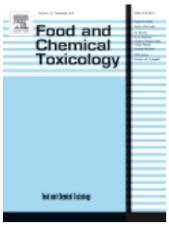

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FOOD CHEMISTRY

AUTHOR INFORMATION PACK

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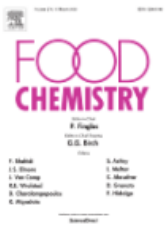

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Mettam, G. R., & Adams, L. B. (2009). How to prepare an electronic version of your article. In B. S. Jones, & R. Z. Smith (Eds.), *Introduction to the electronic age* (pp. 281–304). New York: E-Publishing Inc.
Reference to a website:
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