UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

TESE DE DOUTORADO

BIOPROSPECÇÃO DE PLANTAS BRASILEIRAS MODULADORAS DA ANTIVURULENCIA E DA RESISTÊNCIA À DROGAS CONTRA *Staphylococcus aureus*

CLOVIS MACÊDO BEZERRA FILHO

RECIFE
2018

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Tese de Doutorado apresentada como requisito para obtenção do título de Doutor em Ciências Biológicas pela Universidade Federal de Pernambuco.

Área de Concentração: Ciências Biológicas

Orientadora: Profª Drª Maria Luiza Vilela Oliva

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RECIFE
2018

Catalogação na fonte:
Bibliotecária Claudina Queiroz, CRB4/1752

Bezerra Filho, Clovis Macêdo Bezerra
Bioprospecção de plantas brasileiras moduladoras da antivurulencia e da resistência à drogas contra *Staphylococcus aureus* / Clovis Macêdo Bezerra Filho - 2018.
170 folhas: il., fig., tab.

Orientadora: Maria Luiza Vilela Oliva
Coorientadora: Maria Tereza dos Santos Correia
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Ciências Biológicas. Recife, 2018.

Inclui referências, apêndices e anexos

1. *Staphylococcus aureus* 2. *Eugenia brejoensis* 3. Virulência I. Oliva, Maria Luiza Vilela (orient.) II. Correia, Maria Tereza dos Santos (coorient.) III. Título

615.321 CDD (22.ed.) UFPE/CB-2018-422

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Data: 26/02/2018

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Dedico este trabalho a minha família por todo apoio e fé no meu sucesso. Dedico também a todos os cientistas que acreditam que na busca persistente pelo conhecimento reside a transformação de um país.

AGRADECIMENTOS

A conclusão deste trabalho não seria possível sem a ajuda de tantas pessoas especiais que me fizeram chegar mais parto do sonho de ser cientista. Sou grato aos meus pais, Clovis Macêdo e Antônia Mendes, que desde jovem acreditaram em mim de todas as formas e me deram uma educação libertadora, repleta de amor, respeito e inspiração para realização dos meus objetivos. A educação é o legado maior que se pode deixar para a vida de um filho, cujo valor é impossível mensurar. Agradeço imensamente aos mestres e professores que contribuíram com a minha formação em uma universidade pública que tem como objetivo servir a sociedade nas variadas instâncias e somar com o crescimento desse país, eu não seria a mesma pessoa e de nada valeria toda essa formação privilegiada se não fosse o desejo de transmitir todo o conhecimento desenvolvido.

Agradeço,

Em especial, as minhas orientadoras, à professora Maria Luiza Vilela Oliva que foi uma mãe me acolhendo em seu grupo desde o princípio e que sempre esteve presente me passando seus conhecimentos e entusiasmo incansável na formação de jovens cientistas, à professora Maria Tereza dos Santos Correa pela parceria e acolhimento nos momentos mais difíceis, sempre passando a calma necessária e me permitindo trabalhar ao seu lado nas diversas linhas de pesquisa, à querida professora Márcia Vanusa que ao longo desses 9 anos de amizade sempre foi luz no meu caminho, com suas palavras de perseverança, desejo de crescimento e um carinho contagiante pela pesquisa e pela nossa Caatinga. Não há palavras para expressar meu respeito e bons sentimentos por todas vocês!

Aos professores e parceiros internacionais, Anders Løbner Olsen pela prontidão em me receber na Universidade de Copenhagen e por não medir esforços para ajudar neste projeto e a professora Karen Angeliki Krogfelt que junto ao professor Carsten Struve foram excelentes companhias e uma luz para meus experimentos. O inesquecível jeito dinamarquês, a forma genuína de se fazer ciência e a ajuda dessas pessoas são pontos cruciais para o desenvolvimento dessa tese e foram capazes de aquecer meu entusiasmo mesmo nos congelantes dias de trabalho.

Ao amigo e professor Luís Claudio, pela inspiração e orientação ao longo do projeto e da vida profissional, pelo acolhimento no Brasil e na Dinamarca. Certamente sem o apoio e sem os conselhos do mesmo, esse projeto não teria o mesmo rumo. Muito obrigado!

Ao Doutor Alexandre Gomes da Silva pela coleta de material vegetal, sobretudo pelo conhecimento envolvendo essa espécie de trabalho em comum, que nos trouxe tantas alegrias, chamada *Eugenia brejoensis.*

Aos amigos dos diversos laboratórios que tive a oportunidade de fazer experimentos ao longo dos últimos 4 anos e que me trouxeram alegria, apoio e ajuda na vida pessoal e no trabalho e que levarei dentro coração para a vida inteira. Minha gratidão e respeito a todos os estudantes e funcionários do Departamento de Bioquímica da UFPE, em especial a todos dos laboratórios de Produtos Naturais e Biologia molecular (Professor Nicácio, Joelma, Filipe, Elys, Paula, Cibele, Tiago, Thaise, Bruno Souza, Lívia, Robson, Carol, Tulio, Ana Paula, Seu João, Bruno e Bruna) e ao departamento do Bioquímica e Biofisica da Universidade de São Paulo, em especial a todos do grupo da Professora Maysa (Marlon, Bruno, Mariana, Luciana, Camila Bonturi, Camila Nimri, Rodrigo, Magda, Patrícia, Joana, Yara, Luiza, Tatiana e André) e às professoras Karin do Amaral Riské e Kátia Perez, aos colegas da Universidade de Copenhagen (Maria, Jakob, Chris, Ping Fang, Thomas, Godefroid, Leise e Rasmus) e aos pesquisadores do Staten Serum Institute da Dinamarca (Eric, Michala e Hengameh).

A caminhada foi muito mais prazerosa na companhia Erika, Well e Vinny que sempre foram uma família para mim e me acolheram tão bem em São Paulo. Impossível também esquecer o apoio constante de Raiana e Michely, dois presentes que a ciência me deu e que tenho orgulho de ser amigo e fazerem parte da minha vida!

A Thiago Cahu pelo incentivo que me fez ganhar o mundo, pela lealdade e companhia de sempre e por estar onipresente em todos os momentos desta tese e da minha vida, discutindo ciência a qualquer hora e sempre trocando forças na hora que precisamos aqui e fora do Brasil.

Aos amigos Audrey e Svan por nos receber com tanto carinho na Suíça e por se preocuparem sempre com nosso bem estar e amizade mesmo do outro lado do mundo.

À amiga Marina Marcushi pelo apoio e carinho, às amigas Ligia e Mirelly pela preocupação com minha saúde e aos amigos brasileiros que conhecemos na Dinamarca e levamos pra vida como excelentes companheiros (Erika, Delano, Bruna, Gustavo, Nayara, Laís, Ana Gorete, Natália e Renato).

Aos amigos Layron, Dalyla, Micael e Pedro, que sempre torceram por mim mesmo nos falando raramente e sustentando a amizade pelo whatsapp.

Às amigas Jackeline e Weruska por serem sempre queridas e pela amizade de uma vinda inteira!

Aos senhores Zequinha e Zilda por nos acolher com toda bondade de espirito e permitir o cumprimento da nossa missão.

À Leonardo Marinho por ser querido e representar o carinho pelo sobrinho que nunca tive, que o seu coração continue sendo de uma criança iluminada.

À minha mãe, uma mulher verdadeira e bondosa que enfrenta o mundo comigo e por mim, que suportou a solidão sempre me entusiasmando a seguir meus sonhos, sem medir a barra ou os esforços que teria de enfrentar em nome do meu bem-estar. Nunca estaremos sozinhos neste universo, sou muito grato a Deus por te colocar no meu caminho como um exemplo de força, honestidade e de luz. Amo você para sempre!

‘’Existe uma coisa que uma longa existência me ensinou: toda a nossa ciência, comparada à realidade, é primitiva e inocente; e, portanto, é o que temos de mais valioso.’’

Albert Einstein

RESUMO

O Brasil é detentor de uma grande biodiversidade vegetal e por essa razão desperta interesse devido ao grande potencial biotecnológico. O uso de plantas como fonte de produtos naturais provenientes do metabolismo primário e secundário tem sido exitoso na busca de atividades biológicas. Nesse contexto, estudos prévios do nosso grupo demonstram que Óleos essenciais e Inibidores de Protease constituem agentes poderosos perante microorganismos de interesse clínico, atuando na morte ou redução da virulência dos mesmos. A resistência microbiana de patógenos como *Staphylococcus aureus* à antibióticos comerciais é um problema de saúde mundial, justificando a busca de produtos naturais como novas alternativas ao estudo de diferentes mecanismos de ação e/ou que regulem a virulência e a ação infecciosa desses organismos visando obter moléculas – ou combinação delas - candidatas a novos fármacos. O Óleo essencial de *Eugenia brejoensis* (EbEO), o inibidor de protease recombinante BbKIm e peptídeos desenhados a partir do seu sítio reativo, desenvolvido a partir do inbidor de protease de *Bauhinia bauhinioides,* são capazes de inibir a produção de estafiloxantina, um pigmento produzido por *S. aureus* cuja ação está relacionada a defesa antioxidante desse patógeno, desempenhando um papel fundamental na proteção do ataque oxidativo causado pelas células imunes do hospedeiro. O EbEO demonstrou um grande potencial de ação sinérgica com drogas comerciais. Ambas classes de compostos foram testadas em modelos de virulência *in vitro* e *in vivo* capazes de aumentar o tempo de sobrevivência de larvas de *Caenorhabditis elegans* e *Galleria mellonella* em modelos de sepse. Neste contexto, esta tese de doutorado visa elucidar os mecanismos envolvidos na inibição da virulência e modulação da resistência aos antimicrobianos de *S. aureus* causadas pelo óleo essencial de *Eugenia brejoensis,* uma planta da caatinga pernambucana, e a modalidade recombinante modificada do inibidor de protease de *Bauhinia bauhinioides* (rBbKm) e peptídeos derivados de sua estrutura.

Palavras-chave: *Staphylococcus aureus*. RBbKIm. *Eugenia brejoensis*. Virulência. Antibióticos.

ABSTRACT

Brazil has a great biodiversity of plants and for this reason arouses interest due to the great biotechnological potential. The use of plants as a source of natural products from primary and secondary metabolism has been successful in the search for biological activities. In this context, previous studies from our group demonstrate that Essential Oils and Protease Inhibitors constitute powerful agents against microorganisms of clinical interest, acting on the death or reduction of their virulence. The microbial resistance of pathogens such as *Staphylococcus aureus* to commercial antibiotics is a worldwide health problem, justifying the search for natural products as new alternatives to the study of different mechanisms of action and / or regulating the virulence and infectious action of these organisms in order to obtain molecules - or combination of them - candidates for new drugs. The essential oil of *Eugenia brejoensis* (EbEO), the recombinant protease inhibitor BbKIm and peptides designed from its reactive site, developed from the protease inhibitor of *Bauhinia bauhinioides*, are able to inhibit the production of staphyloxanthin, a pigment produced by S. aureus whose action is related to the antioxidant defense of this pathogen, playing a fundamental role in the protection of the oxidative attack caused by the immune cells of the host. EbEO has shown great potential for synergistic action with commercial drugs. Both classes of compounds were tested in in vitro and in vivo virulence models capable of increasing the survival time of *Caenorhabditis elegans* and *Galleria mellonella* larvae in sepsis models. In this context, this doctoral thesis aims to elucidate the mechanisms involved in the inhibition of virulence and modulation of resistance to S. aureus antimicrobials caused by the essential oil of *Eugenia brejoensis*, a plant from the caatinga of Pernambuco, and the modified recombinant modality of the protease inhibitor *Bauhinia bauhinioides* (rBbKm) and peptides derived from their structure.

Keywords: *Staphylococcus aureus*. rBbKIm. *Eugenia brejoensis*. Virulence. Antibiotics.

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LISTA DE SIGLAS

Acetil-CoA	Acetil coenzima A
ATP	Adenosina tri-fosfato
BbKI	*Bauhinia bauinioides* Kallikrein inhibitor (Inibidor de calicreína de *Bauhinia bauinioides)*
CA-MRSA	Community-acquired methicillin resistant *Staphylococcus aureus* (*Staphylococcus aureus* resistente a meticilina adquirido em comunidade)
CBM	Concentração bactericida mínima
C10, C15 e C20	Monoterpenos, sesquiterpenos e diterpenos
CIM	Concentração inibitória mínima
DNA	Ácido desoxirribonucléico
EbEO	*Eugenia brejoensis* Essential Oil (Óleo essencial de *Eugenia brejoensis)*
ECDC	*European Center for Disease prevention and control*
EROs	Espécies reativas de oxigênio
GUV	Giant Unilamelar vesicles (vesícula unilamelar gigante)
huPK	Calicreína plasmática humana
HA-MRSA	Hospital-acquired methicillin resistant *Staphylococcus aureus* (*Staphylococcus aureus* resistente a meticilina adquirido em hospital)
INICC	*International Nosocomial Infection Control Consortium*
LB	Meio Luria Bertani
LC	Larvicidal concentration (concentração larvicida)
LPS	Lipopolissacarídeo bacteriano
LUV	Large Unilamelar vesicles (vesícula unilamelar grande)
MIC	Minimum Inhibitory concentration (concentração inibitória mínima)
MBC	Minimum bactericidal concentration (concentração bactericida mínima)
MRSA	Methicillin resistant *Staphylococcus aureus* (*Staphylococcus aureus* resistente a meticilina)

MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules (componentes da superfície microbiana que reconhecem moléculas de adesão da matriz)
OE	Óleo essencial
OMS	Organização Mundial da Saúde
POPC	2-oleoil-1-pamlitoil-sn-glicero-3-fosfocoline
POPG	2-oleoil-1-pamlitoil-sn-glicero-3-glicerol
PBS	Phosphate buffered saline (tampão fosfato salina)
rBbKIm	Recombinant *Bauhinia bauinioides* Kallikrein inhibitor modified (inibidor recombinante)
SBTI	Soy bean Trypsin Inhibitor (inibidor de tripsina de soja)
SCP	*Staphylococcus* coagulase positiva
STX	Estafiloxantina
TTC	Cloreto de Trifenil tetrazólio
UV	Radiação ultravioleta

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1 INTRODUÇÃO

O Brasil é um país de dimensões continentais, dotado de uma flora rica e composta por diversas espécies vegetais endêmicas. As variadas condições climáticas, como temperatura, luminosidade e umidade, constituem fatores determinantes para o estudo de plantas exclusivas aos domínios fitogeográficos do território.

Estudos envolvendo plantas medicinais tem ganhado notoriedade no contexto da pesquisa em desenvolvimento de nova alternativas farmacológicas. Pesquisadores brasileiros vêm utilizando metabólitos provenientes do metabolismo primário e secundário em múltiplos contextos e obtido resultados promissores para áreas críticas como antibióticos, problemas circulatórios, cancerologia e síndrome metabólica. O isolamento e a identificação das atividades biológicas presentes em partes de plantas utilizadas na terapêutica constituem um passo fundamental no desenvolvimento de novas drogas ou confirmação do conhecimento popular, muitas vezes utilizado como indicativo em estudos farmacognósicos.

A resistência à antibióticos é considerada um dos maiores problemas em saúde do século XXI devido ao aumento considerável de espécies bacterianas que tem adquirido resistência aos fármacos tradicionais utilizados na terapêutica. A pesquisa não tem conseguido apresentar alternativas funcionais e viáveis na mesma velocidade em que a resistência tem se difundido, limitando a introdução de novos produtos farmacêuticos para o tratamento de infecções.

A infecção hospitalar, atualmente conhecida como nosocomial, é responsável por um grande percentual de mortes relacionadas a esse ambiente em muitos países do mundo. Micro-organismos como *Pseudomonas aeruginosa, Candida albicans e Staphylococcus aureus*, sendo este último considerado um dos principais patógenos do século XXI devido ao seu elevado grau de virulência e fatores de resistência que definem o ataque aos hospedeiros, ganham atenção frente a urgência em seu tratamento. Um estudo conduzido pelo *International Nosocomial Infection Control Consortiu* (INICC), em 2014, constatou que em mais de 43 países há a presença de *Staphylococcus aureus* resistente à meticilina. Trata-se de uma bactéria gram-positiva, com ação oportunista, pertence à família *Staphylococcaceae* e que possui 47 espécies (71 subespécies), das quais 18 podem ser isoladas de amostras biológicas humanas, principalmente de pele e fossas nasais (JI et al 2015; DSMZ, 2015). Compreendem cocos dispostos em arranjo característico em formato de cachos de uva, são Gram-positivos, com diâmetro entre 0,5 e 1,5 μm, anaeróbios facultativos, imóveis e não formadores de esporos

Staphylococcus aureus possui um extenso arsenal de fatores de virulência, que podem ser secretados ou estruturais (GORDON & LOWY, 2008) e contribuem para a colonização e infecção. Esse arsenal é composto por proteínas de parede celular, exoenzimas e exotoxinas, com diversas funções, como promover adesão ao tecido e superfície celular do hospedeiro; auxiliar na evasão ao sistema imune; ou promover a captação de ferro (FOSTER, 2005). Dessa forma, as infecções estafilocócicas são multifatoriais e se apresentam como um desafio a compreensão de seu mecanismo de ação.

Inbidores de protease são compostos com ação catalítica, ou seja, agem degradando as ligações peptídicas por meio de hidrólises no sítio ativo das enzimas, sendo amplamente estudados e utilizados para identificar as mais variadas ações biológicas. Estudos recentes indicam a ação de proteases frente a virulência de *S. aureus* permitindo ora que o microorganismo avance no organismo por meio da lise de enzimas importantes ao funcionamento do mesmo, ora impedindo que enzimas que compõem fatores de virulência do *S. aureus* degradem a membrana celular, drible o sistema imune ou interfira no metabolismo celular.

As plantas aromáticas são ricas em óleos essenciais (OE), têm sido usadas ao longo da história, seja na religião, na medicina ou na cosmética. Os OE são uma mistura complexa de monoterpenos (C10), sesquiterpenos (C15) e diterpenos (C20) que podem ser extraídas dessas plantas e justamente esta mistura complexa que aumenta as possibilidades de ações terapêuticas e torna interessante a busca de novas espécies, principalmente se coletadas em regiões diversas, em épocas diferentes e em diferentes estágios vegetativos. (BERTUCCI et al., 2008; FIGUEIREDO et al 2014). A ação antimicrobiana dos OE somada ao baixo efeito toxicológico, torna-os bons candidatos para atender a busca por novas estratégias no combate aos microrganismos resistentes aos antibióticos clássicos e para se chegar a este objetivo é necessário também avaliar a citotoxicidade e genotoxicidade do OE testado.

1.1 OBJETIVOS

1.1.1 Objetivos gerais

- Avaliar a ação anti-microbiana e moduladora da virulência dos compostos naturais, óleo essencial de *Eugenia brejoensis* e do inibidor recombinante de protease desenvolvido a partir de *Bauhinia bauhinioides*, sobre *Staphylococcus aureus* utilizando modelos *in vitro* e *in vivo* de *Caenohabiditis elegans* e *Galleria melonella*.

1.1.2 Objetivos específicos

- Extrair e caracterizar o óleo essencial das folhas de *Eugenia brejoensis* (EbEO),
- Expressar em vetor pet29a o inibidor de protease rBbKIm,
- Determinar a concentração mínima inibitória do crescimento bacteriano de EbEO e do rBbKIm sobre as linhagens de *S. aureus* ATTC 29213,
- Analisar a resposta no crescimento bacteriano de diferentes concentrações dos compostos (tempo de morte bacteriana),
- Avaliar o efeito sinergético entre EbEO e antibióticos de uso clínico,
- Investigar *in vitro* a ação dos compostos sobre a virulência bacteriana nas linhagens padrões e multirresistentes de *Stahylococcus aureus*,
- Analisar a ação do EbEO na resposta SOS,
- Investigar a ação do EbEO em resposta ao estresse celular causado por peróxido de hidrogênio,
- Estuar o efeito dos compostos na hemólise em sangue total
- Investigar o efeito dos compostos na produção do pigmento estafiloxantina em culturas bacterianas. Método quantitativo e qualitativo,
- Avaliar *in vivo* o efeito no aumento do tempo de vida de *Caenohabditis elegans* e *Galleria mellonella* em modelos de sepse induzido por *S. aureus*,
- Mensurar o efeito de EbEO na produção do pigmento melanina em larvas de *G. melonella* infectadas por *S. aureus*,
- Estimar o crescimento bacteriano na hemolinfa de larvas de *G. melonella* infectadas por *S. aureus* por meio de curvas de crescimento bacteriano,
- Utilizar modelos em vesículas membranares (GUVs) mimetizando a membrana bacteriana (POPG e POPC+ LPS) procurando entender o mecanismo de ação do EbEO, avaliando por microscopia de fluorescência a interação EbEO/GUVs;
- Estudar a interferência no tamanho das vesículas;
- Determinar os parâmetros físico-químicos envolvidos na interação EbEO/vesículas.

2 REFERENCIAL TEÓRICO

O uso de plantas, ao longo do tempo, tem se relacionado com o homem mediante sua necessidade de sobrevivência (ALBUQUERQUE, 2005). Mesmo com o avanço na ciência, a medicina tradicional ainda é praticada em pequenas comunidades carentes onde plantas são a única alternativa disponível nessas comunidades para combater suas enfermidades (MENALE et al., 2016; SILVA et al., 2015). O baixo custo versus o benefício terapêutico e a falta de tratamento para determinadas doenças geralmente são as razões para a manutenção dessas tradições (MENALE et al., 2016).

No Brasil, a base da medicina popular tradicional é derivada de uma mistura das culturas indígenas brasileiras, influências europeias e africanas durante o período de colonização acumulados por pessoas locais com acesso direto à natureza e aos produtos da biodiversidade (ALBAGLI, 2001; OLIVEIRA, L.S, et al 2015). Muitos estudos têm contribuindo na procura por novas substâncias derivadas de plantas medicinais e várias drogas como a aspirina, atropina, camptotecina, digitoxigenina, morfina, podofilotoxina, taxol entre outras já foram obtidas (MUKHTAR et al., 2002)

Afim de se obter novas moléculas ativas, emprega-se diferentes abordagens como ferramenta para seleção de plantas para a triagem farmacológica: a abordagem randômica, a abordagem quimiotaxonômica, a abordagem de amostragem étnico-dirigida e a abordagem baseada em tecidos específicos de plantas (ALBUQUERQUE et al, 2012). A partir destes levantamentos é possível averiguar o potencial dos recursos vegetais sob diferentes aspectos, desde medicinais, alimentares, madeireiros, forrageiros, incluindo também as formas de uso através das gerações. O mais eficaz deles é o levantamento etno-dirigido (SILVA, 2013).

Do metabolismo primário, são produzidas as lectinas e inibidores de protease, os quais estão amplamente distribuídas na natureza e estão presentes em todos os reinos. Por terem a habilidade de se ligar a mono e oligossacarídeos, apresentam uma variedade de efeitos biológicos, dentre eles atividades envolvendo bactérias (KOVÁCS-SÓLYOM et al., 2010; DE LA FUENTE et al., 2014; DEÁK et al., 2015; PATHAN et al., 2017, WESSLER et al 2017)

A complexidade metabólica de plantas é mais extensa que a maioria dos outros organismos, pois além da produção metabólitos primários, as plantas também sintetizam uma vasta gama de metabólitos secundários, os metabólitos primários essencialmente representam substâncias que são produzidas por todas as espécies de plantas e organismos; são geralmente essenciais para sobrevivência. Incluem carboidratos, aminoácidos, nucleotídeos, lipídeos,

enzimas e coenzimas (PICHERSKY, 2000). Os metabólitos secundários são estruturalmente diversificados e algumas estruturas são encontradas, muitas vezes, em espécies específicas (PETSCHENKA et al 2017). Estes compostos desempenham papeis fundamentais na manutenção da planta, atuando na proteção das plantas contra fungos, bactérias, infecções virais, pragas, radiação UV, além de participar em processos de alelopatia, sinalização e atração de polinizadores e frutívoros (SHITAN, 2016).

A atividade antimicrobiana dos óleos essenciais é reconhecida comprovada em toda a literatura especializada (DUARTE et. al., 2006; BECKER et al., 2017), contudo as pesquisas continuam para identificar atividades antifúngicas, antibacterianas, fungistáticas, bacteriostáticas. A maioria dos testes antimicrobianos para OE é feita utilizando a fase líquida. Os estudos na fase vapor vêm ganhando importância nas áreas de alimentos processados ou grãos, descontaminação ambiental e hospitalar, não somente pela sua ação antimicrobiana, mas também pela forma de aplicação. Porém, até agora, não há método padronizado para avaliação dos testes na fase vapor (LANG & BUCHBAUER, 2012).

Apesar de todos os esforços, as infecções adquiridas em hospitais (nosocomiais), têm aumento, bem como a resistência bacteriana aos antimicrobianos usados e o *International Nosocomial Infection Control Consortiu* (INICC), (ROSENTHAL et al., 2014) divulgou seus estudos em mais de 43 países onde encontrou, *Staphylococcus aureus* resistente à meticilina, *Enterococcus faecalis* resistente à vancomicina, *Pseudomonas aeruginosa* resistente à amicacina, entre outros. O relatório do *European Center for Disease prevention and control* (ECDC), (ZARB et al., 2012) indicou que, em 21 países, a média de infecções nosocomiais é de 7,1%. Sendo que os locais no corpo humano mais atingidos são: o trato urinário (27%), seguido do trato respiratório, incluindo pneumonia 24%. Os microrganismos mais frequentemente isolados nas infecções nosocomiais são *E. coli, S. aureus, P. aeruginosa.* Estes dados indicam a necessidade de se buscar novas estratégias para combater a resistência aos antibióticos, bem como a descoberta e desenvolvimento de novos compostos ativos. (FRENCH, 2010).

Como as infecções bacterianas e fúngicas são tratadas por meio da correção de fatores predisponentes e de terapêutica, óleos essenciais, obtidos de plantas medicinais, contra diversas espécies microbianas conhecidas como potencialmente causadoras de infecções oportunistas, incluindo bactérias e fungos, tem se tornado uma alternativa viável no combate a esses patógenos.

A espécie *Staphylococcus aureus* subsp. *aureus* (referida aqui como *S. aureus*) é a principal representante do grupo das espécies coagulase-positivas. Trata-se de um microrganismo gram-positivo que apresenta diversos mecanismos de resistência e elevado grau de virulência. Considerado um dos principais patógenos desse século, *S. aureus*, tem sido alvo de inúmeros estudos devido seu papel em infecções nosoncomiais e a elevada resistência a antibióticos.

2.1 RESISTÊNCIA E ATIVIDADE ANTIMICROBIANA

Com a disponibilidade de vários antibióticos no mercado, a resistência microbiana aumentou significativamente, principalmente devido ao mau uso extensivo dessas drogas e a rápida transferência genética de resistência. É sugerido que a resistência microbiana se desenvolve dentro de sete a oito anos de uso regular de antibióticos, diante disso surge a necessidade de achar novas substâncias antimicrobianas capazes de superar esses obstáculos no tratamento de infecções resistentes (GUIMARÃES et al., 2010; SILVEIRA et al., 2006). Estima-se que 30 a 40% dos antimicrobianos mais disponíveis no mercado são derivados de produtos naturais (CHATTOPADHYAY et al., 2009).

Vários metabólitos secundários de plantas também têm apresentado atividade antimicrobiana. Polifenóis podem combinar-se com as adesinas bacterianas de forma a comprometer a adesão do microrganismo sobre a superfície celular, além de também exercerem atividade antibacteriana provavelmente pela inativação de enzimas hidrolíticas, adesinas e de proteínas transportadoras (LUIS et al., 2014). Taninos podem afetar a síntese da parede celular ao formarem complexos irreversíveis com proteínas (VORAVUTHIKUNCHAI, 2009; CHOLET et al., 2014), enquanto que alguns flavonóides podem atuar como inibidores da topoisomerase tipo II bacteriana. Compostos de natureza terpênica têm sido relacionados com a inibição do crescimento microbiano e estão presentes em óleos voláteis de diversas espécies vegetais, também têm exibido atividade inibitória contra fungos e bactérias (RILEY et al., 2015; BOTSCHUIJVER et al, 2018)

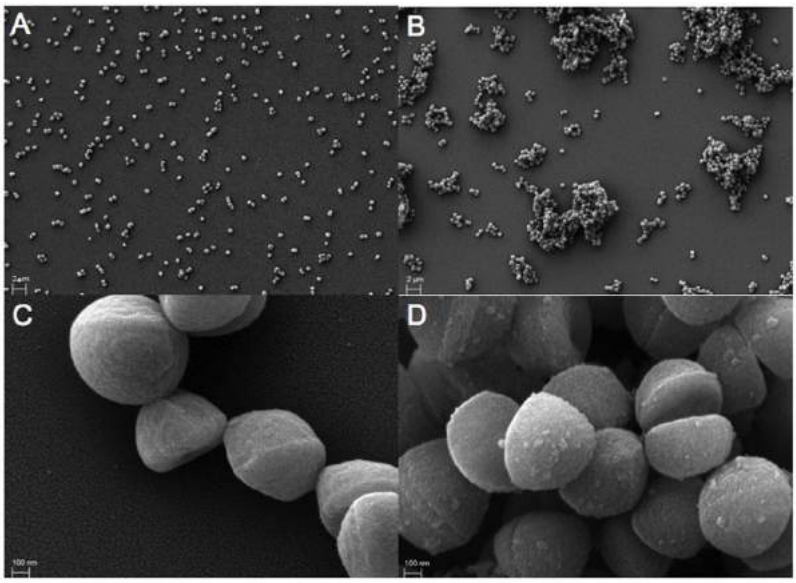
A atividade antimicrobiana de uma substância é geralmente avaliada pela determinação da concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM) *in vitro* após incubação aeróbia por um determinado tempo (LEVISON, 2004). CIM e CMB são consideradas excelentes ferramentas para a determinação da susceptibilidade dos organismos a

microrganismos e são usados para julgar o desempenho de todos os outros métodos de teste de susceptibilidade (SANTOS et al., 2015; NASCIMENTO JUNIOR et al., 2016). Dessa forma, são usados em diagnósticos laboratoriais para confirmar uma resistência anormal, para dar uma resposta definitiva quando um resultado limite é obtido por outros métodos ou quando métodos de teste de difusão em disco não são apropriados.

2.1.1 *Staphylococcus aureus*

O gênero *Staphylococcus* (do grego “staphyle” = cachos e “cocos” = grão), pertence a família Staphylococcaceae, é composto por micro-organismos imóveis, Gram positivos, produtores de catalase, que se agrupam em massas irregulares ou sob a forma de cachos de uva e podem estar associados a uma ampla variedade de infecções oportunistas em seres humanos e em animais. *S. aureus* apresenta-se como um patógeno oportunista presente no meio ambiente e está presente na pele e mucosas de seres humanos e outros mamíferos (FOSTER et al., 2014).

Figura 1 – Microscopia eletrônica *Staphylococcus aureus*



Fonte: Varrone et al 2011

Membros do gênero *Staphylococcus* apresentam-se como cocos Gram-positivos, com 0,5 a 1,5µm de diâmetro, isoladamente, aos pares, em tétrades, em pequenas cadeias (3 ou 4 células) ou irregulares, na forma de cachos (figura 1). Baseado na capacidade de coagular o plasma, através da ação da enzima coagulase, as espécies do gênero são classificadas em coagulase-positivas e coagulase-negativas. A espécie *S. aureus* é a principal representante do grupo das espécies coagulase-positivas (WONG et al., 2017). Essa espécie é reconhecida desde 1883 como agente de infecções, tendo sido responsável, nessa era pré-antibiótico, por taxas de mortalidade em bacteremias de até 82%. Hoje, continua sendo considerada como patogênica e de maior importância entre os estafilococos em infecções humanas, tanto de origem comunitária quanto de origem hospitalar (FRIÃES et al 2015; POORABBAS et al 2015).

Devido à variedade de mecanismos de virulência conhecidos, *S. aureus* é considerado um dos patógenos humanos mais versáteis (KUMAR, 2016). *S. aureus* pode causar desde infecções cutâneas superficiais, como impetigo, celulite e abscessos, até infecções invasivas decorrentes da invasão direta dos tecidos, como bacteriemia, endocardite, pneumonia e meningite (SCHAUMBURG et al 2014, GIDENGIL et al 2015, NAKATSUJI et al 2017). Entre as três principais espécies de cocos Gram positivos patogênicos para o homem estão incluídos *S. aureus, S. epidermidis e S. saprophyticus* (CHAVES et al, 2018). Dentre os *Staphylococcus* coagulase positiva (SCP), *S. aureus* pode produzir mais de 30 fatores de virulência que contribuem para o estabelecimento e manutenção de infecção. A base molecular de

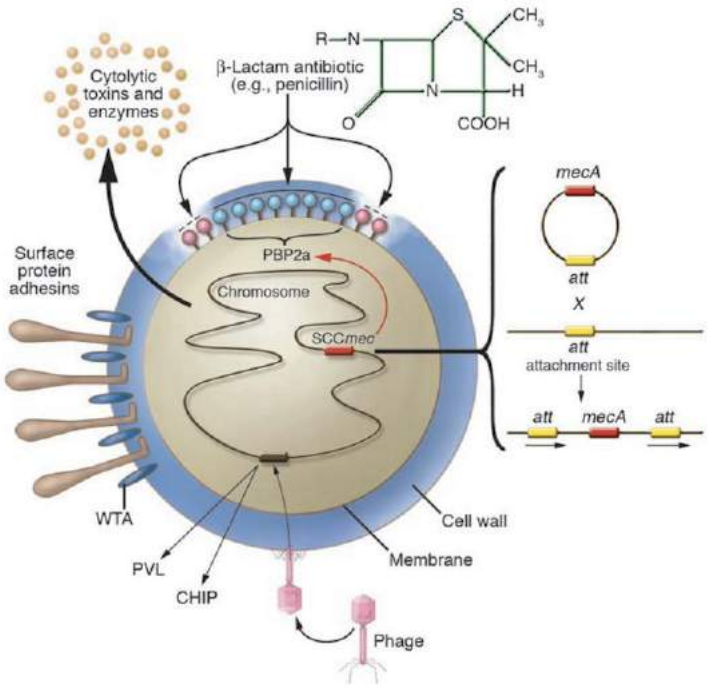
patogenicidade do *S. aureus* é multifatorial e dependente de genes reguladores que têm impacto no comportamento bacteriano (CHAVES et al, 2018).

2.1.2 Fatores de virulência de *S. aureus* e resistência a antibióticos

Dotado de uma grande variedade de mecanismos de virulência identificados, *S. aureus* é considerado um dos patógenos humanos mais versáteis (HODILE et al., 2017). Inúmeros fatores de virulência atuam na patogenia bacteriana. Durante a etapa de colonização está envolvido um conjunto de adesinas que são capazes de se ligar aos componentes da matriz extracelular do hospedeiro, que são designadas pela sigla MSCRAMMs ("Microbial Surface Components Recognizing Adhesive Matrix Molecules") e reconhecidas como importantes receptores para aderência estafilocócica (PROJAN & NOVICK, 1997). A maioria dessas MSCRAMMs possui em comum a característica de estarem ancoradas, covalentemente, à peptideoglicana da parede celular bacteriana através de ligações de transpeptidação (LIANG, X et al 2016). Os MRSA são dotados de uma variedade de genes codificadores de fatores de virulência com os genes da enterotoxinas A-E (sea, seb, sec, sed e see), hemolisinas α, β e δ (hla, hlb e hld), genes relacionados a produção de biofilme (icaAD) e esfoliatinas A, B e C (eta, etb e etd) (GILL et al, 2005; JARRAUD et al, 2012; BRONESKY et al., 2016).

Os estafilococos exibem sensibilidade variável a muitos agentes antimicrobianos. A resistência às antibióticos β-lactâmicos pode ser dividida em várias categorias, sendo as duas principais: (1) produção de β-lactamase, uma enzima codificada por genes plasmidiais, que torna os micro-organismos resistentes a classe das penicilinas (penicilina G, ampicilina, ticarcilina e fármacos semelhantes) cujas características são transmitidas ao longo das gerações. (2) A resistência à Meticilina em Staphylococcus spp. é primariamente mediada pelo gene mecA, que codifica uma proteína que apresenta afinidade reduzida por antibióticos β-lactâmicos (penicillin- binding protein 2 a – PBP2a) (WARREN et al., 2004; GORDON, N. C et al, 2014; BLUMENTHAL, K. G. et al, 2015).

Figura 2 – Mecanismos e alvo de ação para drogas antimicrobianas



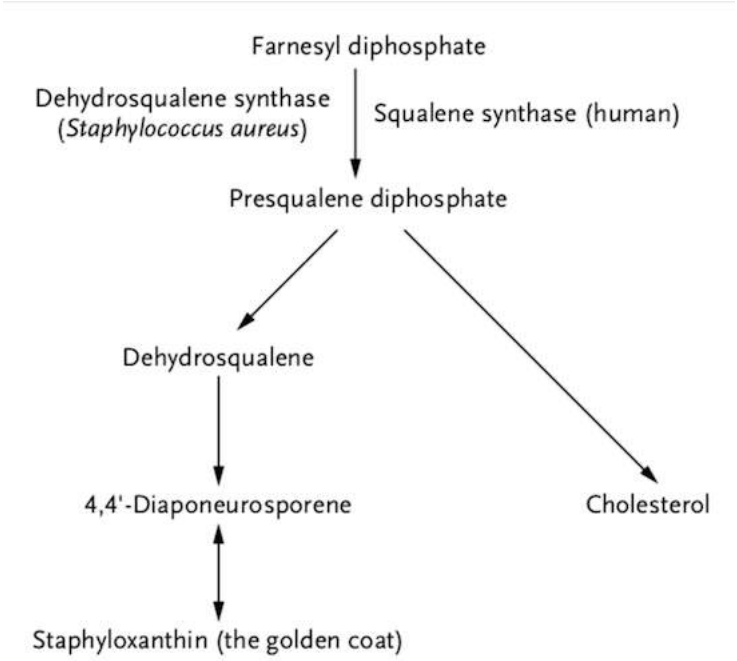
Fonte: FOSTER, T.J (2014)

O alvo de atuação dos antibióticos β-lactâmicos são as chamadas proteínas ligadoras de penicilina (PBP's), proteínas de membrana diretamente envolvidas na biossíntese da parede celular bacteriana. Os β-lactâmicos (figura 2) , que interagem com as PBP's, impedem a formação completa da camada de peptideoglicano da parede celular, desencadeando a morte bacteriana. Nas últimas décadas, *S. aureus* resistente à meticilina emergiram também na comunidade (CA-MRSA, Community-acquired methicillin resistant *Staphylococcus aureus*), trata-se de um patógeno emergente que vem apresentando crescente casos na população de vários países. Os pacientes acometidos por CA-MRSA não tiveram internação em hospitais no ano anterior à infecção, nem foram submetidos a procedimentos médicos como diálise, cirurgia ou cateter, fatos muito comuns em infecção por MRSA (BRATU et al., 2006). Enquanto o MRSA hospitalar (HA-MRSA, Hospital-acquired methicillin resistant *Staphylococcus aureus*) se caracteriza por uma ampla resistência a diversos antibióticos, as cepas CA-MRSA mostram uma sensibilidade (entre 85% e 100%) a drogas como clindamicina, gentamicina, ciprofloxacina, sulfametaxazol, trimetoprim e vancomicina (a droga mais usada em tratamento de sepse de *S. aureus*), mostrando-se resistente apenas à oxacilina e a outros beta-lactâmicos (RIBEIRO et al., 2005).

2.1.3 Mecanismo de ação da staphyloxanthin

Algumas bactérias sintetizam carotenoides mais curtos (C30) de precursores (C15) farnesil difosfato ou produzem carotenoides de cadeia longa de C45 e C50. O exemplo mais conhecido dentre esses carotenoides exóticos é a estafiloxantina (C30) (figura 3), identificada em *Staphylococcus aureus* e poucas outras espécies (PELZ et al., 2005). Atualmente acredita-se que o pigmento amarelo designado estafiloxantina, também revela atividade neutralizadora das espécies reativas de oxigênio (HUANG, 2012). Esse composto encontra-se localizado na membrana celular bacteriana e pode ser considerado um antioxidante biológico contra o peróxido de hidrogênio e radicais hidroxilas (KOSSAKOWSKA-ZWIERUCHO et al, 2016).

Figura 3: Vias de biossíntese do pigmento Estafiloxantina



Fonte: DAUM, R (2008)

Segundo Clauditz et al., em 2006, a estafiloxantina desempenha um papel suplementar na defesa contra danos provocados pelos radicais livres de oxigênio e contribuem de forma ampla para a sobrevivência das células bacterianas durante o estresse oxidativo e a resposta do hospedeiro. Esses achados podem justificar a ausência de diferenças entre as cepas estudadas já que, independente do perfil de resistência antimicrobiana, a espécie bacteriana parece ser capaz de neutralizar os compostos intermediários de oxigênio formado após sua internalização (THAMMAVONGSA, V. et al 2015).

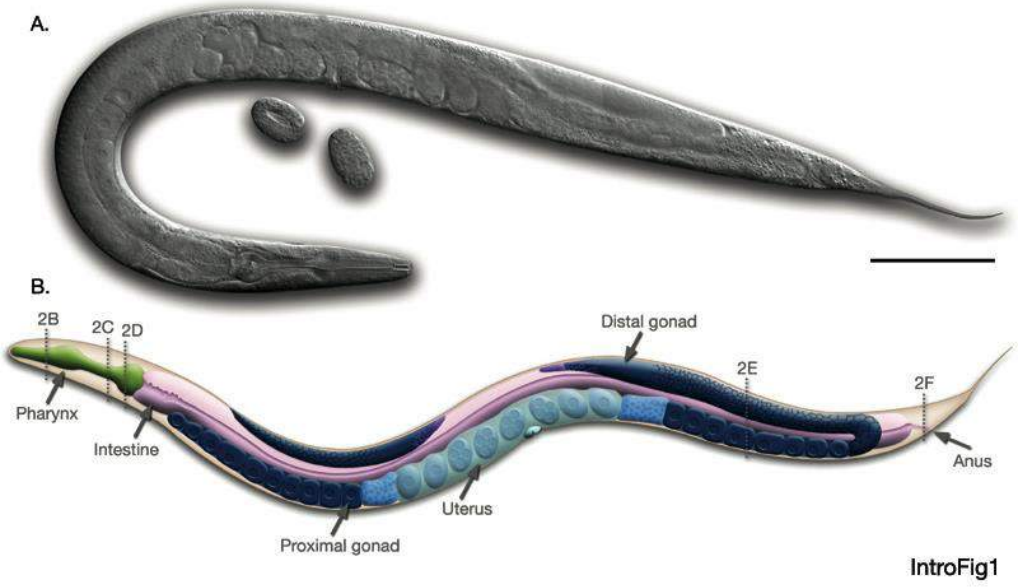
Sabe-se que *S. aureus* se furta do reconhecimento do sistema imune por meio de camuflagem através de cápsulas de polissacarídeos ou biofilme e da produção ou secreção moléculas específicas, que bloqueiam a função de receptor de fagócitos, inibindo a aderência e a fagocitose (DELEO et al., 2009). Após ser englobados, enzimas como a catalase, superóxido dismutase participam da defesa contra as EROs (espécies reativas de oxigênio contra peróxido de hidrogênio e radicais hidroxilas microbicidas FORMADOS (KAISER et al 2016; MARTINELLO et al 2016).

2.2 USO DE MODELOS *IN VIVO* UTILIZANDO NA AÇÃO ANTIMICROBIANA

2.2.1 *Caenohabditis elegans*

O *C. elegans* é um nematoide de vida livre encontrado naturalmente no solo, os vermes apresentam 4 estágios larvais diferentes e quando adultos medem 1,3 mm de comprimento e apresentam diâmetro de 100 μm (KOURTIS & TAVERNARAKIS, 2007). Apesar de anatomia simples, o animal apresenta capacidade de desenvolver atividades como locomoção, alimentação, defecação, postura de ovos, formação de larva Dauer, respostas sensoriais ao toque, cheiro, gosto e temperatura (WORMATLAS, 2017). Existem dois tipos de sexo em *C. elegans*: hermafroditas (XX) e machos (XO) (figura 4a). Os machos são raros, ocorrem numa percentagem de 0,1 % da população e são capazes de acasalar com hermafroditas, facilitando as manipulações genéticas (KOURTIS & TAVERNARAKIS, 2007).

Figura 4a: Anatomia *Ceanohabditis elegans* verme adulto

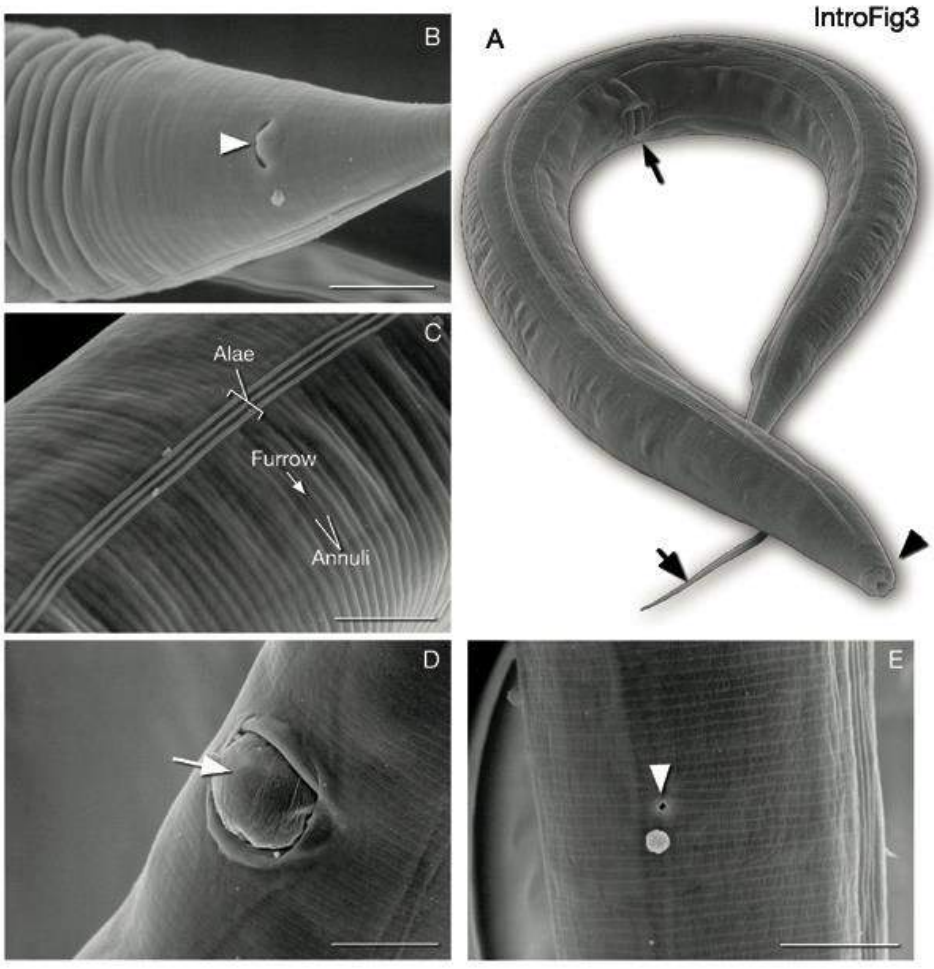


Fonte: Anatomia do verme adulto (WORM ATLAS, 2018). Disponível em: <http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm>

O ciclo evolutivo do *C. elegans* começa pela fase embrionária, logo após a fertilização,

segue por quatro estágios larvais (L1, L2, L3 e L4) até chegar a idade adulta (figura 4b) (HELMCKE et al., 2010). O final de cada fase é marcado por uma muda que consiste na síntese de uma nova cutícula e eliminação da antiga (WORMATLAS, 2018). O nematoide hermafrodita deposita seus primeiros ovos, completando seu ciclo reprodutivo entre 2,5 a três dias de vida e vivem em torno de três semanas (HELMCKE et al., 2010; WORMATLAS, 2018). O adulto hermafrodita autofertilizado tem um período fértil de três a quatro dias e é capaz de produzir cerca 300 descendentes e vive por mais 10 a 15 dias (WORMATLAS, 2018). Sob condições desfavoráveis como falta de alimentos, estresse, elevada densidade populacional, temperatura alta, as larvas podem entrar em um estágio de vida alternativa, chamado larva Dauer.

Figura 4b: Anatomia do verme adulto – Microscopia eletrônica



Fonte: WORM ATLAS (2018)

A utilização de *C. elegans* como organismo modelo para estudos *in vivo* apresenta diversas vantagens por seu tamanho compacto, ciclo reprodutivo e tempo de vida curto, corpo transparente, anatomia conhecida, facilidade de cultivo, genoma pequeno e completamente sequenciado (SI, H et al., 2014). É importante ressaltar que os vermes têm diversos tipos de células semelhantes aos seres humanos, incluindo neurônios, células musculares, intestino e células excretoras, além de compartilhar diversos genes de alta homologia e mecanismos celulares com os seres humanos. Os vermes sofrem de doenças neurodegenerativas que afligem o homem, incluindo doenças infecciosas, distúrbios de controle fisiológico, além do processo de envelhecimento (FURUHASHI & SAKAMOTO, 2014; PALLAUF et al., 2013). Seu pequeno tamanho permite o cultivo em pequeno espaço, na maioria das vezes, em placas de ágar contendo *Escherichia coli* como alimento, em temperatura de refrigeração entre 20 °C a 25 °C a depender da linhagem.

Os genes de *C. elegans* possuem elevada homologia com genes mamíferos, cerca de 60 % a 80 % dos genes humanos foram encontrados nesse nematoide; nematoides transgênicos podem ser facilmente construídos por microinjeção de DNA manipulado *in vitro* na gônada de hermafroditas adultos, onde são embalados em oócitos em desenvolvimento (HELMCKE et al., 2010); a capacidade de produzir nematoides com genes humanos permite o conhecimento de patologias humanas complexas, além disso, possibilita teste com drogas de forma fácil e eficiente (KOURTIS & TAVERNARAKIs, 2007).

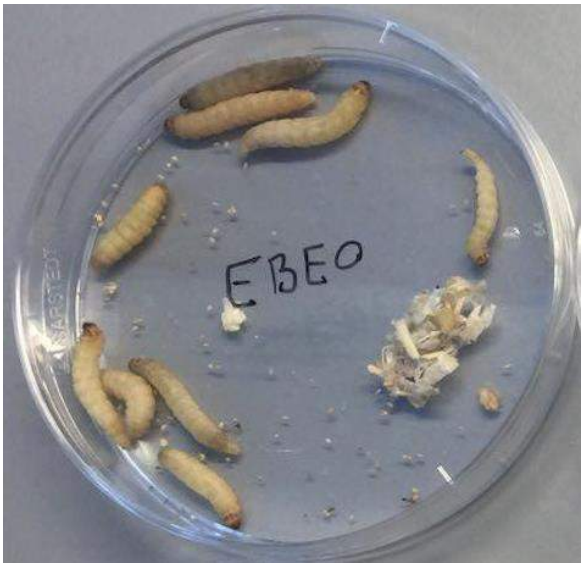
Tais características tornaram o *C. elegans* um atraente e poderoso modelo *in vivo* para estudo de mecanismos patológicos humanos como desordens neurodegenetivas, câncer, envelhecimento e doenças associadas (HELMCKE et al., 2010), através de identificação de genes, mapeamento sistemático de interações genéticas e vias de sinalização implicadas em doenças humanas (HELMCKE et al., 2010; SI et al., 2014; KOURTIS & TAVERNARAKIs, 2007). As mais recentes pesquisas sobre a criação e manutenção de diferentes cepas mutantes e/ou transgênicas, realizadas em diferentes laboratórios ao redor do mundo, estão disponíveis em bases de dados virtuais, como por exemplo, as bases WormAtlas, WormBase e WormBook.

2.2.2 *Galleria melonella*

Uma alternativa aos modelos de infecção em mamíferos é o uso de hospedeiros invertebrados como os nematoides ou insetos. *Caenorhabditis elegans* tem atraído a atenção como um modelo de infecção para uma ampla gama de patógenos bacterianos (MYLONAKIS et al., 2007; PELEG et al., 2009). No entanto, *C. elegans* não pode sobreviver a 37°C e faltam homólogos funcionais de alguns componentes do sistema imune de mamíferos, tais como células fagocíticas especializadas (MYLONAKIS et al., 2007; GLAVISBLOOM et al., 2012). A utilização de insetos como *Drosophila melanogaster* e *Galleria mellonella* como modelo de infecção oferecem a vantagem do uso a 37°C.

Os insetos possuem células fagocíticas especializadas, também conhecidas como hemócitos, que apresentam muitas propriedades em comum com fagócitos de mamíferos. Essas células são capazes de fagocitar patógenos e matá-los usando peptídeos antimicrobianos, melanina e produtos de cascatas proteolíticas (ELEFTHERIANOS & REVENIS, 2011). Desta forma, as larvas de *G. melonella* têm sido utilizadas como modelo de infecção para avaliar a virulência de diversos patógenos humanos e veterinários.

Figura 5: Larvas adultas de *Galleria mellonella*



Fonte**:** Acervo pessoal do autor.

Galleria mellonella é uma espécie de inseto lepidóptero, mais especificamente de

mariposa, pertencente à família *Pyralidae*. Os Lepidópteros são insetos holometabólicos os quais eliminam larvas a partir dos ovos, essas lagartas depois de uma série de transformações, atingem o completo desenvolvimento, realizando a primeira metamorfose, que resulta a pupa e após uma segunda metamorfose, o inseto adulto ou mariposa. Esse lepidóptero ocorre naturalmente em colmeias de abelhas e têm vasta distribuição geográfica, alimentam-se de material presente nelas como cera velha, pólen e mel.

O período de desenvolvimento larval da *G. melonella* é de 40 dias podendo variar devido a condições ambientais e a velocidade de crescimento que é diretamente proporcional à temperatura e ao suprimento alimentar. Em condições ideais de temperatura e alimentação, o peso das lagartas pode dobrar diariamente nos primeiros dez dias (JORJÃO et al 2017). Modelos de infecção de mamíferos ou de fisiologia próxima a deles são fundamentais para elucidar os mecanismos da patogênese bacteriano e também utilizados para avaliar a eficácia de novos agentes antimicrobianos, antes do início de testes em humanos.

2.3 METABOLISMO PRIMÁRIO E SECUNDÁRIO

O metabolismo é o conjunto de reações químicas que ocorrem no interior das células e, no caso das células vegetais, o metabolismo divide-se em primário e secundário. Metabólitos secundários, em contraste com os primários, nem sempre estão envolvidos em funções vitais do vegetal ou mesmo presente em todos eles o que, dentre outras coisas, permite a diferenciação entre as espécies (PICHERSKY &GANG, 2000). Estes produtos têm como função proteger a planta contra herbivoria, ataque de patógenos, bem como beneficiá-la na competição com outros vegetais (PETSCHENKA et al, 2017).

Os metabólitos secundários podem favorecer a atração de polinizadores, de dispersores de sementes e microorganismos simbiontes. Além disso, a produção destes componentes pode proteger a planta de fatores externos como variações de temperatura, umidade, exposição à radiação ultravioleta (UV) e deficiência de nutrientes minerais (ATSA, 1989). Tem sido verificado que muitos dos compostos secundários são benéficos à saúde humana principalmente por suas ações farmacológicas amplamente evidenciada na literatura (PICOLI et al 2010; KABERA et al 2014; ISSAM et al., 2015; WINK et al., 2015).

Uma das estratégias de sobrevivência de plantas em ambientes adversos é o aumento na síntese de produtos do metabolismo secundários. Os metabólitos secundários estão em menor parte envolvidos em funções vitais das plantas, mas destaca-se a atuação nos mecanismos de

defesa dos vegetais (YUE, W. et al 2016). Uma classificação simples para os metabólitos secundários inclui três grandes grupos: compostos fenólicos, terpenos e alcalóides.

Os compostos fenólicos são derivados do ácido chiquímico e do ácido mevalônico. Os terpenos são produzidos a partir do ácido mevalônico ou piruvato e 3-fosfoglicerato. Os alcalóides, compostos secundários nitrogenados, são produzidos a partir de aminoácidos aromáticos, os quais são derivados do ácido chiquímico e de aminoácidos alifáticos. Flavonoides, taninos e ligninas fazem parte dos compostos fenólicos; saponinas, carotenóides e a maioria dos hormônios vegetais são terpenos; nicotina e cafeína são alguns exemplos de alcalóides. (SIMÕES et al, 2010).

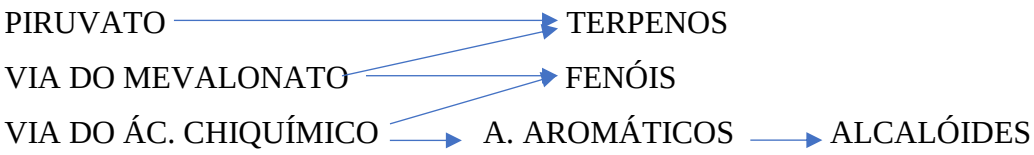
Embora classificadas em metabolismo primário e secundário, as reações não ocorrem independentemente umas das outras. Atualmente, são conhecidas mais de 200.000 estruturas (SIMÕES et al., 2010). De uma forma geral, os metabólitos primários essencialmente representam substâncias que são produzidas por todas as espécies de plantas e organismos; são geralmente essenciais para sobrevivência. Incluem, principalmente, a base universal e essencial dos carboidratos, aminoácidos, nucleotídeos, lipídeos, incluindo as enzimas e coenzimas.

As vias de síntese dos metabólitos primários são muito semelhantes entre os organismos (PICHERSKY & GANG, 2000). O armazenamento de carboidratos nos vegetais ocorre sob a forma de amido, feita para disponibilizar energia através de um processo que converte o piruvato e o acetato em acetil-coenzima A, o qual entrará no ciclo do ácido tricarboxílico que resultará na formação de ATP, dióxido de carbono e água. Os aminoácidos ocorrem tanto sob a forma livre como unidades formadoras de proteínas e outros metabólitos. A maioria dos aminoácidos contém apenas carbono, hidrogênio, oxigênio e nitrogênio em sua estrutura, mas outros átomos podem estar presentes, como o enxofre e o iodo. (LUCIANO et al. 2017). As enzimas são constituídas de proteínas ou contém proteína como parte essencial. Usualmente agem sobre uma categoria de substância, já que são específicas para um grupamento químico ou ligação química, podendo agir como lipases, proteases, oxidases, redutases, hidrolases e entre outras funções. Os lipídeos são triglicerídeos com cadeias longas saturadas ou insaturadas como constituintes importantes de reserva animal e vegetal, particularmente nas sementes. A partir dos ácidos graxos é produzido o acetil-CoA pela remoção de unidades com dois carbonos (EVANS, 2002).

A origem de todos os metabólitos secundários pode ser resumida a partir do metabolismo da glicose, via dois intermediários principais, o ácido chiquímico e o acetato. O

ácido chiquímico dá origem aos precursores da maioria dos metabólitos secundários aromáticos, como os taninos hidrolisáveis, alcaloides derivados de aminoácidos aromáticos e fenilpropanóides. O acetato fornece unidades de acetila que compõem o intermediário acetil-tio-coenzima A (acetil-CoA), o precursor de vários grupos de substâncias, como os aminoácidos alifáticos e os alcalóides derivados destes, terpenóides, esteróis, ácidos graxos e triglicerídeos. Os aminoácidos alifáticos originam-se no ciclo do ácido cítrico, enquanto que os demais metabólitos derivam do mevalonato ou da condensação de unidades de acetato. Alguns compostos derivam não apenas de um desses intermediários, mas da combinação de uma unidade do ácido chiquímico e uma ou mais unidades de acetato ou derivados deste, como ocorre com as antraquinonas, dos flavonóides e dos taninos condensados (SIMÕES, 2010; OLIVEIRA et al., 2017).

Figura 6 - Esquema biossintético dos metabólitos secundários.



Fenóis constituem o maior grupo de metabólitos secundários vegetais. São amplamente distribuídos na natureza, são encontrados possuindo estrutura simples com um anel aromático até polímeros altamente complexos, como os taninos e ligninas (CARVALHO et al., 2002; EVANS, 2002). Compostos fenólicos, servem como atrativo ou repelentes para predadores, pigmentos florais e componentes estruturais das plantas (HARBORNE, 1998). Também são importantes constituintes de algumas plantas medicinais e na indústria alimentícia são usados como agentes aromatizantes, corantes e antioxidantes. Algumas categorias de polifenóis são de interesse farmacêutico: fenóis simples, taninos, cumarinas e seus glicosídeos, antraquinonas e seus glicosídeos, naftoquinonas, flavonas e seus flavonóides glicosídicos, antocianidinas e antocianinas, lignanas e ligninas. Quanto à síntese destes compostos, estes podem ser formados pela via do ácido chiquímico, também conhecida como via do chiquimato, ou pela via do ácido acético. A via do ácido chiquímico parece ser uma via importante para a biossíntese

de unidades C6-C3 (derivados de fenilpropano) a partir de hidrocarbonetos. (OLIVOTO et al., 2017).

2.3.1 Óleos essenciais

Define-se Óleo essencial como um produto vegetal obtido por hidrodestilação, destilação a vapor ou destilação a seco (RUBIOLO, 2010; SU et al., 2016). É um termo empregado para designar líquidos oleosos voláteis dotados de aroma forte, quase sempre agradável, extraídos de plantas por processos específicos (MOUCHREK FILHO,2001). O termo "óleo volátil", tem sido mais usado devido à relação com as propriedades físico-químicos da planta, já que "óleo essencial" pode designar também produtos odorantes não formados anteriormente no vegetal, que são as "essências heterosídicas" obtidas através da hidrólise enzimática de heterosídeos (GONÇALVES et al., 2003 ; SILVA et al., 2003). Os Óleos Essenciais não são substancias puras, sendo, portanto, constituídos por variadas classes de compostos apolares, são misturas complexas de substâncias lipofílicas, geralmente odoríferas e líquidas (OLIVEIRA et al., 2017). Seus constituintes variam desde hidrocarbonetos terpênicos, alcoóis simples e terpênicos, aldeídos, cetonas, fenóis, ésteres, óxidos, peróxidos, furanos, ácidos orgânicos, lactonas, cumarinas, entre outros, sendo que a grande maioria, é constituída de derivados fenilpropanóides ou de terpenóides, Principalmente de monoterpenos e sesquiterpenos (RODRIGUES et al 2013; SIMÕES et al., 2010)

Os componentes químicos dos óleos essenciais podem ser classificados em dois grupos de origem distinta: o grupo dos terpenos e o grupo de compostos aromáticos derivados do fenilpropano. Sendo que, apenas os terpenos mais voláteis estão presentes nos óleos essenciais, ou seja, aqueles cujo peso molecular não é tão alto: mono e sesquiterpenos. Os monoterpenos estão quase sempre presentes, podendo ser acíclicos, monocíclicos ou bicíclicos chegando a constituir acima de 90% do óleo essencial (SIMÕES et al., 2010). Os Óleos Essenciais geralmente são obtidos a partir de folhas, flores, frutos, raízes, caule, ervas, madeira e ervas daninhas sendo armazenados em células secretoras, células epidérmicas, tricomas, dutos e canais. (BAKKALI, 2008; BURT, 2004). Segundo SIMÕES & SPITZER (2010), embora todos os órgãos de uma planta possam acumular óleos essenciais, sua composição pode variar conforme a localização (a exemplo, o óleo das cascas de canela é rico em aldeído cinâmico, enquanto, as folhas e raízes dessa planta são ricas, respectivamente, em eugenol e cânfora). A aplicação de óleos essenciais varia de lugar para lugar, e parecem depender da disponibilidade,

o tipo e a eficácia das plantas adequadas em diferentes localizações geográficas. Dentre as aplicações genéricas, destacam-se o uso na arte da perfumaria e aplicações odoríferas industriais, aplicações baseadas no sabor, importantes na indústria de alimentos e bebidas e as propriedades físico-químicas são aproveitadas no uso de solventes e emulsivos industriais. No entanto, do ponto de vista biológico, um grande número de plantas tem sido relatado por possuir as mais variadas propriedades tais como inseticida, anti-nutricional e repelência para insetos de produtos armazenados, assim como atividade herbicidas (JAYASEKARA et al., 2005; POONPAIBOONPIPAT et al.; 2013; DA SILVA et al 2015). Os óleos essenciais além de desempenharem papéis ecológicos importantes na proteção das plantas, atuam como antibacterianos, antioxidante e citotóxicas, antivirais, antifúngicos, inseticidas, dentre outras. Devido a essas propriedades, principalmente a antimicrobiana, o uso desses óleos nas indústrias farmacêuticas, cosmética e alimentícia está se difundindo cada vez mais como alternativa aos produtos sintéticos (YE et al., 2013; NAVARRA et al 2015; DA SILVA et al 2015)

Do ponto de vista tecnológico, a busca de novos fármacos oriundos de plantas é um processo interativo de descoberta de protótipos moleculares (lead compounds ou templates), a partir do fracionamento biomonitorado de espécies vegetais, seguido de melhoramento dos protótipos pelo planejamento e síntese de análogos, visando incrementar propriedades farmacológicas (BASER, K. H. C., & BUCHBAUER, G., 2015). Acoplado a esse paradigma pode ser observado o aumento no emprego dos óleos essenciais como fontes alternativas a outros produtos dispostos no mercado. O Brasil é um grande produtor de óleos essenciais. Embora as plantas aromáticas, condimentares e medicinais constituam fontes reais e potenciais matérias-primas de crescente demanda, seu cultivo não recebe a devida atenção. Quanto à produção brasileira, cerca de 80% dos óleos essenciais se destina ao mercado externo. Sendo que esta provém do cultivo de um limitado número de espécies exóticas introduzidas e de espécies nativas em processo de puro extrativismo (CORAZZA, 2002).

2.4 INIBIDORES DE PROTEASE

Os inibidores são compostos de origem vegetal ou animal que bloqueiam a hidrólise por uma enzima de um determinado substrato (SILVA-LUCCA et al., 2013). Inibidores de natureza proteica são capazes de produzir complexos com enzimas sendo assim capazes de inibir competitivamente as atividades catalíticas. Atuam em um grupo específico de enzimas, sendo os mais conhecidos os inibidores de cisteíno-proteinases, serino

proteinases, metaloproteinases, aspártico-proteinases e os multifuncionais que têm a capacidade de inibir diferentes classes de enzimas ao mesmo tempo (RICHARDSON, 1991).

Esses inibidores de proteinases são classificados em famílias, segundo sua estrutura primária molecular. A família de inibidores do tipo Kunitz apresentam massa molecular aproximada de 20 kDa. O inibidor mais estudado deste grupo é o SBTI, um inibidor de tripsina extraído de feijão de soja (OLIVA *et al.*, 2000).

Inibidores de proteinase inibem enzimas proteolíticas ou aumentam os níveis de antiproteinase endógena e podem contribuir para a prevenção da progressão da doença (BARNES & STOCKLEY, 2005). As proteinases são moléculas de sinalização envolvidas na hemostasia (SALU et al 2018), a morte celular, proliferação de células, replicação de DNA na resposta inflamatória e remodelamento tecidual (TURK, 2006; ALVES et al, 2016). Os inibidores de protease estão também presentes em plantas e estão envolvidos em funções biológicas de plantas e de condições patológicas. Por conseguinte, a prevenção de proteólise indesejada tem sido extensamente estudada (OLIVA et al, 2009).

2.5 PLANTAS MEDICINAIS E ESTUDOS ETNOBOTÂNICOS

O homem em toda sua trajetória registrada na história comportou-se como um agente manipulador do ambiente no qual se encontra, usufruindo do mesmo em benefício próprio mediante suas necessidades de sobrevivência (ALBUQUERQUE, 2005). Apesar dos constantes aprimoramentos na medicina, a medicina tradicional ainda é praticada em pequenas comunidades onde existem carências diversas e a agricultura é a principal economia, as plantas medicinais acabam sendo a única alternativa disponível nessas comunidades para combater suas enfermidades (MENALE et al., 2016; SILVA et al., 2015). As razões para a manutenção dessas tradições incluem o baixo custo versus o benefício terapêutico e a falta de tratamento para determinadas doenças. (MENALE et al., 2016).

No Brasil, a base da medicina popular é o conhecimento tradicional relacionado às plantas medicinais derivado de uma mistura de culturas indígenas brasileiras e influências europeias e africanas durante o período de colonização acumulados por pessoas locais com acesso direto à natureza e aos produtos da biodiversidade (OLIVEIRA, L.S 2015).

Dadas as circunstâncias sobre a resistência adquirida por microorganismos causadores de problemas de saúde pública, a necessidade da descoberta de soluções medicamentosas pra

doenças que não possuem tratamento eficaz, mas apenas um controle paliativo, faz-se relevante a bioprospecção por novas moléculas bioativas (ALMEIDA et al., 2012; GUIMARÃES et al., 2010; NASCIMENTO et al., 2000). De acordo com a Organização Mundial da Saúde (OMS), uma planta medicinal é definida como qualquer planta que contenha substâncias que possam ser usadas para fins terapêuticos ou que sejam precursoras da semi-síntese quimiofarmacêutica. O desenvolvimento de novos produtos de fontes naturais também é encorajado porque estima-se que das 300.000 espécies de plantas existentes no mundo, apenas 15% foram avaliadas para determinar seu potencial farmacológico (DE LUCA et al., 2012). Muitos estudos pioneiros contribuíram na procura por novas substâncias derivadas de plantas medicinais e várias drogas como a aspirina, atropina, camptotecina, digitoxigenina, morfina, podofilotoxina, taxol entre outras foram obtidas (BROSSI, 1985; MUKHTAR et al., 2002).

Diferentes abordagens são utilizadas como ferramenta para seleção de plantas para a triagem farmacológica. Esta abordagem trata-se de uma estratégia baseada no consenso de informações obtidas através de vários informantes sobre uma mesma espécie (CANALES et al., 2005). Assim, estudos étnico-dirigidos são importantes para entender o relacionamento entre a comunidade e a dinâmica de uso das plantas (ARAÚJO et al., 2007), conhecer os recursos terapêuticos das plantas encontradas em seu ambiente natural bem como os alvos fisiológicos , servindo, portanto, como um instrumento promissor para a indústria farmacêutica na elaboração de novos medicamentos (SILVA et al., 2015) e no gerenciamento para conservação e sustentabilidade (ALMEIDA et al., 2010; SILVA et al., 2010).

Classificando os estudos etnobotânicos de acordo com os domínios fitogeográficos naturais encontrados no Brasil, o principal estudado é a Floresta Atlântica (35,25%), seguido pelo domínio Caatinga (30,13%), o Cerrado (16.67%), a Amazônia (12.18%), a Pampas (4.49%) e o Pantanal (1.28%). Até 2012, no mínimo 248 estudos etnobotânicos examinaram a vegetação da Caatinga, onde mais de 65% deles foram conduzidos no estado de Pernambuco (ALBUQUERQUE et al., 2012).

No nordeste brasileiro é possível encontrar dois domínios majoritários: a Floresta Atlântica e a Caatinga (DI STASI et al., 2002; ALBUQUERQUE et al., 2007). Nas florestas tropicais estão abrigadas da metade das 500.000 espécies de planta no mundo e há vinte anos atrás, menos de 1% tinham suas propriedades medicinais investigadas. Nesse contexto a Caatinga pareceu ser mais promissora para estudos de bioprospecção que outros domínios fitogeográficos corroborando a ideia que a diversidade vegetal brasileira é uma fonte

promissora para diversas finalidades terapêuticas (SILVA et al 2015; DE MESQUITA et al 2018).

2.5.1 *Eugenia brejoensis*

Pertencente à família Myrtaceae, é conhecida popularmente como cutia e de recente descrição botânica (MAZINE, 2008). A família Myrtaceae possuí cerca de 3.100 espécies divididas em aproximadamente 140 gêneros, separados em duas subfamílias: Leptospermoideae e Myrtoideae (segundo APG II modificado por JUDD et al. 1999; WATSON & DALLWITZ 2007). A sub-família Leptosmermoideae distribui-se principalmente nas Américas do Sul e Central, apresenta apenas a tribo Myrteae e três subtribos, Eugeniinae, Myrciinae e Myrtinae, totalizando 70 gêneros e 2400 espécies; Já a sub-família Myrtoideae concentra-se na Austrália com 70 gêneros e 700 espécies no total. (BRIGGS & JOHNSON, 1979). No Brasil ocorrem 23 gêneros e aproximadamente 1.000 espécies da subfamilia Myrtoideae (CARDOSO & SAJO 2006). A família Myrtaceae corresponde a 1,32% do total das Angiospermas conhecidas, o que é bastante representativo, considerando-se um total de 400 famílias (JUDD et al 1999) e apresenta-se distribuída na maioria das formações vegetais do Brasil (SOARES-SILVA, 2000). O maior gênero da família Myrtaceae, é o *Eugenia L*. estimado em 1.009 espécies, distribuídos a partir do Sul do México, Cuba, Brasil, Antilhas para o Uruguai e Argentina, com um pequeno número de espécies na África (GOAVAERTS et al. 2008).

As espécies de *Eugenia* apresentam um importante papel ecológico nas florestas tropicais (LANDRUM& KAWASAKI 1997; CARDOSO& SAJO 2006; RAMOS et al. 2010). Elas dispersam suas sementes por proporcionar frutos comestíveis, os mesmos são atrativos para muitos tipos de animais. As diversas espécies *Eugenia* são apreciadas por seus frutos comestíveis, tais como a pitanga, *E. uniflora* (LEE et al. 1997) e jacuntinga, *E. edulis* (HUSSEIN et al. 2003).

Eugenia brejoensis é uma das espécies encontradas na caatinga do estado de Pernambuco, encontrada em regiões de brejo, localizadas na ‘caatinga’ (figura 2) (MAZINI & CASTRO 2008). Um estudo de Giaretta & Peixoto (2014), demonstrou que essa espécie também está presente em regiões como Paraíba, Alagoas, Sergipe e Nordeste do Espírito Santo. O uso popular da mesma tem sido apontado para as ações antidiarreica, antifebril e anti-

reumática a partir de folhas, frutos, casca e caule porém por apresentar descrição botânica recente podem apresenta uma série de possibilidades ainda inéditas na espécie e que vem sendo avaliadas em nosso grupo. Estudos recentes comprovam a presença de atividade antimicrobiana contra bactérias a partir de diversas partes da planta. (SILVA et al. 2011; AZEVEDO et al 2012).

Figura 7 – *Eugenia brejoensis (folha)*

Fonte: *Herbarium Network (2018)*

2.5.1.1 O óleo essencial de *Eugenia brejoensis* (EbEO)

O óleo essencial foi extraido a partir de folhas da planta recentemente pelo método de hidrodestilação utilizando o aparato de Clevenger. Foram contabilizados 31 compostos
Para 89,3% dos componemtes detectados por cromatografia gasosa (GC/MS).

O óleo continha principalmente sesquiterpenos (62,66%) e sesquiterpenos oxigenados (26,64%) e os principais constituintes foram δ-cadineno (22,6%), β-cariofileno (14,4%), α-muurolol (9,34%) a-cadinol (8,49%) e bicyclogermacrene (7,93%) (figura 8). As identidades de cinco constituintes, que representam 9,92% do total, não puderam ser confirmadas. Estudos anteriores de óleos essenciais das folhas de membros das Myrtaceae, incluindo Neofabricia myrtifolia, Asteromyrtus angustifolia, E. dysenterica, E. caryophyllata (síncope E. caryophyllus), revelaram a presença comum de β-cariofileno como componente principal **(**BROPHY, J.J. & CLARKSON, J.R. (1994); FICHI, G et al 2006; JIROVETZ L.E et al 2006)

Figura 8: Composição química do Óleo essencial de *Eugenia brejoensis*

Component [a]	Retention index		Percentage of
	Calculated [b]	Literature [c]	total oil
α-Cubebene	1351	1348	0.25
α-Copaene	1377	1374	0.79
β-Elemene	1393	1389	1.22
α-Gurjunene	1411	1409	2.83
β-(E)-Caryophyllene	1421	1417	14.4
β-Copaene	1431	1430	0.36
β-Gurjunene	1434	1431	0.05
Aromadendrene	1441	1439	1.2
trans-Muurola-3,5-diene	1452	1451	0.11
α-Humulene	1455	1452	1.5
Caryophyllene <9-epi-(E)->	1463	1464	2.33
trans-Cadina-1(6),4-diene	1475	1475	0.1
γ-Muurolene	1478	1478	1.14
β-Selinene	1490	1489	0.34
trans-Muurola-4(14),5-diene	1494	1493	0.02
Bicyclogermacrene	1502	1500	7.93
γ-Cadinene	1516	1513	5.94
δ-Cadinene	1526	1522	22.6
trans-Cadina-1,4-diene	1534	1533	0.63
α-Cadinene	1540	1537	1.06
α-Calacorene	1545	1544	0.19
Palustrol	1570	1567	0.13
Spathulenol	1580	1577	3.28
Viridiflorol	1594	1592	0.08
Guaiol	1600	1600	1.61
Cubenol <1,10-di-epi->	1618	1618	0.31
Cubenol <1-epi->	1631	1627	0.92
α-Muurolol	1644	1644	9.34
α-Cadinol	1657	1652	8.49
Bulnesol	1670	1670	0.08
Shyobunol	1693	1688	0.07
Sesquiterpenes hydrocarbons			62.66
Oxygenated sesquiterpenes			26.64
Unidentified compounds			9.92
Total			99.22

Fonte: SILVA et al. (2015)

Dentro do gênero *Eugenia*, os óleos foliares mostram várias semelhanças, como exemplificado pela abundância dos monoterpenos α e β-pineno e do sesquiterpeno β-cariofileno (COLE, R.A et al 2007). Magina et al. (2000) detectaram estes terpenos, juntamente com biciclogermacreno e δ-cadineno, nos óleos essenciais de *E. brasiliensis*, *E. beaurepaireana e E. umbelliflora*, embora o viridiflorol e o espathulenol fossem componentes principais em duas das espécies. A congruência entre óleos da mesma família provavelmente está associada às

características dos solos e climas das regiões, cujas condições ambientais eram semelhantes às da localização em que *E. brejoensis* foi coletada no presente estudo. Esses fatores apoiam as identidades descritas dos constituintes do óleo essencial de *E. brejoensis*, uma planta outrora conhecida endêmica apenas do estado de Pernambuco (GIARETTA & PEIXOTO, 2014).

O óleo foliar de *E. brejoensis* exibiu uma atividade larvicida moderada A. aegypti, na medida em que a mortalidade foi observada com soluções contendo mais de 100 ppm de óleo. As concentrações necessárias para matar 50% das larvas de mosquitos variaram entre 160 e 280 ppm, apresentando um valor médio de LC50 de 214,7 ± 12,3 ppm. As propriedades larvicidas do δ-cadineno e do bicyclogermacrene não foram descritas na literatura, embora o p-cariofileno seja conhecido por exibir atividade larvicida e valores de LC50 variando entre 50 e 1.202 ppm foram relatados. No entanto, Santos et al. afirmam que o β-cariofileno fornece apenas uma pequena contribuição para atividade larvicida (150-400 ppm, mortalidade <10%). A discrepância entre esses relatórios provavelmente reflete graus variáveis de susceptibilidade das diferentes cepas de mosquito empregadas nos ensaios. A alta porcentagem de β-cariofileno (14,4%) detectada no óleo foliar de *E. brejoensis* sugere que outro componente pode ser responsável pelos efeitos larvicidas observados. As atividades larvicidas contra *A. aegypti* de vários extratos solventes de folhas de *E. uniflora* foram registradas em vários estudos **(**FAMUYIWA & ADEBAJO, 2012). Os extratos de folhas de *E. jambolana* obtidos usando diferentes solventes apresentaram valores de CL50 na faixa de 40 a 400 ppm contra A. aegypti e outras espécies de mosquitos 29 e exibiram efeitos sinérgicos contra o mosquito da febre amarela quando combinados com a piretróide de deltametrina sintética **(**RAGHAVENDRA et al, 2013).

Embora os óleos essenciais de uma série de espécies de Eugenia sejam conhecidos por apresentar uma grande variedade de atividades biológicas úteis (SING, J et al 2012), a identificação de componentes em novas fontes de óleo essencial pode levar à descoberta de novos compostos bioativos e inseticidas naturais.

2.5.2 *Bauhinia bauhinioides*

Entre as inúmeras espécies vegetais de interesse medicinal, encontram-se as plantas do gênero *Bauhinia*, pertencentes à família Leguminosae, as quais são encontradas principalmente nas áreas tropicais do planeta, compreendendo aproximadamente 300 espécies. Muitas destas plantas são usadas como remédio na medicina popular em várias regiões do mundo, incluindo

a África, Ásia e América Central e do Sul (BREVIGLIERI, 1997). Os estudos fitoquímicos e farmacológicos realizados com estas plantas indicam que as mesmas são constituídas principalmente de glicosídios esteroídicos, triterpenos, lactonas e flavonoides (CECHINEL, 2000).

No Brasil, as plantas do gênero *Bauhinia* são conhecidas como "Pata-de-vaca" ou "Unha-de-boi". As folhas, caules e raízes das espécies de *Bauhinia*, especialmente *B. manca, B. rufescens, B. forficata, B. cheitantha e B. splendens*, são amplamente utilizadas no Brasil e em outros países em forma de chás e outras preparações fitoterápicas para o tratamento de várias enfermidades, principalmente infecções, processos dolorosos e diabetes.

2.5.2.1 O inibidor de calicreína recombinante *Bauhinia bauhinioides* (rBbKIm)

Bauhinia é um gênero de plantas da subfamília *Caesalpinoideae* que compreende mais de 600 espécies nativas de florestas tropicais e subtropicais (RICHARDSON, 1991). Numerosos inibidores de proteases têm sido isolados a partir deste género, particularmente a partir das espécies de *Bauhinia bauhinioides.*

BbKI é um inibidor da tripsina, quimotripsina e plasmina. É também um potente inibidor da calicreína plasmática humana (huPK). BbKI é o primeiro inibidor derivado da planta com estrutura primária descrita e que é um inibidor de calicreína tissular (CAGLIARI C, CAROLI, et al 2003; OLIVA, 2003).

Recentemente, novas estratégias terapêuticas visando o controle da produção e/ou inativação das proteinases estão sendo discutidas para o tratamento da doença pulmonar obstrutiva crônica (GOLD, 2015). Desde que as proteinases deixaram de ser consideradas apenas como proteínas de degradação enzimas e tornaram-se importantes moléculas sinalizadoras envolvidas em vários processos biológicos vitais, os inibidores da proteinase estão sendo intensamente investigados (KORKMAZ, 2010).

3 RESULTADOS

3.1 ARTIGO 1 - *BAUHINIA BAUHINIOIDES* KALIKEIN RECOMBINANT INHIBITHOR AFFECTS *STAPHYLOCOCCUS* VIRULENCE

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Manuscript to be submitted to the journal Peptides

ISSN: 0196-9781

Impact factor: 2.7

Qualis CAPES: B1 – Área: Ciências Biológicas I

Abstract

The study of antimicrobial peptides has been in the focus of the fight against resistant microorganisms like multirresistent *Staphylococcus aureus*. This work aimed to evaluate the anti-*S. aureus* action of the recombinant peptide rBbKIm, a protease inhibitor from *Bauhinia bauinioides* and has already been identified as cytotoxic to cancer cells. This compound did not demonstrate microbicidal activity at concentrations of 50 to 250 μg/mL, but indicated a reduction in the production of virulence factors *in vivo* and *in vitro* models. The concentration of 250 μg/mL was responsible for the highest percentage reduction in staphyloxanthin production, a carotenoid pigment produced by *S. aureus that acts fighting against* free radicals of host immunity that can lead bacterials to death. Concentrations of 25 μg/mL to 100 μg/mL decreased bacterial biofilm production in accordance with the action of this peptide against the hla/ spa genes, demonstrating that it constitutes a potent QSI (*quorum sensing* inhibitor). Synthetic peptides were designed and also applied in the same models used. In vivo models in *Caenohabditis eleg*ans demonstrated that the rBbKIm was able to increase the worm lifespan in a infection model. *Galleria melonella* larvae showed around 70% survival after 96 hours of infection and concomitant treatment of 250 μg/mL when compared to larvae treated with *S. aureus* only, these peptides were not able to decrease bacterial load in hemolymph of this insect but apparently decreased the virulent effect of *S. aureus*. rBbKIm and derived peptides showed similar effects in *Galleria melonella*. It was concluded that rBbKI and derived peptides are important candidates for the treatment of infections caused by *Staphylococcus aureus* by affecting the virulence of this bacterium without decreasing its plasma amount using mechanisms of action that reduce the aggression of host cells and can be applied in human cells.

Keywords: *Staphylococus aureus*, virulence, rBbKIm, nosocomial inffections

Introduction

Antibiotic resistance is considered one of the major health problems of this century due to the considerable increase in bacterial species that has acquired resistance to traditional drugs used in therapeutics. Nosocomial infections are responsible for a large death percentage related to

hospital environment in many countries of the world. Microorganisms such as *Pseudomonas aeruginosa, Candida albicans and Staphylococcus aureus,* the latter being considered one of the main pathogens of the 21st century due to its high degree of virulence and resistance factors that define the attack on the hosts, gain attention before the urgency in treatment. *S. aureu*s is considered one of the most versatile human pathogens due to the variety of known virulence mechanisms (HODILE et al., 2017).

Antimicrobial peptides (AMPs) are studied as viable alternatives to combat antimicrobial resistance that currently involves practically all available antibiotics (WHO, 2015). AMP's are also found in bacteria, protozoa, bacteria and plants and present various mechanisms of action against human cells such as virulence reduction, interference in membrane synthesis and disruption (FARKAS, A., 2017). There are different types of AMP whose structure varies according to their function and action, are distinct secondary structures formed by amino acid sequence, that interact with each other during the mechanism of action. (HANCOCK & SAHL, 2006).

The main objective of this work was to use the pet29a vector to obtain by means of heterologous expression the protease inhibitor rBbKIm (FERREIRA et al 2013) to determine the minimum inhibitory concentration of bacterial growth on the strains of S. aureus ATTC 29213. Additionally, the effects of this compound and derived peptides on *Staphylococcus aureus* virulence were tested, these results were further confirmed using in vivo *Galleria melonella* and *Ceanohabditis* models.

Metodology

Antimicrobial analysis

The antimicrobial activity of rBbKIm were confirmed by determination of Minimum Inhibitory Concentration (MIC) against several *S. aureus* antibiotic resistant strains by the method described elsewhere (SILVA, L. C. N. et al, 2013).

Staphyloxanthin quantitative assay

Overnight cells were re-inoculated at 1:100 dilution in LB medium and incubated for 16 h at 37°C with or without tested compounds. One mL of cells was then collected by centrifugation at max speed for 10 min and washed with 1 mL of phosphate-buffered saline (PBS). At this point, cell pellets were photographed to compare the staphyloxanthin production (SPAULDING, A.R et al., 2014). For caratenoid pigment extraction, cell pellets were resuspended in 0.2 mL of methanol by vortexing, and this mixture was heated at 55º C for 3 min. Pigment extraction was separated from cell debris by centrifugation at max speed for 10 min. This pigment extraction step was repeated 3 times, and the optical densities of collected extractions were measured at 465 nm.

C. elegans anti-infective assay

Caenorhabditis elegans was used to evaluate the anti-infective effect protease inhibitor. This free-living terrestrial nematode is an advantageous model organism because it is multicellular and exhibits conserved physiological systems, short life span, fully sequenced genome and about 60-80% of genes homologous to humans. It is also considered a fast, cheap and efficient model for in vivo testing of antimicrobial substances.

Chronological synchronization of *C. elegans* strains

EbEO increased the lifespan of *C. elegans* (AU37 strains) when compared to untreated *S. aureus* control. The worms were monitored for 7 days and at the end of this period the viability between the concentrations used (0,11 and 0,05 mg/mL. MIC and MIC/2 respectively) varied between 70 and 80% (Figure 6).

Strain synchronization was performed by the alkaline lysis method, based on the treatment of pregnant hermaphrodite adults with lysis solution (50% sodium hypochlorite, 2.5 mM NaOH). Embryos resistant to this treatment were collected and placed in M9 liquid medium. After 12 h, the L1 worms obtained were seeded on plates containing the NGM medium (seeded with *E. coli* OP50) for 48 h at 25°C, time required to reach the L4 stage.

In vivo virulence assays using *Galleria mellonella*

G. melonella larvae (~ 200 mg) were randomly selected in experimental groups (n = 10/ group), PBS was used as a control for rBbKIm non-treated larvae and compared treated with only MIC larvae and larvae infected with 10 µl of a bacterial *S. aureus* suspension (1.0 x 10^5 CFU) plus rBbKIm and then in the ventral region and subsequently incubated at 37° . After 1 and 3 hours, larvae hemolymph was collected to measure melanization degree and was compared with PBS control and only rBbKIm 100 µg/mL group reincubated at 37°C during all experiment time. Mortality wasn't observed after infection and incubation time.

Biofilm formation

A *Staphylococcus aureus* bacterial suspension (80 µL suspension, 0.1 measured at OD600) was added to 80 µL solution containing 25 to 100 µg/mL rBbKIm and the volume was completed to 200 µL with LB medium in a 96-well plate. All the samples was well mixed and incubated at 37°C. After 24 h, wells was washed gently three times with PBS to remove non-adherent cells. Remaining attached bacteria should be heat-fixed at 60°C for 1 hour. The adherent biofilm layer formed was stained with 0.4% crystal violet for 15 min at room temperature and solubilized with DMSO (Sigma-Aldrich Co.USA) and absorvance measured at 570 nm. The Biofilm formation control was considered culture without peptide treatment and represent 100% of biofilm formation.

Statistical analysis

Statistical analyses were performed using the software GraphPad Prism version 5 (www.graphpad.com). Data from were analyzed by two-way analysis of variance (ANOVA), 1-way and 2-way and Tukey test. A *p*-value of < 0.05 was considered to be statistically significant. Differences in the survival of *G. melonella* larvae were determined using the Kaplan-Meier method to calculate survival fractions and log-rank test was used to compare survival curves.

Results and Discussion

Antimicrobial analysis

The antimicrobial activity of rBbKIm were confirmed by determination of Minimum Inhibitory Concentration (MIC) against *S. aureus* ATCC 29312 several antibiotic resistant strains using microdilution assay. It was used the method described elsewhere (da Silva, L. C. N. et al, 2013) with some modifications and using TTC to revelation of microbial growth. This peptide didn't shown any bactericide activity even in ATCC strains.

***Staphyloxanthin* quantitative assay**

The protease inhibitor rBbKIm showed no microbicidal activity when tested with *S. aureus* strains, however, was shown to be a potent inhibitor of the virulence of this microorganism. The production of the caratenoid pigment Staphyloxanthin was substantially affected qualitatively in the 250 μg / mL concentration but in quantitative terms there was dose-dependent.

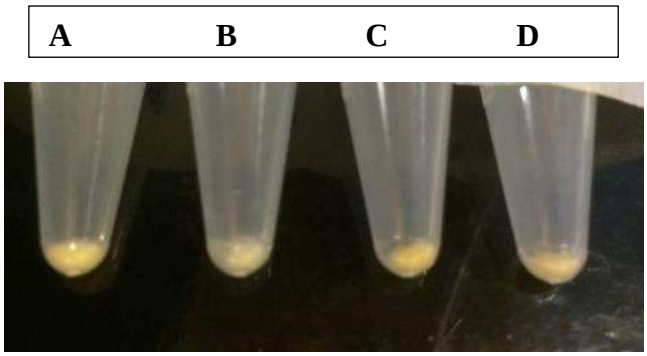

Figure.1a: (A) Control, overnigh culture with ~ 10^6 CFU **(B)** overnight culture treated with 250 μg/mL, **(C)** overnight culture treated with 100 μg/mL and **(D)** overnight culture treated with 50 μg/mL

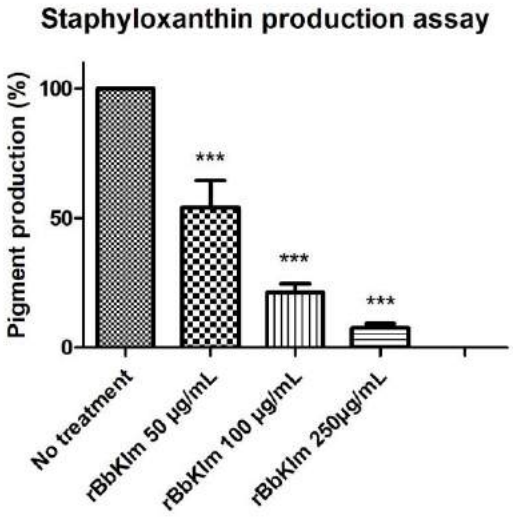

Figure 1b: (A) Control, overnigh culture with ~ 10^6 CFU **(B)** overnight culture treated with 100 μg/mL, **(C)** overnight culture treated with 250 μg/mL and **(D)** overnight culture treated with 50 μg/mL.

A quantitative assay was used to determine the level of reduction of the production of this carotenoid (Lee et al., 2012). Suspensions of *S. aureus* were treated with different concentrations of rBKIm and staphyloxanthin pigment (STX) contained in the bacterial pellet was evaluated qualitatively and quantitatively. The figure shows the relation staphyoxanthin/concentration of rBbKim quantitatively (Figure 1b), where even at lower concentrations as 50 μg/mL, the protease inhibitor is still able to inhibit the production of the pigment to values around 50% reducing the production of the pigment in a dose-dependent way.

***C. elegans* lifespan assay**

rBbKIm increased lifespan of *C. elegans* larvae infected with *S. aureus*. The concentration of 100 μg/mL was able to increase the survival of this worm even under conditions of infection in which the nematode was cultivated in contact with S. aureus allowing the ingestion and proliferation of bacterial cells from the ingestion of the medium in which it was cultivated . The difference between the survival percentage of the worms became more pronounced and statistically significant from the second day of treatment, remaining relevant until the last day

of the experiment (figure 2)

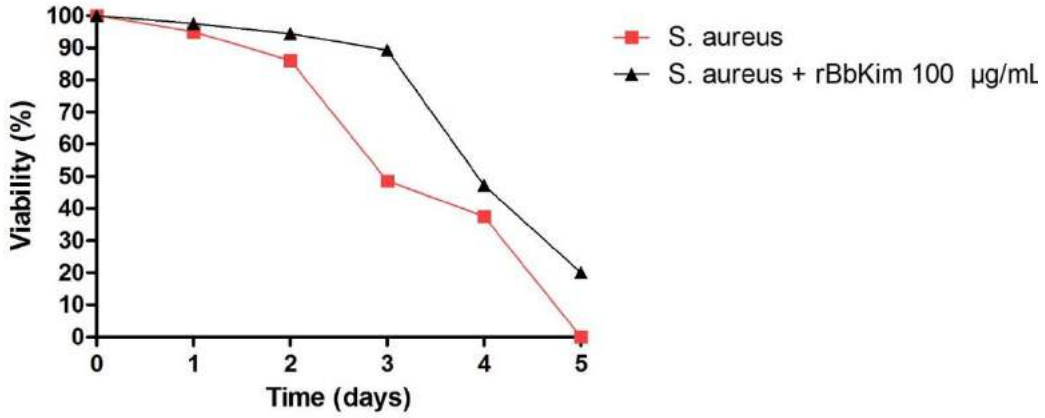

Figure 2:. *Caenohabditis elegans* (AU37 strain) was maitained for 5 days. Control group was considerated the plates containing only *C. elegans* treated with S. aureus (red line in figure). Experimental group include worms infected with S. aureus and feeded with 100 µg/mL of rBkIm

Protease inhibitor toxicity assay in *Galleria melonella*

The toxicity of 250 µg/ mL rBbKIm was also evaluated in *Galleria melonella* models and at the highest concentration tested before did not cause any changes in the larval life when compared with a PBS control showing that this peptide isn't toxic to larvae in this concentration

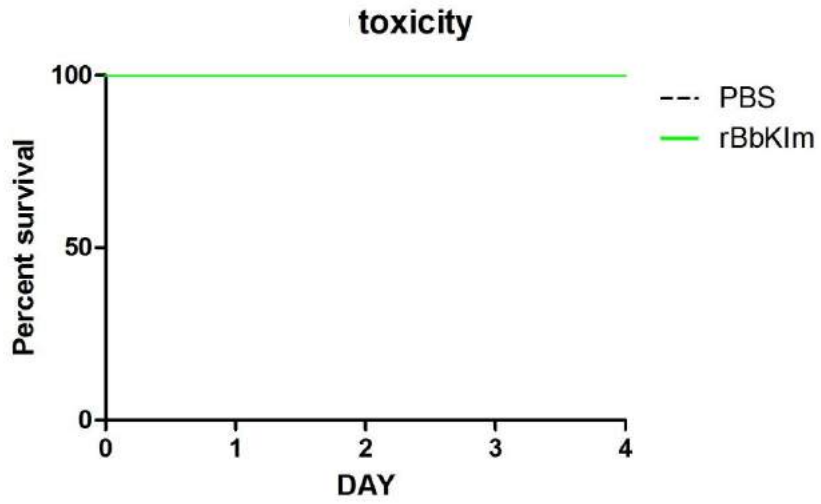

Figure 3:. The rBbKIm protease inhibitor was tested at the same concentration as that for the experimental design and, like the PBS control, showed no toxicity in *G. melonella* model

***Galleria mellonela* survival assay**

The action of the compound was evaluated in the *S. aureus* infection model *in G. melonella* larvae. rBbKIm did not show any toxicity to larvaes in 4 days time period (figure 4). Survival of the larvae was monitored over 4 days and those treated with 100 μg/mL rBbKIm showed a survival of 70% at the end of the period, while the Infected larvae showed 100% mortality at the end of the third day. In order to verify the bacterial load of the larvae throughout the treatment day's, number of colonies present in the plasma (n = 5 individuals) was collected, seeded and counted. The figure 5 shows the difference (in potency of 10) between *S. aureus* growth over the first 3 days between larvae treated and not with protease inhibitor. Melonization is an indicator of the anti-virulence effect of compound on *G. melonella* models. Inhibitor significantly reduced stress, resulting in less melonization when 10^6 CFU / mL were given to larvae and treated with 250μg/mL.

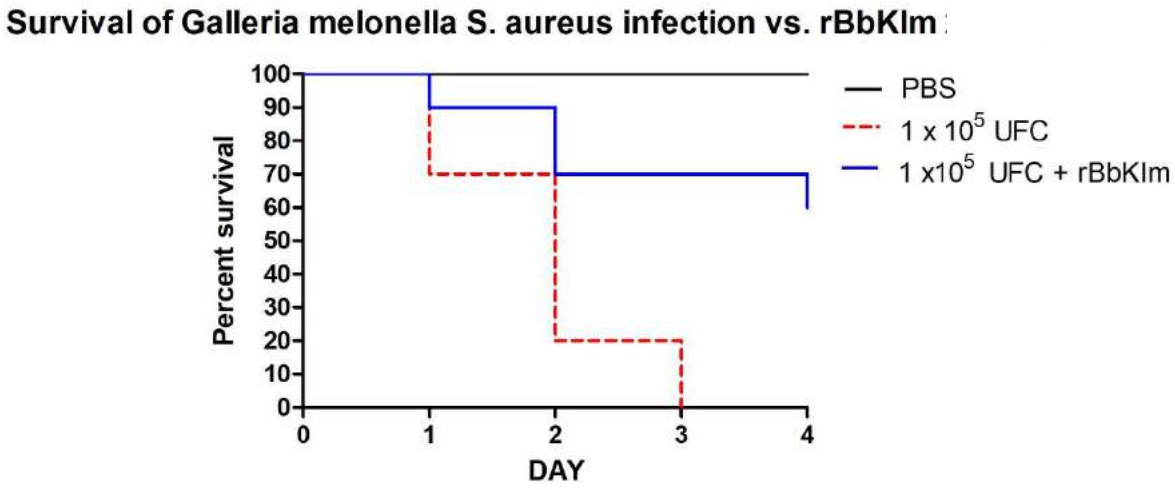

Figure 4:. ***Galleria melonella* survival.** Ten G.melonella larvae (~ 200 mg) were randomly selected and divided into experimental groups (n = 10 for each group), each larvae received rBbKIm 100 μg/mL injected with a Hamilton syringe, the larvae were incubated at 37°C and compared with a control group that received sterile PBS and maintained in the same conditions. Mortality was observed by counts up to the fourth day after infection.

rBbKIm prolonged the survival of *Galleria melonella* larvae in *S. aureus* sepsis models, even though previous results indicate that this peptide shows no microbicidal action alone. This indicates that's somehow this peptide increase larve lifespan

S. aureus in *Galleria mellonella* plasm content

The survival of a large number of larvae is not linked to the death of the percentage of bacteria available in the hemolymph of *Galleria mellonela*. The action of the inhibitor is could be linked to inhibition of the proteases produced by *S. aureus* mode of action and/or virulence reduction

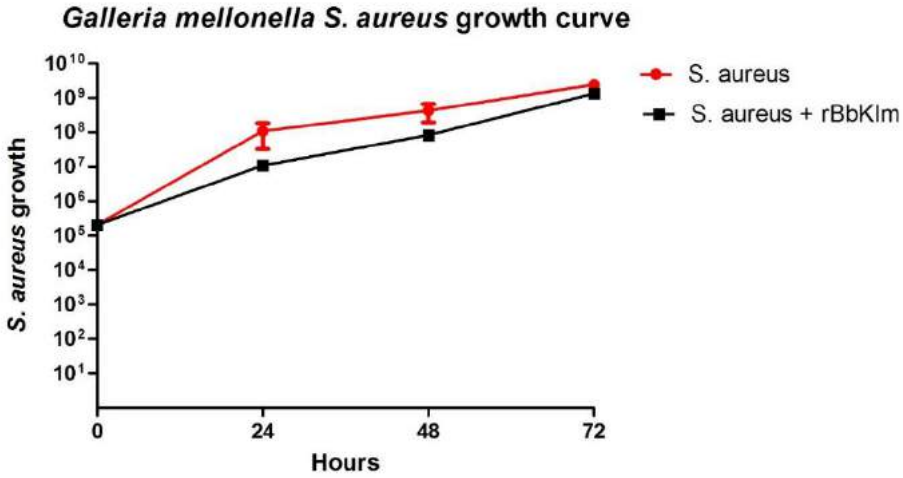

Figure 5. *S. aureus* hemolynph load was measured up to 72 hours and compared with same initial bacteria concentration (10^5 CFU/mL) treated with 100 μg/mL

Beta galactosidade assay (hla/spa gene expression)

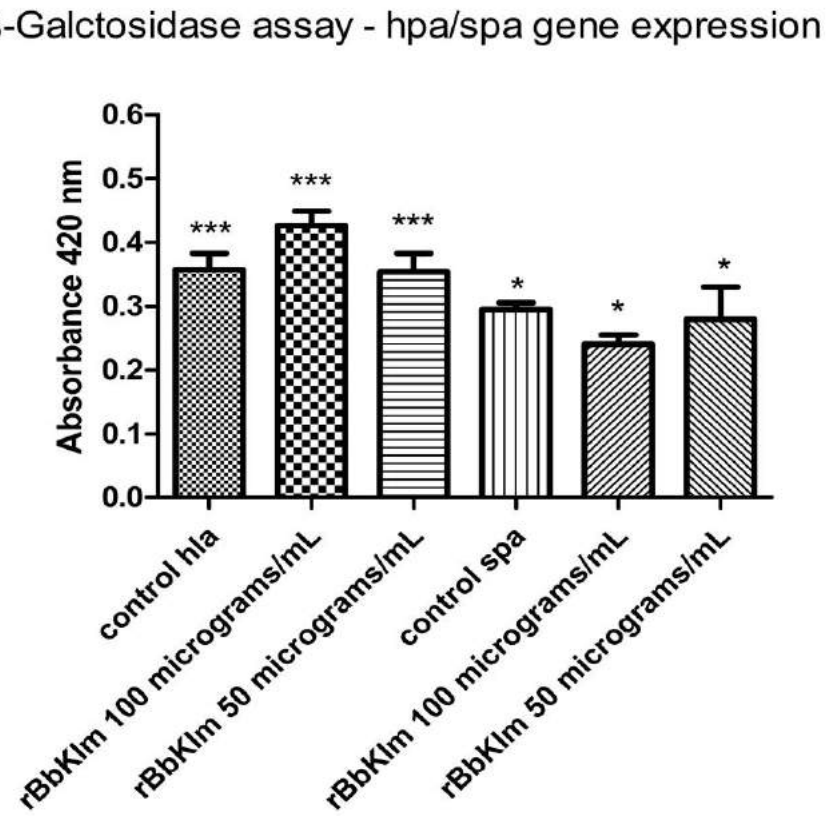

Figure 6:. rBbkIm in 100 μg/mL is capable simultaneously increase hla gene expression and decrease spa gene expression when compared with control without peptide treatment, constituting a probable Quorum Sensing Inhibitor (QSI).

rBbKIm increases hla gene expression and decreases spa gene expression, thus being a possible inhibitor of Quorum sensing (QSI)

The synthesis of extracellular proteins is related to the mode of action of *Staphylococcus aureus*, which uses this mechanism to guarantee virulence in infections. This protein synthesis is regulated by a gene region known as *agr*, which is responsible for the expression and suppression of specific genes (VANDENESCH, F. ET AL 1991).

The genes responsible for the production of a-hemolysin (hla) and protein A (spa) are activated and repressed by agr and are crucial factors for virulence of this microorganism. (VANDENESCH, F. ET AL 1991). The hla gene is the main virulence factor of S. aureus since

the alpha toxin is capable of acting as a key factor in the pathogenesis of S. aureus in infections of skin, bloodstream and pneumonia (BECKECK et al., et al. 2007, BUBECK & PATEL, 2007; BUBECK & SCHNEEWIND, 2008). Protein A is associated with the capture of IgG antibody molecules that prevents bacterial cell phagocytosis by the immune system cells (FOSTER, TJ, 2005; FOURNIER & PHILLPOT, DJ, 2005; VOTINTSEVA, A. A et al., 2014) .

The rBbKIm peptide was tested at concentrations of 50 and 100 μg/mL against the expression of the two genes (hla and spa) and at the highest concentration (100 μg/ ml) was able to increase hla gene expression and suppress spa gene expression spa. Such behavior suggests that this peptide is able to influence the agr region of the bacterial genome and interfere in the S. aureus agr QS system (Quorum sensing) (NIELSEN, A et al 2010).

Biofilm formation

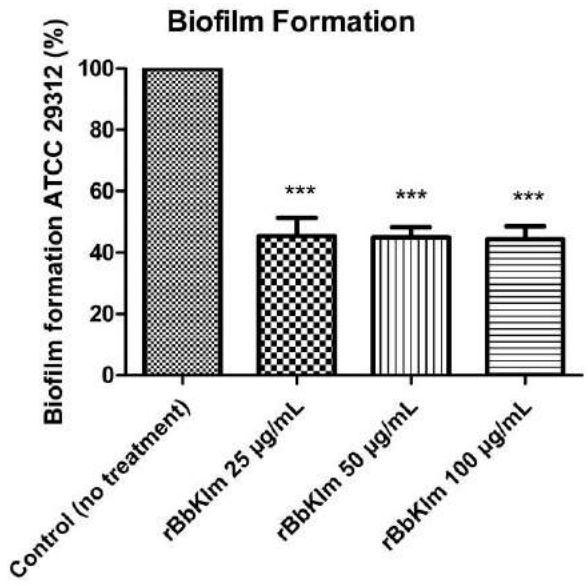

Figure7:. Overnight cultures at 37º C were tested with or without rBbKIm treatment in *S. aureus* biolfiolme production. 25 μg/mL to 100 μg/mL showed reduction of biofilm production with an approximate percentage of 40%

Different concentrations of rBbKIm were tested for biofilm formation. Staphylococcus aures ATCC 29312 was used as a control and all concentrations were responsible for a reduction of biofilm formation around 40%. Even at different concentrations the reduction of the biofilm percentage formed remained constant from 25 μg / mL up to 100 μg/mL and statistically

significant. According to the literature, there is a relationship between hla / spa genes as possible QSI (*quorum sensing* inhibitor), observed in the experiments performed.

Peptides derivated from rBbKIm also showed anti-virulence action

Peptides derived from the same inhibitor showed similar action with reduction of staphyloxanthin production. Peptides named P09, P10 and P11 (unpublished data) were tested. The P10 peptide was able to reduce the production of the pigment Staphyloxanthin at concentrations of 250 μg/mL, 100 μg/mL, 50 μg/mL and 25 μg/mL.

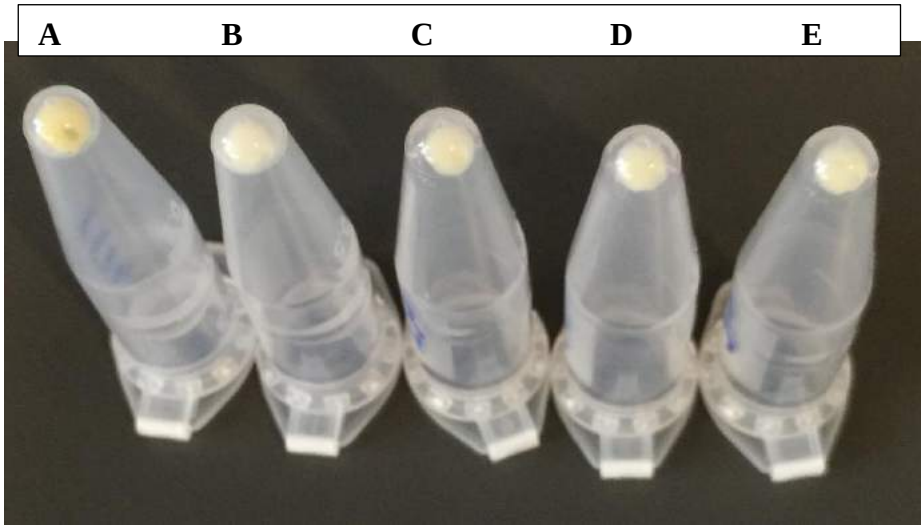

Figure 8:. The peptide identified with P10 showed good decrease of Staphyloxanthin production. Tubes identified (A) control sample without treatment (B) bacterial overnight culture treated with 250 μg/mL (C) bacterial overnight culture treated with 100 μg/mL, (D) bacterial overnight culture treated with 50 μg/mL e (E) bacterial overnight culture treated with 25 μg/mL.

***Galleria melonella* peptide assays**

The P11 peptide had the best action in increasing the survival of *G. melonella* in *S. aureus* sepsis models. Although the peptide designated as P11 showed better results for the survival of *G. melonella* infected with *S. aureus*, peptides P09 and P10 showed a high percentage of survival at the end of the fourth day, resulting in a survival percentage of at least 40% for P09 and a percentage of 50% for P10.

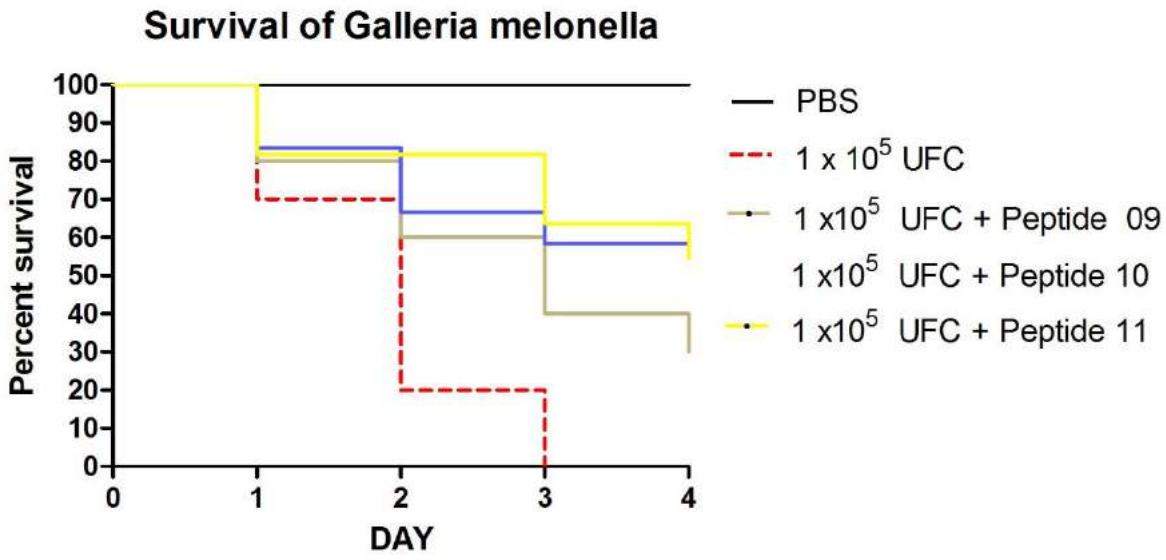

Figure 9:. *Galleria melonella* peptide survival. Ten G.melonella larvae (~ 200 mg) were randomly selected and divided into experimental groups (n = 10 for each group), each larvae received peptides injected with a Hamilton syringe, the larvae were incubated at 37°C and compared with a control group that received sterile PBS and maintained in the same conditions. Mortality was observed by counts up to the fourth day after infection.

These results indicates that both rBbKIm and peptides whose structure was inspired by the original structure of the original molecule have a mechanism of action capable of increasing the survival time of the treated larvae since the untreated animals present a mortality rate of 100% at the end of only 3 days. These observations indicate that it is possible to use these peptides as traditional antimicrobials (BENINCASA, M et al 2017).

Conclusion

The recombinant version of rBbKIm proved to be relevant to the need to combat multiresistant microorganisms such as *Staphylococcus aureus*. AMP's have been used as important tools against the mode of action of bacteria that extends from membrane alterations to the synthesis of genetic material and molecules involved in enzymatic action. The discovery of new drugs that in addition to guaranteeing the therapeutic efficacy preserve the cells of the host without causing toxicity has motivated the use of molecules of origin and synthetic for this purpose.
The peptide and sintetic peptides was active in mechanisms usually associated with virulence even without bactericidal or bacteriostatic activity. Even at lower concentrations, rBbKIm

significantly reduced the production of staphyloxanthin in a dose-dependent manner, which reveals a role in the microbial synthesis of this pigment attributed to the production of free radicals in the cell attacked by *S. aureus*. Thus, a lower production of the caratenoid implies the reduction of virulence factor of high impact in the pathogenesis of the infectious process.

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3.2 ARTIGO 2 - AÇÃO ANTIMICROBIANA E ANTIVURULÊNCIA DO ÓLEO ESSENCIAL DE *EUGENIA BREJOENSIS* EM MODELOS *IN VITRO* E *IN VIVO* USANDO *CEANOHABDITIS ELEGANS* E *GALLERIA MELONELLA*

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Manuscript to be submitted to Journal of Natural Products

ISSN: 0163-3864

Impact factor: 3.281

Qualis CAPES: A2 – Área: Ciências Biológicas I

Abstract

The fight against pathogenic microorganisms demands the use of new alternatives for control of resistant species. The use of essential oils from plant species against *Staphylococcus aureus* has been effective. *Eugenia brejoensis* is an endemic Brazilian flora and in this work the action of the essential oil (EbEO) from this Brazilian caatinga species against *S. aureus* lineages was identified. The minimum inhibitory concentration of bacterial growth was 0.11 mg / mL and maintained to at least 0.88 mg / mL (8xMIC), EbEO had antibacterial activity similar to antibiotics such as ampicillin, chloramphenicol, kanamycin (synergism); ciprofloxacin and erythromycin (partial synergism). EbEO was able to induce genes linked to bacterial SOS response and to biofilm production such as recA, hlA and spA. The oil was able to modulate and reduce the production of staphyloxanthin pigment related to the production of free radicals by bacterial cultures, additionally resulting in death of *S. aureus* when subjected to conditions of stress caused by H2O2, revealing an anti-virulence action of this oil. EbEO was able to increase the lifespan of *Ceanohabditis elegans* larvae even at sub-MIC concentrations at concentrations of 0.11 mg / mL (MIC) and 0.05 mg / mL (MIC / 2). Virulence models in *Galleria melonella* have demonstrated that in animal models EbEO decreases both the bacterial plasma load and reduces the stress caused by larvae infection, leading to a survival increase of around 70%. EbEO consists of a natural alternative that acts against *S. aureus* modulating the virulence of this microorganism with bacteriostatic effect and that may be a future adjuvant to the treatment against resistant microorganisms, with actions in the immune system and in the joint action with traditional antibiotics.

Keywords: *Eugenia brejoensis*, *Staphylococcus aureus*, virulence, *Galleria melonella*, antibiotics

Introduction

Staphylococcus aureus infections are often fatal and in several cases are associated with resistance to various antibiotics such as β-lactams, drugs widely used in clinical treatment in hospitals. **1,27** Infections, previously limited to hospitals, have also spread to outside environments and have victimized patients in various disease states. **2** The excessive and sometimes indiscriminate use of antibiotics, both inside and outside hospitals, has induced the acquisition of resistance by different molecular mechanisms. As a result, we have the emergence of multiresistant *S. aureus* strains such as MRSA, methicillin resistant, VRSA, PRSA, beta-lactam resistant strains in general or erythromycin. **3,21,28** The phenotype of these bacterial strains is highly variable, depending on factors such as geographic area, drug resistance, risk factors that together constitute varying degrees of virulence **4,5,30** The proliferation of these infections can be considerably reduced by rational use of medicines and strict prophylactic measures of infection **6** The main drugs currently used against MRSA are Vancomycin, Teicoplanin, Linezolid and Daptomycin. with vancomycin being the first choice antibiotic against MRSA infections **3, 31.**

Although the efficacy of some drugs is still satisfactory, it is not uncommon to record the susceptibility of multiresistant strains to *S. aureus* **7**, which remains one of the major pathogens of the 21st century, responsible for a broad spectrum of diseases such as endocarditis, cirrhosis infantile, among them to cause generalized hospital infection or in organs like lung and the skin. In recent decades, several reasons justify the search for new alternatives for the treatment of infections caused by *S. aureus*, such as the increase of mutant lines with marked virulence, reduced susceptibility to glycopeptides, important in the action of drugs such as vancomycin, and the increase of the amount of drugs with reduced efficiency in relation to MRSA lineages, being these innovative alternatives found by these bacteria whose classic drugs did not predict in its mechanism of action. **9**

Acquisition of new resistance genes is capable of encoding new antibiotic binding proteins such as penicillin that makes *S. aureus* refractory to all available lactam antibiotics, such acquisition in its physiology allows an increase in the pathogenicity of *S. aureus*, **10**. The production of staphyloxanthins, alterations in alpha-hemolysin expression and cellular responses and in immunities have been associated with the virulence of this pathogen **29**. *S. aureus* has been reported throughout the literature as an important pathogen with severe consequences to the

organism due mainly, its ample ability to produce various virulence factors ranging from enzymes to toxins. The term "anti-MRSA drugs" currently refers to a large class of resistant antibiotics such as penicillin, which includes amoxicillin, oxacillin, methicillin and the like. MRSA also developed resistance to other antibiotics such as erythromycin, streptomycin and tetracyclines with much shorter time intervals ranging from 1 to 3 years **11, 12**

Plants are known to produce a large variety of small antibiotic molecules and in recent years several studies have shown advances in the discovery of potential new agents **13,14,15,16**. Essential oils are obtained by hydrodistillation and have been studied as pharmaceutical alternatives to the traditional antimicrobial agents, constituting an interesting and unprecedented alternative against pathogens not combated by traditional antibiotics. **17**

Eugenia brejoensis is a plant belonging to the Mytaceae family and has recently been described as an endemic species in the Northeast (Pernambuco, Sergipe, Alagoas, Paraíba) and in a small region of Espírito Santo **18**. The essential oil of E. brejoensis was extracted and characterized by the first time by our group. **19** There are still few records of biological activities of this essential oil in the literature and the special conditions of soil and temperature to which Caatinga plants are submitted promotes adequate conditions for a deeper study of its potential, especially biotechnological and antimicrobial.

This work aims to demonstrate the bacteriostatic and anti-virulence action of the compounds present in the essential oil of *Eugenia brejoensis in vitro* and *in vivo* models. It is the first record in the literature of the action of this Brazilian plant

Materials and Methods

Plant material

The plant material for the referred study was collected in Pernambuco state. O parque Nacionaldo Catimbau (PARNA) has an 62,554 ha area, tropical semiarid climate, average annual temperature of 23°C, and average annual rainfall ranging from 500 to 1098 mm (APAC 2013). The essential oil of *E. brejoensis* (EbEO) was obtained from leaves by hydrodistillation and characterized by GC / MS chromatography **19**. All the material used was processed following the usual techniques in taxonomy, being deposited in the IPA Herbarium (voucher access number: IPA 84.033), from the Agronomic Institute of Pernambuco.

Isolation analysis of essential oil

Fresh leaves (342 g) were chopped and submitted to hydrodistillation for 4 hours in a Clevenger-type Apparatus. The oil was dried over anhydrous sodium sulfate and stored at -5°C in tubes well sealed until required for analysis and bioassay. The oil was analyzed by gas chromatography (GC) using a Thermo Fisher Scientific (Waltham, MA, USA) Trace GC Ultra chromatograph. The compounds were identified by comparison with previously reported Values of retention indices (RI) obtained by coinjection of oil samples and linear C9-C30, hydrocarbons were identified and confirmed by matching the MS acquired for Components with those stored in the library of the GC-MS system (MassFinder 4, NIST08 and Wiley Registry ™ 9th edition) and was published recently **1**

Antimicrobial analysis

The antimicrobial activity of EbEO were confirmed by determination of Minimum Inhibitory Concentration (MIC) against *S. aureus* ATCC 29312 **23**. The bacteriostatic combinatory effects of EbEO and several antibiotics used in clinic treatments were also evaluated. The fractional inhibitory concentration index (FIC) was assessed algebraically by the sum of the MIC of oil, drug and combined for sample present in the well: ΣFIC: (MIC Oil + D/ MIC Oil) + (MICD + Oil/ MICD), where was considered **$0 \leq \Sigma FIC \leq 0.5$ = Synergetic effect** and **$0.5 < \Sigma FIC \leq 1.0$ = Additive or partial synergetic effect** according to Vuuren & Viljoen, 2011 **32**

Growth curves

Overnight cultures of *S. aureus ATCC 29213* were diluted 1:100 in LB medium and placed under shaking at 37ºC. Cultures were reached an OD_{600} (optical density at 600 nm) of 0.1 they were distributed in fresh LB tubes containing increasing concentrations of EbEO (2X and 8XMIC) or ciprofloxacin (2XMIC) alone. The cell growth was monitored by spotting 4 µL of 10-fold-diluted suspensions from each tube in quadruplicate at 0, 1 h, 2.0 h, 3.0 h, 4.0 h and

5.0h. The plates were incubated at 37ºC for 24 h. After this period, the colonies were counted for the calculation of CFU/mL.

SOS response

SOS response activation has been related as promoter of antibiotic resistance, persister cells and virulence genes expression **34**.

The induction of SOS response were measured using a derivative *S. aureus* 8325-4 strain carrying a *recA::lacZ* fusion. Bacteria cells were grown exponentially in LB medium until reach an OD_{600} between 0.1 and 0.2. Then, the cells were treated with oil or ciprofloxacin (both at 0.5XMIC) for 3 hour and β-galactosidase activity was measured as described by Miller (1972) after permeabilization by toluene. **24**

Staphyloxanthin assay (quantitative and qualitative)

Overnight cells were re-inoculated at 1:100 dilution in LB medium and incubated for 16 h at 37°C with or without tested compounds. One mL of cells was then collected by centrifugation at max speed for 10 min and washed with 1 mL of phosphate-buffered saline (PBS). At this point, cell pellets were photographed to compare the staphyloxanthin production.**25** For caratenoid pigment extraction, cell pellets were resuspended in 0.2 mL of methanol by vortexing, and this mixture was heated at 55º C for 3 min. Pigment extraction was separated from cell debris by centrifugation at max speed for 10 min. This pigment extraction step was repeated 3 times, and the optical densities of collected extractions were measured at 465 nm. **26**

Hydrogen peroxide resistance assays

Overnight cultures grown for 16 h in LB were re-grown to mid-log phase in LB (turbidity at 600 nm of 1). Then, 0.1 mL of each culture was incubated with H_2O_2 at a final concentration of 1.5 % (v/v) for 60 min with shaking at 250 rpm. The percentage of cells surviving the stresses was calculated as the number of colony-forming units CFU/mL remaining after each stress divided by the initial CFU/mL. Three independent experiments were conducted.

***C. elegans* survival and anti-infective assay**

The lifespan assay was made using a stereli AU37 strain, avoid overgrowth of offspring during the experiment. After sicronisation, L1 larvae were transferred to 25°C (non-permissive fertility temperature) until reaching the L4 state. At this time, the larvae (about 15) were transferred to 24-well plates containing the liquid medium M9 and *S. aureus* (grown in LB medium containing 10 μg / mL of cholesterol) in a ratio of 4: 1 (v / v). Different concentrations of EbEO (MIC and MIC / 2) have been added and longevity will be assessed every day and animals will be classified as dead when they do not present spontaneous movement or response after stimulation with a platinum loop.

G. melonella survival assay

To evaluate if EbEO could protect against *S. aureus* infection we perform a *Galleria melonella* infection assays. *G. melonella* larvae (~200 mg) were randomly distributed in three experimental groups (n=10) with or without oil treatment. Two groups were infected by injection of 10 μL of a recent *S. aureus* suspension (1.0 x 10^5 CFU), optimized previously, into the last left proleg, followed by incubation at 37ºC. After 2 hours, one of these groups received 10 μL of EbEO at MIC dissolved in PBS (resulting an in dose of 0.02 μg EbEO to each 100 mg of larvae). Larvae treated with PBS were used as positive control. The larvae were also treated with *beta-caryophyllene* (oil major compound) and both were incubated at 37ºC, and the larval viability was verified dialy for 4 days but only for oil treated had quantified of bacteria measured.

Quantification of bacteria in _G. melonella_ hemolymph

G. melonella larvae were infected with *S. aureus* and treated as described above. Each day, a total of 5 larvae (only for oil treated larvae, not for *beta-caryophyllenebeta-caryophyllene)* were cut in the cephalocaudal direction with a scalpel blade and squeezed to remove the hemolymph. Each sample was 10-fold-diluted in PBS and 4 μL was plated on LB agar. After 24h-incubation at 37ºC, the colonies were enumerated and the results were expressed as CFU/mL.

Hemolymph early melonization

Hemolymph melanization assay was performed using 10 larvae that were infected with 10^6 cells/larva of *G. melonela* and incubated at 37°C for 1 and 3 hours. After the incubation

period hemolymph were collected and diluted 1:10 in cold PBS and centrifuged at 12,000 rpm in a centrifuge. Supernatants were placed in a 96 well microdilution plate, and optical density determined at 465 nm.

Statistical analysis

Statistical analyses were performed using the software GraphPad Prism version 5 (www.graphpad.com). Data from were analyzed by two-way analysis of variance (ANOVA), 1-way and 2-way and Tukey test. A *p*-value of < 0.05 was considered to be statistically significant. Differences in the survival of *G. melonella* larvae were determined using the Kaplan-Meier method to calculate survival fractions and log-rank test was used to compare survival curves.

Results

EbEO bacteriostatic and synergistic activity

The antimicrobial activity of compounds was evaluated by agar microdilution and diffusion method **33**. EbEO showed antimicrobial activity with a MIC (Minimum Inhibitory Concentration) value of 0.11 mg / mL. This oil was shown to be a bacteriostatic agent even at the concentration of 0.88 mg / mL (8xMIC). Nearly 3 hours after T0, treated samples reduced almost 50% colony number although both EbEO concentration did not show statistic differences. In addition, essential oil concentrations have antibacterial activity similar to ciprofloxaxin, CIP, (Figure 1). Additionally, EbEO increased the action of drugs such as ampicillin, chloramphenicol, kanamycin (synergism); ciprofloxacin and erythromycin (partial synergism), Figure 2.

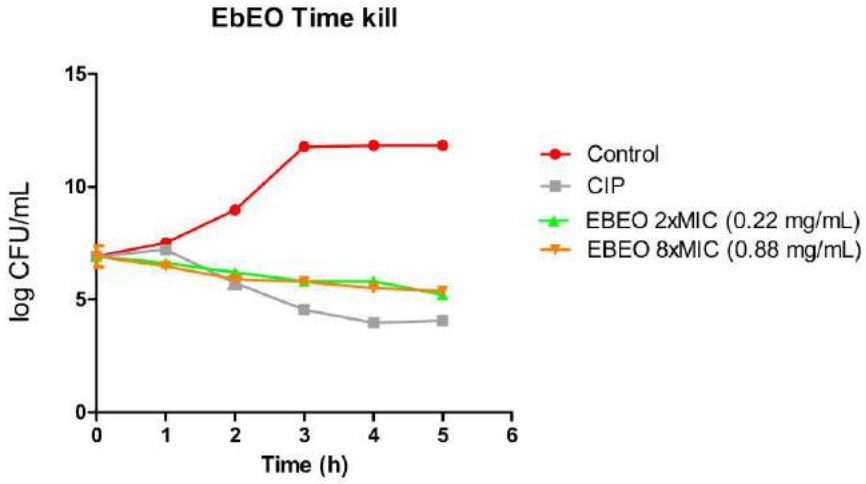

Figure 1: Inhibition curve of *S. aureus* treated with different EbEO concentrations: 0.22 mg / mL (2xMIC) and 0.88 mg / mL (8xMIC).

Table 1- Effect of EbEO in association to antibiotics:

Plant	**MIC (alone)**	**MIC_{D+E} (drug in presence of EO)**	**MIC_{E+D} (EO in presence of each drug)**	**ΣFIC***
EbEO†	117 µg			
Ampicilin‡	25 µg	5	0.0625	0.45
Chloramphenicol‡	12.5 µg	1.25	1.25	0.15
Ciprofloxacin‡	0.78 µg	0.312	0.5	0.6
Erythromycin‡	0.39 µg	0.156	0.156	0.6
Kanamycin‡	6.25 µg	0.3125	0.3125	0.075

The fractional inhibitory concentration index (FIC) was assessed algebraically by the sum of the MIC of oil, drug and combined for sample present in the well:

†mg ** µg/mL

‡Antibiotic used agains nosoncomial infections

*ΣFIC: (MICE+D/ MICE) + (MICD+E/ MICD)

0≤ΣFIC≤0.5 = Synergetic effect

0.5<ΣFIC≤1.0 = Additive or partial synergetic effect

EbEO synergistic effect with the antibiotics ampicillin, chlorofenicol and kanamycin (with 0≤ΣFIC≤0.5) and additive effect (partially synergistic) in combination with minimal concentrations of ciprofloxacin and erythromycin (with 0.5 <ΣFIC≤1.0).

EbEO induce SOS response

Our results indicates the participation of EbEO in SOS response in *S. aureus* strains, demonstrated by qualitative induction of the recA gene and increased volume of the treated cells. To quantify induction of the recA gene, we used a line derived from *S. aureus* 8325-4 containing the recA gene fused to the reporter gene lacZ (recA :: lacZ) (Gottschalk et al., 2013). This strain was incubated with different concentrations of the EbEO (0.11 mg/mL) at certain time intervals and the β-galactosidase level was determined according to Kjelstrup et al. (2013) (figure 2).

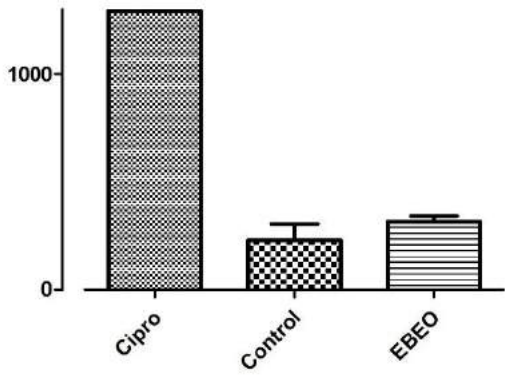

Figure 2: recA bacteria gene expression is reduced when bacteria culture is treated with EbEO (0.11 mg/mL) and Ciprofloxacin (0.78 μg) conduces cells to overexpression and high SOS response activation

Natural products effects on expression of *S. aureus* agr locus

S. aureus strains with hla and spa genes integrated with lacZ reporter gene was used to search for compounds with quorum sensum inhibitor properties - agr QSI (Nielsen et al., 2010). LB agar plates were prepared containing a fresh (10-3) diluted culture of each reporter strain, the appropriate antibiotic and X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), as a reagent to detect β-galactosidase activity. After incubation, plates containing the hla :: lacZ line become intensely blue due to high HLA expression, while the spa :: lacZ plates become slightly bluish. Positive results can be visualized through blue / white screening of the rings surrounding the halos containing putative QSIs. The intent of this approach is that the compounds added to the plate prevent HLA expression, leading to white rings on the X-gal plates. Simultaneously, a QSI will induce expression of protein A, leading to a blue ring in the reca :: lacZ lineage. Results indicates that EbEO shows a qualitative spa gene activity. EbEO was capable to increase *spa* gene expression around 50% and decrease *hla* gene expression around 33% that means a potentials *quorum sensing inhibitor* (QSI) (figure 3b insert)

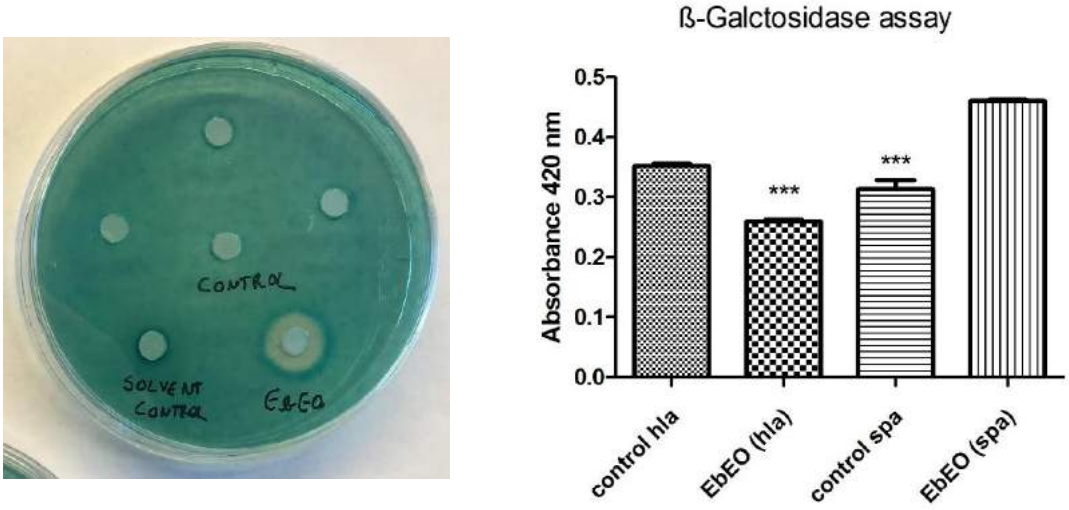

Figura 3a : EbEO is capable of inhibiting hla expression, showed by formation of white rings in strains that contais spa :: lacZ

Figura 3b: EbEO is capable simultaneously reducing hla gene expression and increasing spa gene expression, constituting a probable Quorum sensing Inhibitor (QSI).

***Staphyloxanthin* quantitative assay**

A quantitative assay was used to determine the level of reduction of the production of this carotenoid **25**. Suspensions of *S. aureus* were treated with different concentrations of EbEO and staphyloxanthin pigment (STX) contained in the bacterial pellet was evaluated qualitatively and quantitatively. The figure shows the relation staphyoxanthin/concentration of EbEO quantitatively (Figure 4), where even at lower concentrations as 0.013 mg/mL (MIC/8), the oil is still able to inhibit the production of the pigment to values around 10% and approximates 0% indicating that EbEO reduced the production of the pigment in a dose-dependent way.

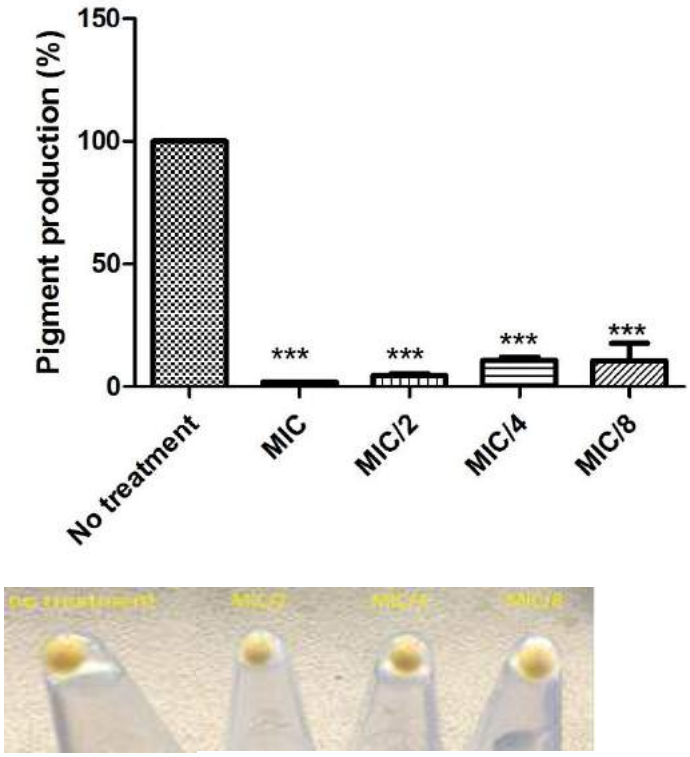

Figure 4: Color of pigments was read at 465 nm. It has been observed that the production of STX presents dose dependence and is critically reduced when exposed cultures treated with concentrations ranging from 0.11 to 0.013 mg/mL in overnight cultures.

S. aureus ATCC 29213 culture diluted (1: 100 in Luria-Bertani-LB medium) and incubated with the compounds tested for 16 h at 37°C with or without the concentrations assessed. Cells (1 ml) were collected by centrifugation at full speed for 10 minutes and washed with 1 ml of phosphate buffered saline (PBS). At this time, cellular sediments were photographed for comparison of staphyloxanthin production. For the extraction of the carotenoid pigments, the cell pellets were resuspended in 0.2 ml of methanol by vortexing, and this mixture was heated

at 55°C for 3 min. The pigments were separated from the cell debris by centrifugation at full speed for 10 min. This pigment extraction step was repeated 3 times, and the optical densities of the extractions measured at 465 nm for detection of staphyloxanthin. The color of pigments was read at 465 nm. It has been observed that the production of STX presents dose dependence and is critically reduced when exposed cultures treated with concentrations ranging from 0.11 to 0.013 mg/mL in overnight cultures.

Resistence to hidrogen peroxide

Staphyloxanthin is recognized for acting as an antioxidant agent defending *S. aureus* against the attacks of reactive oxygen species generated by the immune cells of the host during infection. To assess the effect of reducing staphyloxanthin production and EbEO action on the intracellular antioxidant behavior of bacterial cells (Figure 5). EbEO in high concentrations is capable of reversing the effect of the production of reactive oxygen species by *S. aureus* (represented by the degradation of hydrogen peroxide) responsible for the virulence of this microorganism. In this way, a lower bacterial growth was observed in the concentrations used (0.11 to 0.013 mg/mL, MIC to MIC/4).

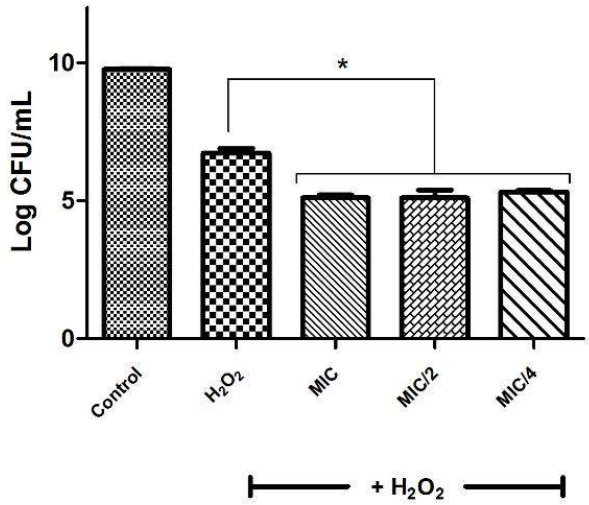

Figure 5: Resistence to hidrogen peroxide *S. aureus* cultures (ATCC 29213) were cultured in the presence of different subinhibitory concentrations of each compound for 16 h after that. The absorbance was adjusted to 1 at 600 nm. Thereafter, each culture (0.1 mL) was incubated with H2 O2 at a final concentration of 1.5% (v / v) for 60 min with shaking at 250 rpm. The percentage of surviving cells was calculated as the number of colony forming units (CFU) / mL remaining after each strain divided by the initial CFU/mL. Three independent experiments were performed.

Evaluation of antimicrobial activity of EbEO in vivo model

***C. elegans* anti-infective assay**

Caenorhabditis elegans was used to evaluate the anti-infective effect of the oil. This free-living terrestrial nematode is an advantageous model organism because it is multicellular and exhibits conserved physiological systems, short life span, fully sequenced genome and about 60-80% of genes homologous to humans. It is also considered a fast, cheap and efficient model for in vivo testing of antimicrobial substances.

Chronological synchronization of *C. elegans* strains

EbEO increased the lifespan of *C. elegans* when compared to untreated *S. aureus* control. The worms were monitored for 7 days and at the end of this period the viability between the

concentrations used (0,11 and 0,05 mg/mL, MIC and MIC/2 respectively) varied between 70 and 80% (Figure 6).

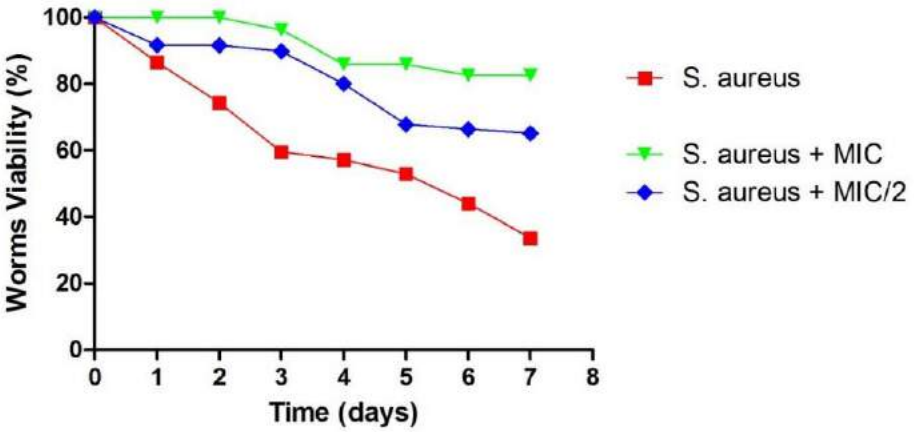

Figure 6a: Viability of ***C. elegans*** **infected with *S. aureus* in medium contained EbEO.** Strain synchronization was performed by the alkaline lysis method, based on the treatment of pregnant hermaphrodite adults with lysis solution (50% sodium hypochlorite, 2.5 mM NaOH). Embryos resistant to this treatment were collected and placed in M9 liquid medium. After 12 h, the L1 worms obtained were seeded on plates containing the NGM medium (seeded with *E. coli* OP50) for 48 h at 25°C, time required to reach the L4 stage.

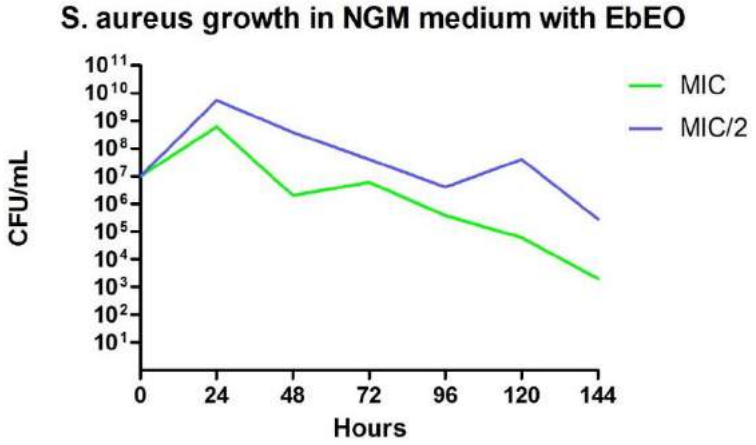

Figure 6b: Growth profile of *S. aureus* exclusively from medium containing NGM and EbEO, over days

In vivo* virulence assays using *Galleria mellonella

The action of the compounds was evaluated in the *S. aureus* infection model *in G. melonella* larvae. EbEO did not show any toxicity to larvaes in 4 days time period (figure 7A). Additionally, was tested also beta-caryophyllene compound and survival of the larvae was monitored over 4 days and those treated with EbEO showed a survival of 70% at the end of the period, while the Infected larvae showed 100% mortality at the end of the third day (figure 7B). In order to verify the bacterial load of the larvae throughout the treatment day's, number of colonies present in the plasma (n = 5 individuals) was collected, seeded and counted. The figure 8 shows the difference (in potency of 10) between *S. aureus* growth over the first 3 days between larvae treated and not with EbEO. Melonization is an indicator of the anti-virulence effect of compound on *G. melonella* models. EbEO significantly reduced stress, resulting in less melonization when 10^6 CFU / mL were given to larvae and treated with MIC.

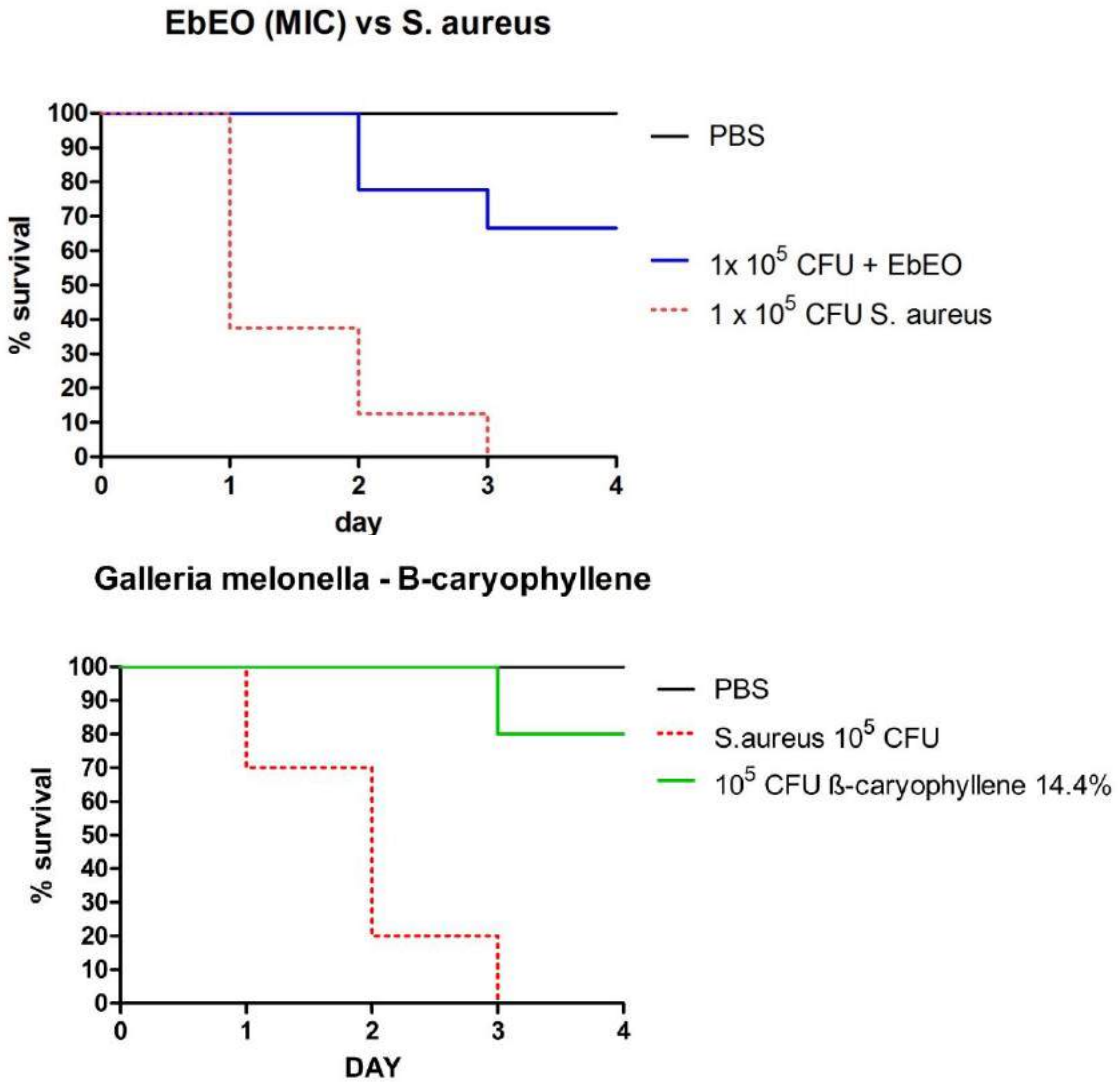

Figure 7A: *Galleria melonella*-B-caryophyllene survival. 10 G.melonella larvae (~ 200 mg) were randomly selected and divided into experimental groups (n = 10 for each group), each larvae received this compound injected with a Hamilton syringe, the larvae were incubated at 37°C and compared with a control group that received sterile PBS and maintained in the same conditions. Mortality was observed by counts up to the fourth day after infection.

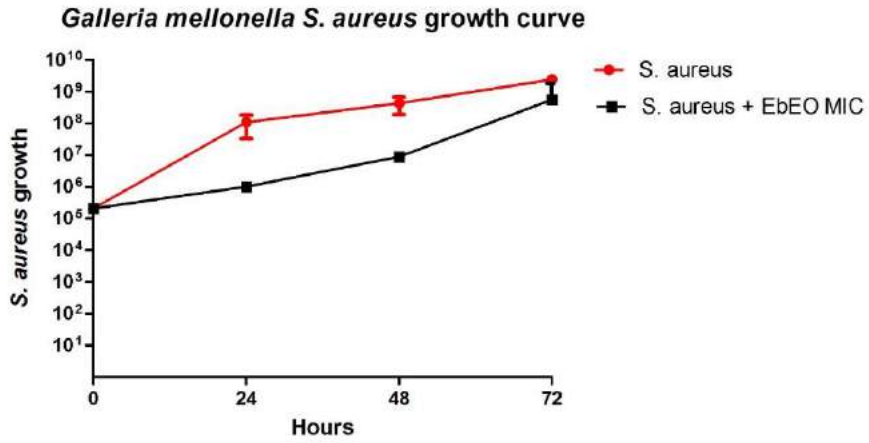

Figure7B: 1x10^5 CFU was pre-defined as ideal for tests with the ATCC 29312 strain. *G. melonella* larvae (~ 200 mg) were randomly selected in experimental groups (n = 10 / group), and then infected with 10 µl of a bacterial suspension (1.0 x 10^5 CFU) in the ventral region and subsequently incubated at 37°. After 2 hours, the larvae received EbEO MIC concentration, as well as the vehicle PBS to the control tubes and were reincubated at 37°C. Mortality rate was observed for 4 days after infection.,

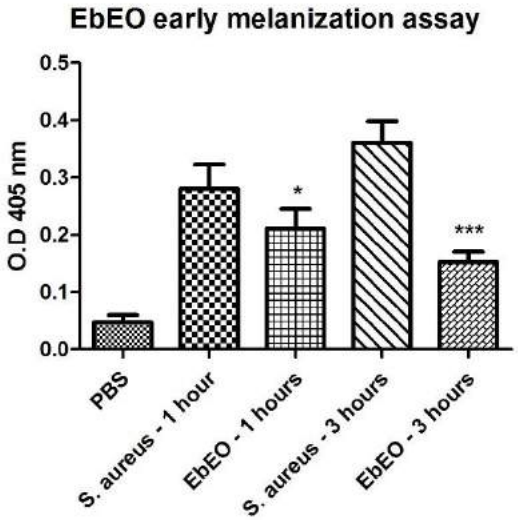

Figure 8: Early melaniation assay of EnEO against 106 CFU *S. aureus* (2and3 hours after exposition)

G. melonella larvae (~ 200 mg) were randomly selected in experimental groups (n = 10 / group), PBS was used as a control for EbEO non-treated larvae and compared treated with only MIC

larvae and larvae infected with 10 µl of a bacterial *S. aureus* suspension (1.0 x 10^5 CFU) plus EbEO and then in the ventral region and subsequently incubated at 37° . After 1 and 3 hours, larvae hemolymph was collected to measure melanization degree and was compared with PBS control and only EbEO MIC group reincubated at 37°C during all experiment time. Mortality wasn't observed after infection and incubation time.

Discussion/Conclusion

The essential oil of *Eugenia brejoensis* is a potent antimicrobial agent with significant anti-virulence action, presenting unprecedented results for this species *in vitro* and *in vivo* models1. The work suggests that EbEO is able to significantly reduce the action of *S. aureus* by mechanisms involved in cell oxidation and bacterial immunity, an interaction that also promotes the protection of hemocytes 3 as well as in vivo defense action in models using *C. elegans* and Well established models such as larvae of *Galleria melonela.* The oil showed an association with the reduction of the virulence profile associated with the production of pigments such as staphyloxanthin and melanin (in *G. melonella* larvae), as well as reducing the bacterial serum levels of *G. melonella* larvae. 11,12The results demonstrate that EbEO also acted increasing and prolonging the survival of Caenorhabditis elegans, at least preserving the same serum content of bacteria in the medium.

EbEO has been shown to be extremely effective when used in synergism with commercial drugs of wide clinical use such as ampicillin, chloramphenicol and kanamycin and a partially synergistic effect for ciprofloxacin and erythromycin.

The evaluation of the immune response and gene expression can be developed from the clues found here through the responses to the hla and spa genes, involved in the SOS response, like other genes not studied linked to virulence and antimicrobial peptides5, The genetic response of the organism to treatment with EbEO.

The anti-virulence action of EbEO against MRSA must also be evaluated in other parameters developed herein, taking into account individual variations both in individuals and in response to resistance of *S. aureus*, a microorganism that has undergone constant adaptations to the drugs used in the therapy. **3,8,9,11**. In view of the urgent need to discover molecules with antimicrobial properties that reduce the action key factors of *S. aureus* virulence 6, as well as elucidation of their mechanism of action, studies involving molecules from extracts, isolated molecules or volatile plant oils And produced by biotechnology, **6.7.8**.

The essential oil of *Eugenia brejoensis*, a Brazilian species, is an important source of molecules that combined can provide a new drug option for *S. aureus* infection. It is the first time that these compounds extracted by hydrodistillation of Brazilian caatinga species are reported with this function in the literature. Our work is a pioneer in the study of the antimicrobial action, mechanism of action and virulence making an accurate screening of activities relevant to the pathologies that these microorganisms are associated with.

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3.3 ARTIGO 3 - *EUGENIA BREJOENSIS* ESSENTIAL OIL (EBEO) ACTION ON MIMETIC CELL MEMBRANE MODELS

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Manuscript to be submitted to Biochemical and Biophysical Research Communications
ISSN: 0006-291X

Abstract

In the present work, *Eugenia brejoensis* essential oil (EbEO) antimicrobial effect was evaluated to elucidate the mechanism for bactericidal activity with biomembrane mimetic systems of POPC, POPG, GUVs and LUVs. EbEO biological activity was investigated to determinate the MIC and the effect on *S. aureus* hemolysin production (hemolysis assay) and susceptibility in wholeblood in the presence of EbEO. We further analyzed the effect of EbEO concentrations in the presence of lipophilic compounds like bacteria membrane, including LPS. Additionally,

physic-chemical parameters like melting temperature and enthalpy were evaluated together with fluorescence microscopy equipped with electrid field to evaluate the interaction between essential oil and vesicles. EbEO showed effectiveness as an antimicrobial against clinical isolated *Staphylococcus aureus* strains. Concentration of EbEO ranged from 7 µg to 0,9 mg in each treatment, suggesting high potential against this pathogen, specially towards antibiotic therapy resistant strains, which indicates that the treatment with essential oil can contribute with used antibiotics. Sub-MIC concentrations of EbEO successfully inhibited red blood cells hemolysis by *S. aureus*. The presence of essential oil was also capable of lowering bacterial counts in whole-blood assay. LUVs leaking test showed that the addition of EbEO did not promoted membrane disruption, even in the presence of 5% LPS. Isothermal titration calorimetry results indicated that EbEO did not affected the stability of POPC membranes and the melting temperature in DSC thermograph was determined to be 39°C. Fluorescence microscopy revealed that EbEO (1000x diluted) interacted with LUVs in a time dependent manner adding volume to the vesicles without disruption. These results indicate that EbEO is an effective antibacterial product and the antimicrobial mode of action is not related to cell membrane disruption but possible has intracellular effects, promoting bacterial vulnerability.
Keywords: Antimicrobial, Large unilamelar vesicles, *Staphylococcus aureus*, hemolysis, Giant unilamelar vesicles, POPG, POPC

Introduction

Essential oils (EO) are recognized for their physical-chemical properties as low molecular weight lipophilic compounds present in many plants. Their composition is heterogeneous, varying widely in quantity and types of terpenes. Their hydrophobic biological characteristics have shown the potential as antimicrobials, possible by interfering in the cell membrane dynamics and stability. The mechanisms to counter-balance the microbial virulence is due to the interactions of terpenoids and bacterial membrane. Many virulence factors affect the bacteria pathogeny, and during the colonization step an array of molecules, e.g. adhesins, are present covalently linked to the cell wall, binding to the host extracellular matrix components, which is one of the most important receptors for staphylococci attachment [1] (Liang et al. 2016). Different *Staphylococcus aureus* strains are capable of synthetize microbial components recognizing adhesive matrix molecules (MSCRAMMs), which are specific molecules

responsible for the virulence capacity of some strains of causing different infections [2] (Francis, 2015).

Eugenia brejoensis Mazine (Myrtacae) is an herbaceous plant recently described [3] (Giaretta et al. 2014) as an endogenous species from Caatinga Domain, in Northeastern Brazil. Essential oil from *E. brejoensis* (EbEO) was extracted and characterized for the first time by da Silva et al (2015) [4], and there is still little information about the its biological activity, regardless the well-known potential as antioxidants, antimicrobials and others [5,6,7](Savoia, 2012; Jordan et al, 2013; Radulovic et al., 2013).

Recently, it was shown that EbEO has potential antimicrobial activity against *S. aureus* strains, including multi-resistant clinical isolates (unpublished data). Some of the activity including synergism effect with antibiotics can be explained by the effect on virulence factors naturally present in bacteria. However, it was not clear if the interaction of essential oil with bacterial membrane, interacting with lipophilic compartments of bacteria is the mode of action. The objective of his work was to evaluate the EO on mimetically biomembranes, to analyze the effect regarding the interaction between unilamelar membranes and EbEO.

Materials

The phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), egg (chicken) sphingomyelin (SM), and cholesterol (chol) were purchased from Avanti Polar Lipids (Birmingham, AL). SM is a mixture of lipids of different fatty acids chains, with prevalence of 16:0 (86%) and traces of 18:0 (6%) and 22:0 and 24:1 (3% each). The fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate was from Life Technologies (Carlsbad, CA).

Essential oil of *E. brejoensis* (EbEO) was obtained from dry leaves by hydrodistillation and previously characterized by GC/MS chromatography [4](Silva, et al., 2015). All the material used was processed following the usual techniques in taxonomy, being deposited in the IPA Herbarium (voucher access number: IPA 84.033), from the Agronomic Institute of Pernambuco.

All other reagents and chemicals were of analytical grade.

Antimicrobial analysis

The antimicrobial activity of EbEO was performed by determination of Minimum Inhibitory Concentration (MIC) against several *S. aureus* antibiotic resistant strains by the method described Santos, I. P. D. et al, 2015[11]. Composition analysis of EbEO was determined by GC-MS by Silva et al. (2015) [4].

<Table 1>

Hemolysis Assay

The lysis efficacy of human red blood cells was measured with whole cultures of *S. aureus* grown in the presence of EbEO. The efficacy of red blood cell lysis by *S. aureus* was evaluated using in the presence of the compounds using the methodology reported by Lee et al. (2013) [8]. Briefly, *S. aureus* cells were diluted at 1:100 from an overnight culture in LB and cultured with or without tested compounds at 37°C for 16 h with shaking at 250 rpm. Cell cultures (50 μL including cells and culture supernatant) were added into diluted human red blood cells that had previously been separated by centrifugation at 10000 RPM for 5 min, washed with sterile PBS buffer three times and diluted at 3 % of red blood cells in PBS buffer. For hemolytic activity, the mixture was incubated at 37 C for 1 h with 250 rpm shaking. The supernatant was collected by centrifugation at 16,6009g for 10 min, and the optical density was measured at 543 nm. Lysis was determined by reading at 543 nm and the control consists of hemolysis caused by the virulence of this microorganism.

Whole blood killing assay

S. aureus cells from overnight cultures were inoculated in 25 mL LB medium (1:100 dilution), with or without tested compounds, were cultured for 6 h at 37°C with shaking of 250 rpm. Freshly drawn human whole blood (0.5 mL) was then mixed with *S. aureus* cultures (62.5 μL) together with EbEO at MIC/2 and MIC/4 (0.055 and 0.0275 mg/mL, respectively), and mixtures were incubated at 37°C for 2 h with agitation at 250 rpm. *S. aureus* survival was measured by counting surviving CFU.

Preparation of Large Unilamellar Vesicles (LUVs)

A lipid film was formed on the walls of a test tube from a solution of lipids (POPG:POPC 3:7, mol:mol) in chloroform, dried with a stream of N2 and left in vacuum for 2 h. A buffer solution containing 30 mM HEPES, pH 7.4, with 100 mM NaCl was added, and multilamellar vesicles

(MLVs) were formed by mechanical agitation. Subsequently, to obtain LUVs, this lipid dispersion was extruded at least 11 times through polycarbonate membranes with a pore size of 100 nm. In all experiments, the phospholipid concentration was measured by indirect determination of phosphorus content, according to the methodology described by Rouser at al 1970 [9].

Isothermal Titration Calorimetry and Differential Scanning Calorimetry

The isothermal titration calorimetry (ITC) measurements were performed with a Microcal VP-ITC from Microcal (Northampton, MA). The reference cell was filled with water and the reaction cell with the EbEO solution (~ 5 μM). The volume of the cells was 1.46 mL. The syringe was loaded with a suspension of LUVs (12 mM POPG:POPC 3:7 mol:mol). Differential Scanning Calorimetry (DSC) scans were performed in a Microcal VP-DSC Microcalorimeter (Microcal Inc., Northampton, MA, USA) equipped with 0.5 mL twin total-fill cells. Heating rates were 20°C/h (10°C/h yielded identical scans). Scans were performed at least in duplicate. Thermograms correspond to second upscan.

Entrapment of Carboxyfluorescein (CF) in LUVs and Leakage Assay.

LUVs were prepared as described above with a buffer solution of 30 mM HEPES, pH 7.4, with 50 mM CF and 85 mM glucose, added to adjust the osmolarity of the solution. To remove the free CF outside vesicles, the suspension of LUVs was eluted with 30 mM HEPES buffer, pH 7.4, with 100 mM NaCl through a Sephadex resin G-25 Medium column where vesicles with entrapped CF (CF-LUVs) were collected in the void volume of the column. An aliquot of CF-LUVs was diluted to yield ~50 μM final lipid concentration (the exact value was determined later by determination of the phosphorus content) in a cuvette. The fluorescence emission of CF fluorescence was monitored at $\lambda = 520$ nm using $\lambda = 490$ nm as excitation wavelength with a spectrofluorimeter F-2500 from Hitachi (Washington, DC). Different concentrations of EbEO (1, 2.5, 5, and 10 μM) were added to the LUVs suspension. At the end of each experiment, Triton X-100 was added to promote full CF leakage. The percentage of CF leakage was given by $100(Ft - Fo)/(Fmax - Fo)$, where Ft is the fluorescence at a selected time, Fo is the initial fluorescence (before addition of peptide), and Fmax is the maximum fluorescence obtained after addition of Triton X-100 [10].Martins et al 2008

Optical and epifluorescence microscopy

GUVs were observed with an inverted microscope Zeiss Axiovert 200 (Jena, Germany) equipped with a digital camera PCO.edge 4.2 (Kelheim, Germany). For fluorescence

microscopy, the GUVs contained 1:1000 EbEO/POPC, and illumination was done with an HBO 103W mercury lamp and filters with excitation at 540–552 nm and emission band at 575–640 nm. An observation chamber was filled with 95 mL of a 0.2 M glucose solution containing EbEO at the desired concentration. A 5 mL aliquot of GUV suspension was added to the observation chamber, and the observation with a 63 x air objective was started. The objective lens was previously used in phase contrast mode to locate the electrodes and vesicles and further switched to fluorescence mode for the application of electric fields. The experiments were performed at room temperature (22°C).

Edge tension measurements

GUVs of each lipid composition (POPC, POPC/chol, 7:3, SM/chol 7:3) were prepared in 0.2 M sucrose and dispersed in 0.2 M glucose with 0.1 mM NaCl (this ensures vesicle deformation into a prolate shape (Riske and Dimova, 2005). The GUVs were accommodated in an electrofusion chamber (Eppendorf, Germany) with 92-mm diameter cylindrical electrodes spaced at 500 mm. The electrofusion chamber was connected to a multiporator (Eppendorf, Germany). Observation was carried out with a 20x air objective in phase contrast or epifluorescence mode, as described in the previous section. For each selected GUV located between the electrodes, a single square DC pulse of 100 V amplitude and 300 ms duration was applied and the vesicle relaxation dynamic was recorded at 100 or 300 fps. The experiments were performed at room temperature (~22°C). The radii of the GUVs were measured on the images using the software ImageJ (NIH, USA), and fits to the axis measurements were done with Origin 8.0.

Graphs and Statistical analysis

Statistical analyses and graphs were performed using the software GraphPad Prism version 5 (www.graphpad.com). Data were analyzed by one-way analysis of variance (ANOVA), Tukey test. A p-value < 0.05 was statistically significant.

Results and Discussion

In order to confirm the anti-staphylococci activity, EbEO was tested against different *Staphylococcus aureus* strains clinical isolates. The effective minimal inhibitory concentrations (MIC) towards resistant microbes were 7μg/mL to 0.9 μg/mL, even to strains resistant to antibiotic therapy. These results show the potential action of EbEO against standard strains (ATCC 29213 and UFPEDA 02) (Table1) and to other stains, as an effective bactericidal.

The effect of natural products on the production of hemolysin by *S. aureus* is shown in Figure 1. EbEO reduces blood cells hemolysis undergoing *S. aureus* with MIC / 2 and MIC / 4 concentrations (0.055 and 0,0275 mg/mL, respectively) (Figura 1A). EbEO is able to reduce the hemolytic action of *S. aureus* even in whole blood samples (without separation of the figurative elements). The figure //B shows that the antimicrobial action and antivirulence in conditions closer to the physiological conditions is equally significant. The test demonstrates a reduction in the survival of *S. aureus* when the plasma levels of the microorganism are in the presence of EbEO.

Once *S. aureus* infection takes place, the bacteria can spread through the blood circulation and cause hemolysis, due to the production of α-hemolysin. In sub-MIC concentrations, EbEO was able to inhibit isolated human red blood cells lysis in vitro in concentrations such as MIC/2 and MIC/4 (Figure 1A). Additionally, the whole blood assay gives information about the bacteria growth in whole blood in the presence of EbEO through time, from MIC to MIC/4 concentrations. The results indicate that EbEO is able to significantly lower the bacteria counts (Figure 1B).

The mode of action of EbEO was evaluated using unilamelar cell membrane models. Firstly, it was observed the capacity of interaction of EbEO with POPC vesicules (large unilamelar vesicules – LUVs) prepared with entraped carboxyfluorescein. There was no dye leakage from vesicles, even when EbEO added greatly excced the MIC (Figure 2A), suggesting that it do not causes membrane disrupting even in high concentrations and directly interaction with POPC. Figure 2B indicates that in concentrations up to 10 μM and high volume adde of bacterial lipopolysaccharide (LPS) did not contribute to the leak, being only equivalent to the control without any EbEO added.

I order to determinate the interaction temperature when EbEO and POPC is favred, the mixture was submitted to calorimetry evaluation. The Isothermal titration calorimetry (ITC) is shown in Figure 3A. In maximum interaction, approximately -500 cal/mol is released at 40°C. DSC thermograph (Figure 3B) shows that most stable temperature of 39°C (melting), which is near the physiological temperature in humans.

In Figure 4 is shown the experiments carried out in a fluorescence microscope equipped with AC Field using giant unilamelar vesicles (GUV) with glucose to increase contrast. The vesicles were submitted to electric field and added of EbEO to a ratio of 1:1000. The changes in morphology was observed in Figure 4A. Photomicrographs showed that in tested conditions no

loss of contrast was observed, indicating that there was no leaking from GUVs. There were at least three conformational stages in the presence of EbEO: 1°: spherical vesicles free of deformities and stable when submitted to the electric field; 2°: added EbEO caused deformation on GUVs generating different sized axis, with no loss of contrast (Figure 4B); and 3°: vesicles turn to the original shape with presence of light spots, suggesting the rearranging of lipidic components in the membranes. In phase two, it was possible to measure the variation of vesicle axis size ratio, to estimate the variation and proportion between them and then the alteration with the injected volume throughout the experiment, in same conditions, with repetitions.

Using this information, it is possible to determine a axis change constant as seen in Figure 4C, according to the ITC data the constant for axis modification when EbEO is added is 4x10-4M-1. The preservation of contrast and visible enlargement of GUV volume when compared to initial volume suggest that EbEO was capable of interact with membrane lipids without.

EbEO showed effects against standard and multiresistant *S. aureus* strains. It was possible to demonstrate for the first time the application of essential oil in mimetic membrane models to correlate the antimicrobial activity, and *E. brejoensis* is here presented and potential source of antimicrobial effectors, a property never before evaluated for antibacterial and antivirulence. Mimetic model analysis suggests that the mode of action of the EO is related to the incorporation in the cell membrane, triggering at the same time the inducers for cell death and counter-acting the virulence factors in pathogenic *S. aureus*. More studies are necessary to further observe the membrane alterations in resistant microbes as well as other virulence factors related to *S. aureus*.

Acknowledgments

The authors would like to thank CNPq, CAPES and FAPESP to bench grants and scholarship.

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List of Figure/Table legends

Table 1: Minimal inhibitory concentration (MIC) of EbEO towards multi-resistant clinical isolated *S. aureus* strains

Figure 1: Hemolysis assay and human whole blood killing assay on effect of EbEO concentration on *S. aureus* counts.

Figure 2: **POPC LEAKAGE PERCENT** Leakage assay of LUV vesicles. A: in the presence of different EbEO and volume added (from 5 to 50 μL); B: LUV in the presence of 5% (w/v) LPS and EbEO 10 μM (95% POPC + 5% LPS leakage percentual)**.**

Figure 3: **ITC EbEO – POPC DSC** Isothermal tritation calorimetry (ITC) of POPC increasing concentrations in the presence 5 μM EbEO. DSC experiments – Melting temperature (39ºC)

Figure 4: Fluorescence microscopy with AC Field. A: a time lapse of LUV vesicles added of EbEO; There is no leakage and deforming followed by enlargement is observed. B: Constant k versus the concentration of EbEO. C: Area increase (%) dependent of EbEO concentration.

Table 1

S. aureus strain	MIC (µM)	MIC(mg/mL)
UFPEDA 70	**2,15**	**0,45**
UFPEDA 731	**2,15**	**0,45**
ATCC 29213	**0,52**	**0,11**
UFPEDA 02	**1,075**	**0,22**
UFPEDA 705	**1,075**	**0,22**
UFPEDA 671	**2,15**	**0,45**
UFPEDA 59	**4,3**	**0,90**
UFPEDA 726	**0,537**	**0,11**
UFPEDA 1802	**2,15**	**0,45**
UFPEDA 679	**0,537**	**0,11**
UFPEDA 691	**1,075**	**0,11**
UFPEDA 683	**0,067**	**0,00708**
UFPEDA 659	**1,075**	**0,11**

Figure 1

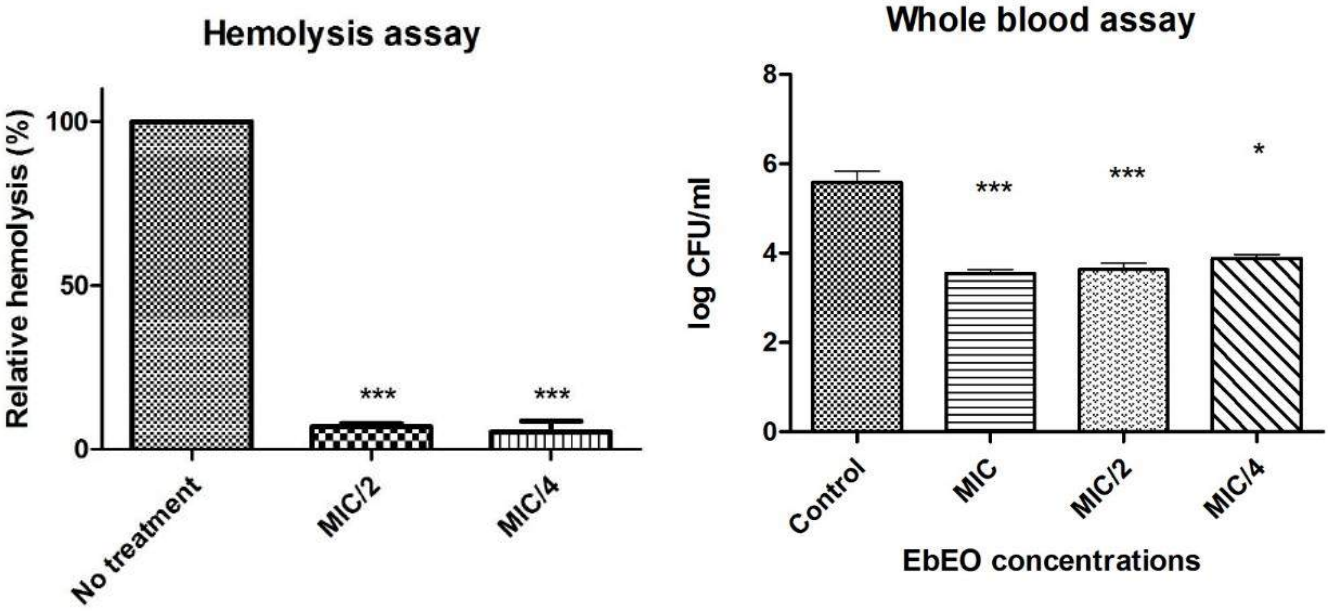
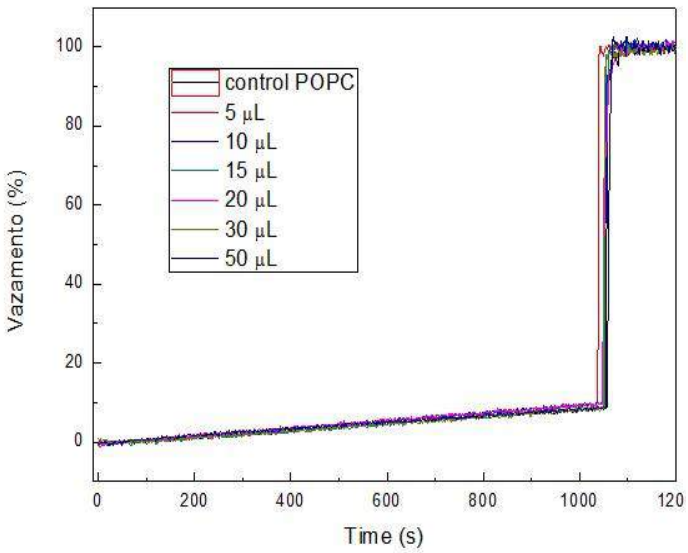

Hemolysis assay
Relative hemolysis (%)
100
50
0
***
***
No treatment
MIC/2
MIC/4
Whole blood assay
log CFU/ml
8
6
4
2
0
***
***
*
Control
MIC
MIC/2
MIC/4
EbEO concentrations

Figure 2


control POPC
5 µL
10 µL
15 µL
20 µL
30 µL
50 µL
Vazamento (%)
Time (s)
0
20
40
60
80
100
0
200
400
600
800
1000
120

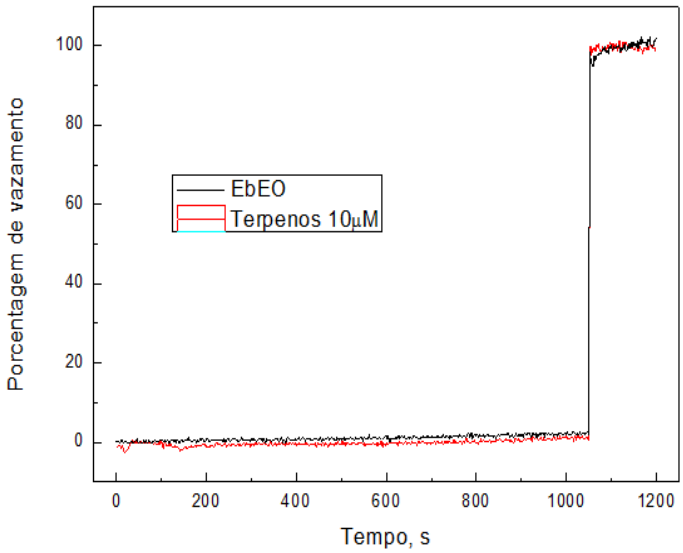

EbEO
Terpenos 10µM
Porcentagem de vazamento
Tempo, s
0
20
40
60
80
100
0
200
400
600
800
1000
1200

Figure 3

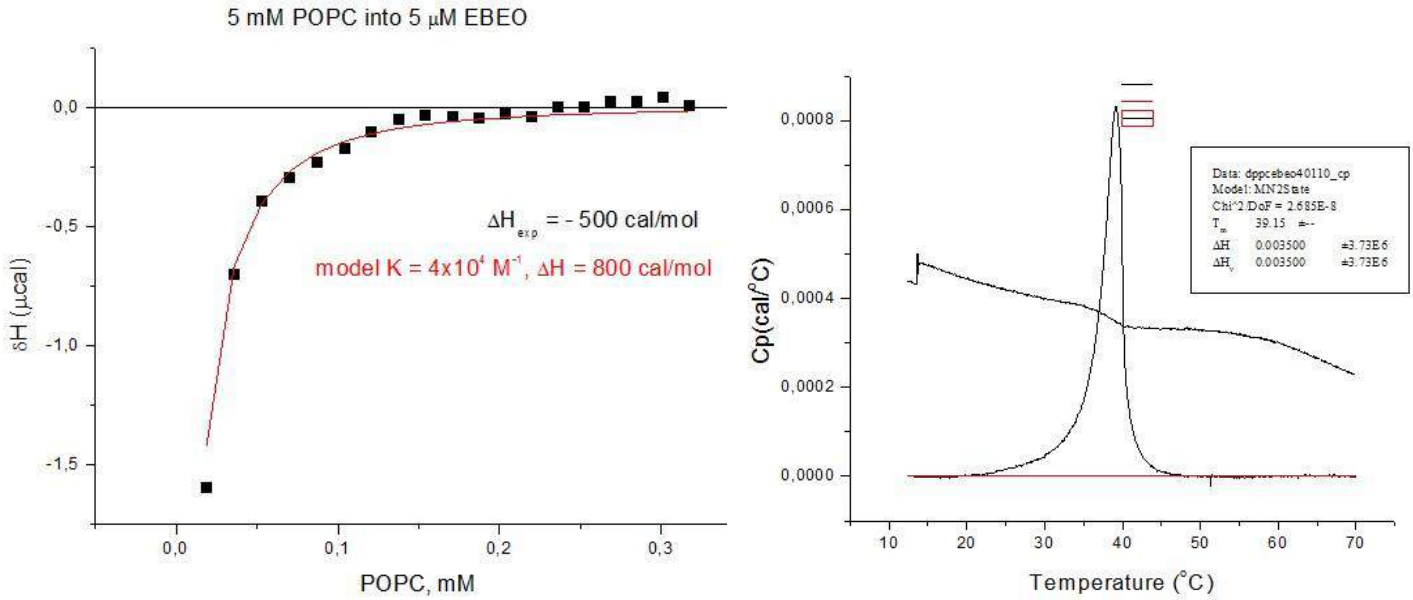
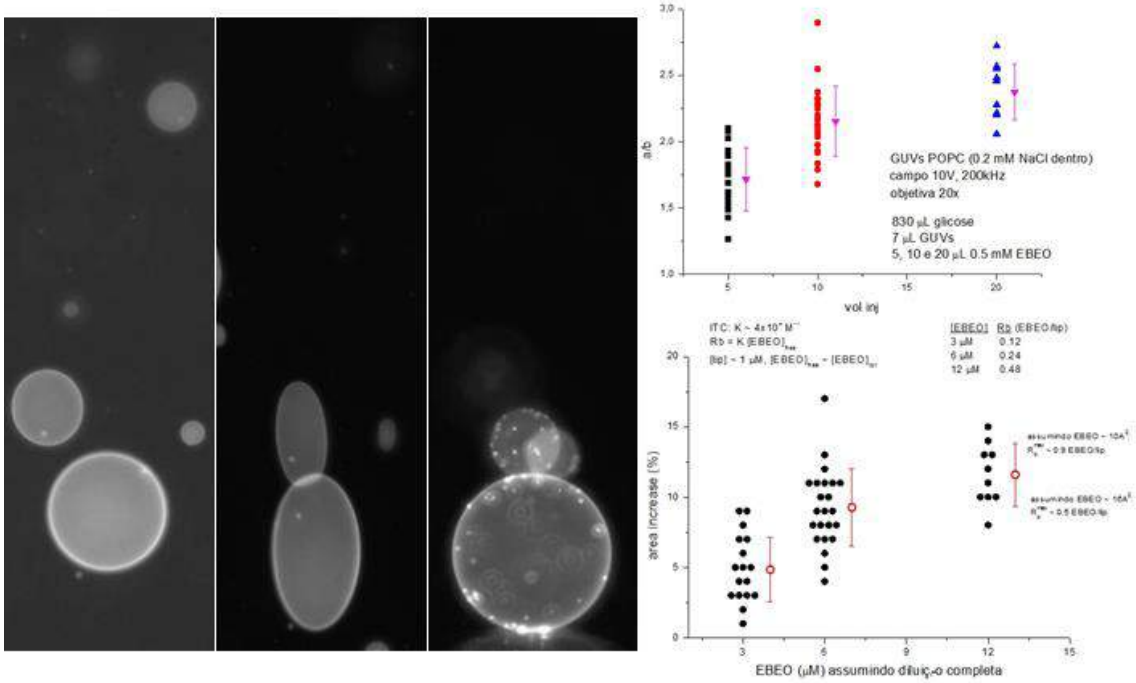

5 mM POPC into 5 μM EBEO
δH (μcal)
POPC, mM
ΔH exp = - 500 cal/mol
model K = 4x10⁴ M⁻¹, ΔH = 800 cal/mol
Cp(cal/°C)
Temperature (°C)
Data: dppcebeo40110_cp
Model: MN2State
Chi^2/DoF = 2.685E-8
T∞ 39.15 +/-
ΔH 0.003500 ±3.73E6
ΔHv 0.003500 ±3.73E6

Figure 4


GUVs POPC (0.2 mM NaCl dentro)
campo 10V, 200kHz
objetiva 20x
830 µL glicose
7 µL GUVs
5, 10 e 20 µL 0.5 mM EBEO
a/b
vol inj
ITC: K ~ 4x10^7 M^-1
Rb = K [EBEO]free
[lip] ~ 1 µM, [EBEO]free ~ [EBEO]in
[EBEO] Rb (EBEO/lip)
3 µM 0.12
6 µM 0.24
12 µM 0.48
area increase (%)
EBEO (µM) assumindo diluição completa

4 CONCLUSÃO

- rBbKIm não apresenta atividade microbicida;
- Este inibidor é capaz de reduzir importantes fatores de virulência de *S. aureus* como a produção de estafiloxantina e expressão dos genes hla e spa.
- Tanto a proteína íntegra quanto os peptídeos derivados do seu sítio reativo foram capazes de aumentar a sobrevida das larvas de *Galeria mellonella*
- O peptídeo retro-inverso apresentou maior atividade quando comparado ao peptídeo padrão derivado da proteína
- Plantas brasileiras constituem excelentes fontes de compostos com ação antimicrobiana (anti-*S. aureus*)
- O óleo essencial de *Eugenia brejoensis* é capaz de alterar a virulência e apresenta efeito microbicida.
- O inibidor, embora não apresente efeito microbicida, é capaz de interferi em fatores da virulência e auxiliar na sobrevida de espécies susceptíveis a ação desse microorganismo

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ARTIGOS PUBLICADOS DURANTE O CURSO DE DOUTORADO

1 - POTTIER, M. ; BEZERRA-SILVA, P. C. ; **Bezerra Filho, CM** ; SILVA, A. G. ; SILVA, M. V. ; CORREIA, M. T. S. ; NAVARRO, DANIELA MARIA DO AMARAL FERRAZ . Chemical Composition of the Essential Oil of Buchenavia tetraphylla Leaves. Journal of Essential Oil-Bearing Plants JCR, v. 20, p. 1-7, 2017.

2 - SILVA, L. C. N. ; **Bezerra Filho, CM** ; PAULA, R. A. ; SILVA, C. S. S. E. ; SOUZA, L. I. O. ; SILVA, M. V. ; CORREIA, M. T. S. ; FIGUEIREDO, R. C. B. Q. . In vitro cell-based assays for evaluation of antioxidant potential of plant-derived products. Free Radical Research JCR, v. 50, p. 801-812, 2016.

3 - FERRO, T. A. F. ; ARAUJO, J. M. M. ; PINTO, B. L. S. ; SANTOS, J. S. ; SOUZA, E. B. ; SILVA, B. L. R. ; COLARES, V. L. P. ; NOVAIS, T. M. G. ; **Bezerra Filho, CM** ; STRUVE, C. ; CALIXTO, J. B. ; MONTEIRO-NETO, V. ; SILVA, L. C. N. ; FERNANDES, E. S. . Cinnamaldehyde inhibits *Staphylococcus aureus* virulence factors and protects against infection in a Galleria mellonella model. Frontiers in Microbiology (Online) JCR, v. 7, p. 236147, 2016.

4 - NASCIMENTO DA SILVA, LC ; ROBSON NEVES CAVALCANTI FILHO, J ; **MACEDO BEZERRA FILHO, C** ; FONSECA DA SILVA, T ; VANUSA DA SILVA, M ; DOS SANTOS CORREIA, MT ; LØBNER-OLESEN, A . New insights into anti-*S. aureus* action of Buchenavia tetraphylla and Libidibia ferrea: inhibition of DNA replication. Planta Medica JCR, v. 81, p. S1-S381, 2016.

5 - LINS NETO, J. R. ; UCHOA, A. D. A. ; MOURA, P.A.D ; **Bezerra Filho, CM** ; TENORIO, J. C. G. ; SILVA, A. G. ; SILVA, M. V. ; CORREIA, M. T. S. . Phytochemical screening, total phenolic content and antioxidant activity of some plants from Brazilian flora. Journal of Medicinal Plant Research JCR, v. 10, p. 409-4016, 2016.

6 - DA SILVA, ALEXANDRE GOMES; ALVES, RENATA CARLA CORRÊA; **FILHO, CLOVIS MACÊDO BEZERRA** ; BEZERRA-SILVA, PATRÍCIA CRISTINA ; SANTOS, LEILANE MARINA MORAIS DOS ; FOGLIO, MARY ANN ; NAVARRO, DANIELA MARIA DO AMARAL FERRAZ ; SILVA, MÁRCIA VANUSA DA ; CORREIA, MARIA TEREZA DOS SANTOS . Chemical Composition and Larvicidal Activity of the Essential Oil from Leaves of *Eugenia brejoensis* Mazine (Myrtaceae). Journal Of Essential Oil Bearing Plants JCR, v. 18, p. 1441-1447, 2015.
Citações: WEB OF SCIENCE™ 1 | SCOPUS 3

7 - **Bezerra Filho, CM**; FRANCA, C. T. ; OLIVEIRA, M. B. M. . Compliance tobiosafety standards of the principal laboratories generating chemical waste of the pharmaceutical department of UFPE. International Journal of Pharma Medicine and Biological Sciences, v. 3, p. 57-63, 2014.

8 - SILVA, L. C. N. ; **Bezerra Filho, CM** ; PAULA, R. A. ; COELHO, L. C. B. B. ; SILVA, M. V. ; CORREIA, M. T. S. . Cratylia mollis Lectin: A Versatile Tool for Biomedical Studies. Current Bioactive Compounds, v. 10, p. 44-54, 2014.

APÊNDICE A - ARTIGO PUBLICADO NO PERIÓDICO JOURNAL OF ESSENTIAL OIL BEARING PLANTS

POTTIER, M.; BEZERRA-SILVA, P. C.; **Bezerra Filho, CM**; SILVA, A. G.; SILVA, M. V.; CORREIA, M. T. S.; NAVARRO, DANIELA MARIA DO AMARAL FERRAZ. Chemical Composition of the Essential Oil of *Buchenavia tetraphylla* Leaves. Journal of Essential Oil Bearing Plants JCR, v. 20, p. 1-7, 2017.

Taylor & Francis

Journal of Essential Oil Bearing Plants

ISSN: 0972-060X (Print) 0976-5026 (Online) Journal homepage: http://www.tandfonline.com/loi/teop20

Chemical Composition of the Essential Oil of Buchenavia tetraphylla Leaves

Maud Pottier, Patrícia Cristina Bezerra-Silva, Clovis Macêdo Bezerra Filhos, Alexandre Gomes da Silva, Márcia Vanusa da Silva, Maria Tereza dos Santos Correia & Daniela Maria do Amaral Ferraz Navarro

To cite this article: Maud Pottier, Patrícia Cristina Bezerra-Silva, Clovis Macêdo Bezerra Filhos, Alexandre Gomes da Silva, Márcia Vanusa da Silva, Maria Tereza dos Santos Correia & Daniela Maria do Amaral Ferraz Navarro (2017): Chemical Composition of the Essential Oil of Buchenavia tetraphylla Leaves, Journal of Essential Oil Bearing Plants, DOI: 10.1080/0972060X.2016.1253506

To link to this article: http://dx.doi.org/10.1080/0972060X.2016.1253506

Published online: 09 Mar 2017.

APÊNDICE B - ARTIGO PUBLICADO NO PERIÓDICO FREE RADICAL RESEARCH

SILVA, L. C. N.; **Bezerra Filho, CM**; PAULA, R. A.; SILVA, C. S. S. E.; SOUZA, L. I. O.; SILVA, M. V.; CORREIA, M. T. S.; FIGUEIREDO, R. C. B. Q. *In vitro* cell-based assays for evaluation of antioxidant potential of plant-derived products. Free Radical Research JCR, v. 50, p. 801-812, 2016.

Free Radical Research

ISSN: 1071-5762 (Print) 1029-2470 (Online) Journal homepage: http://www.tandfonline.com/loi/ifra20

In vitro cell-based assays for evaluation of antioxidant potential of plant-derived products

Luís Cláudio Nascimento da Silva, Clovis Macêdo Bezerra Filho, Raiana Apolinário de Paula, Cristiane Santos Silva e Silva, Larissa Isabela Oliveira de Souza, Márcia Vanusa da Silva, Maria Tereza dos Santos Correia & Regina Célia Bressan Queiroz de Figueiredo

To cite this article: Luís Cláudio Nascimento da Silva, Clovis Macêdo Bezerra Filho, Raiana Apolinário de Paula, Cristiane Santos Silva e Silva, Larissa Isabela Oliveira de Souza, Márcia Vanusa da Silva, Maria Tereza dos Santos Correia & Regina Célia Bressan Queiroz de Figueiredo (2016): In vitro cell-based assays for evaluation of antioxidant potential of plant-derived products, Free Radical Research

To link to this article: http://dx.doi.org/10.1080/10715762.2016.1193668

Accepted author version posted online: 24 May 2016.
Published online: 24 May 2016.

APÊNDICE C - ARTIGO PUBLICADO NO PERIÓDICO FRONTIERS IN MICROBIOLOGY (ONLINE)

FERRO, T. A. F.; ARAUJO, J. M. M.; PINTO, B. L. S.; SANTOS, J. S.; SOUZA, E. B.; SILVA, B. L. R.; COLARES, V. L. P.; NOVAIS, T. M. G.; **Bezerra Filho, CM**; STRUVE, C.; CALIXTO, J. B.; MONTEIRO-NETO, V.; SILVA, L. C. N.; FERNANDES, E. S. Cinnamaldehyde inhibits *Staphylococcus aureus* virulence factors and protects against infection in a *Galleria mellonella* model. Frontiers in Microbiology (Online) JCR, v. 7, p. 236147, 2016.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 December 2016
doi: 10.3389/fmicb.2016.02052

Cinnamaldehyde Inhibits *Staphylococcus aureus* Virulence Factors and Protects against Infection in a *Galleria mellonella* Model

*Thiago A. F. Ferro[1], Jéssica M. M. Araújo[1], Bruna L. dos Santos Pinto[1], Jéssica S. dos Santos[1], Eliene B. Souza[1], Bruna L. R. da Silva[1], Valderlane L. P. Colares[1], Tânia M. G. Novais[1], Clovis M. B. Filho[2], Carsten Struve[3], João B. Calixto[4], Valério Monteiro-Neto[1,5], Luís C. N. da Silva[1] and Elizabeth S. Fernandes[1]**

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OPEN ACCESS

Edited by:
Octavio Luiz Franco, Universidade Católica de Brasília, Brazil

Reviewed by:
Atte Von Wright, University of Eastern Finland, Finland
Andre Moraes Nicola, University of Brasília, Brazil

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 12 October 2016
Accepted: 07 December 2016
Published: 21 December 2016

Citation:
Ferro TAF, Araújo JMM, dos Santos Pinto BL, dos Santos JS, Souza EB, da Silva BLR, Colares VLP, Novais TMG, Filho CMB, Struve C, Calixto JB, Monteiro-Neto V, da Silva LCN and Fernandes ES (2016) Cinnamaldehyde Inhibits Staphylococcus aureus Virulence Factors and Protects against Infection in a Galleria mellonella Model. Front. Microbiol. 7:2052. doi: 10.3389/fmicb.2016.02052

Bacterial resistance to the available marketed drugs has prompted the search of novel therapies; especially in regards of anti-virulence strategies that aim to make bacteria less pathogenic and/or decrease their probability to become resistant to therapy. Cinnamaldehyde is widely known for its antibacterial properties through mechanisms that include the interaction of this compound with bacterial cell walls. However, only a handful of studies have addressed its effects on bacterial virulence, especially when tested at sub-inhibitory concentrations. Herein, we show for the first time that cinnamaldehyde is bactericidal against *Staphylococcus aureus* and *Enterococcus faecalis* multidrug resistant strains and does not promote bacterial tolerance. Cinnamaldehyde actions were stronger on *S. aureus* as it was able to inhibit its hemolytic activity on human erythrocytes and reduce its adherence to latex. Furthermore, cinnamaldehyde enhanced the serum-dependent lysis of *S. aureus*. *In vivo* testing of cinnamaldehyde in *Galleria mellonella* larvae infected with *S. aureus*, showed this compound improves larvae survival whilst diminishing bacterial load in their hemolymph. We suggest that cinnamaldehyde may represent an alternative therapy to control *S. aureus*-induced bacterial infections as it presents the ability to reduce bacterial virulence/survival without promoting an adaptive phenotype.

Keywords: essential oil, cinnamaldehyde, infection, bacterial virulence, *S. aureus*

INTRODUCTION

Bacterial pathogens have evolved several mechanisms to acquire resistance to drug and hereby survive antibiotic treatment in eukaryotic hosts, including mutations, plasmid acquisition, amongst others (Blair et al., 2015; Lin et al., 2015). In fact, multidrug resistant strains have been observed with increasing frequency and their spreading has been recognized as one of the most

APÊNDICE D - ARTIGO PUBLICADO NO PERIÓDICO PLANTA MEDICA

NASCIMENTO DA SILVA, LC; ROBSON NEVES CAVALCANTI FILHO, J; **MACEDO BEZERRA FILHO, C**; FONSECA DA SILVA, T; VANUSA DA SILVA, M; DOS SANTOS CORREIA, MT; LØBNER-OLESEN, A. New insights into anti-*S. aureus* action of *Buchenavia tetraphylla* and *Libidibia ferrea*: inhibition of DNA replication. Planta Medica JCR, v. 81, p. S1-S381, 2016.

LC Nascimento da Silva[1,2], J Robson Neves Cavalcanti Filho[3], C Macedo Bezerra Filho[3], T Fonseca da Silva[3], M Vanusa da Silva[3], MT dos Santos Correia[3], A Løbner-Olesen[1]

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Further Information

Publication History

Publication Date:
14 December 2016 (online)

- Congress Abstract
- Full Text

DNA replication is an essential process carried out by protein machinery with β-clamp (DnaN) playing a crucial role, which make it an attractive target for potential new antibiotics with high therapeutic index [1]. This work evaluated the ability of 30 extracts from 22 plants to prevent the dimerization of β-sliding clamp (DnaN) of S. aureus. The antimicrobial activity was evaluated against strain S. aureus 8325 – 4. DnaN-DnaN interaction was performed using a Bacterial two-hybrid system (BTH) and confirmed using S. aureus 8325 – 4 derivative strain overexpressing DnaN and flow cytometry [1]. The effects of SOS response was evaluated using a S. aureus strain with integrated recA:lacZ reporter [1]. The combinatory effects with drugs (ciprofloxacin, ampicilin and chloramphenicol) and the mutagenic potential were also determined. The initial antimicrobial screening revealed that eight extracts were able to inhibit S. aureus, but only three extracts inhibited the in vivo interaction of DnaN-DnaN: methanolic (BTME) and ethyl acetate (BTEE)extracts from Buchenavia tetraphylla leaves, and aqueous extract from Libidibia ferrea fruits (LFAE). The antimicrobial activity of these two plants has been reported by our group [2,3]. Overexpression of DnaN in S. aureus resulted in resistance towards all these plant extracts. The active extracts induced an increase in cell mass without increase their DNA content, as expected for DNA replication inhibitors. LFAE and BTME showed synergistic and additive effects with chlorampenicol, respectively; and noninteractive effects with the other drugs. BTEE showed additive effects with all tested drugs. The extracts had mutagenic frequencies smaller than ciprofloxacin. This work provides more details about the anti-S. aureus actions of B. tetraphylla and L. ferrea which have DNA replication as potential target. The purification and structural characterization of active compounds from all these plants are the next step of our research.

Acknowledgements: Program Science without borders CAPES/Brazil.

Keywords: DnaN, Caatinga, natural products.

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APÊNDICE E - ARTIGO PUBLICADO NO PERIÓDICO JOURNAL OF MEDICINAL PLANT RESEARCH

LINS NETO, J. R.; UCHOA, A. D. A.; MOURA, P.A.D; **Bezerra Filho, CM**; TENORIO, J. C. G.; SILVA, A. G.; SILVA, M. V.; CORREIA, M. T. S. Phytochemical screening, total phenolic content and antioxidant activity of some plants from Brazilian flora. Journal of Medicinal Plant Research JCR, v. 10, p. 409-4016, 2016.

academicJournals

Vol. 10(27), pp. 409-416, 17 July, 2016
DOI: 10.5897/JMPR2015.5979
Article Number: F679AE659546
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Phytochemical screening, total phenolic content and antioxidant activity of some plants from Brazilian flora

João da Rocha Lins Neto[1], Amanda Dias de Araújo Uchôa[1,2], Priscila Andrade de Moura[1], Clovis Macêdo Bezerra Filho[1], Juciara Carneiro Gouveia Tenório[1], Alexandre Gomes da Silva[2], Rafael Matos Ximenes[3], Márcia Vanusa da Silva[1,2] and Maria Tereza dos Santos Correia[1,2]*

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[2]Núcleo de Bioprospecção e Conservação da Caatinga, Instituto Nacional do Semiárido/Ministério da Ciência, Tecnologia e Inovação (INSA/MCTI), Campina Grande, Paraíba, Brazil.
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Received 19 October 2015; Accepted 4 April, 2016

The present study evaluated the total phenolic and flavonoid content as well as the antioxidant activity of methanolic leaf extracts of five plants from Brazilian flora: *Abarema cochliacarpos*, *Croton corchoropsis*, *Myroxylon peruiferum*, *Stryphnodendron pulcherrimum* and *Tanaecium cyrtanthum* by 2,2'-azino-bis- (3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and total antioxidant capacity assays. A thin layer chromatography analysis of all plant extracts has also been performed and it showed the presence of different types of secondary metabolites, namely saponins, phenylpropanoids and flavonoids. Among the studied plants, *A. cochliacarpos* and *S. pulcherrimum* showed considerable antioxidant radical scavenging activity on all the tested assays and they also exhibited substantial amounts of phenolic compounds. In addition, a positive correlation was found between total phenols and both ABTS radical scavenging activity and total antioxidant capacity assays, thus indicating the major role of phenols on the antioxidant activity of these plants. To the best of the authors' knowledge, this is the first approach where the phenolic content and antioxidant activity of *A. cochliacarpos*, *C. corchoropsis*, *M. peruiferum*, *S. pulcherrimum* and *T. xanthophyllum* were explored.

Key words: Brazilian medicinal plants, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), flavonoid content, phenolic content.

INTRODUCTION

Oxidation and reduction of molecules are essential to life; they represent normal phenomena that occur in cell metabolism. Among substances involved in oxidation-reduction reactions of molecules are free radicals, which are organic or inorganic compounds having one or more unpaired electrons on their valance shell, they are chemically unstable and very reactive (Lushchak, 2014).

In organism, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in metabolic processes such as energy production, regulation of cell

APÊNDICE F - ARTIGO PUBLICADO NO PERIÓDICO JOURNAL OF ESSENTIAL OIL - BEARING PLANTS

DA SILVA, ALEXANDRE GOMES; ALVES, RENATA CARLA CORRÊA; **FILHO, CLOVIS MACÊDO BEZERRA**; BEZERRA-SILVA, PATRÍCIA CRISTINA; SANTOS,

LEILANE MARINA MORAIS DOS; FOGLIO, MARY ANN; NAVARRO, DANIELA MARIA DO AMARAL FERRAZ; SILVA, MÁRCIA VANUSA DA; CORREIA, MARIA TEREZA DOS SANTOS. Chemical Composition and Larvicidal Activity of the Essential Oil from Leaves of *Eugenia brejoensis* Mazine (Myrtaceae). Journal Of Essential Oil - Bearing Plants JCR, v. 18, p. 1441-1447, 2015.
Citações: WEB OF SCIENCE ℠ 1|SCOPUS3

Taylor & Francis

Journal of Essential Oil Bearing Plants

ISSN: 0972-060X (Print) 0976-5026 (Online) Journal homepage: http://www.tandfonline.com/loi/teop20

Chemical Composition and Larvicidal Activity of the Essential Oil from Leaves of Eugenia brejoensis Mazine (Myrtaceae)

Alexandre Gomes da Silva, Renata Carla Corrêa Alves, Clovis Macêdo Bezerra Filho, Patrícia Cristina Bezerra-Silva, Leilane Marina Morais dos Santos, Mary Ann Foglio, Daniela Maria do Amaral Ferraz Navarro, Márcia Vanusa da Silva & Maria Tereza dos Santos Correia

To cite this article: Alexandre Gomes da Silva, Renata Carla Corrêa Alves, Clovis Macêdo Bezerra Filho, Patrícia Cristina Bezerra-Silva, Leilane Marina Morais dos Santos, Mary Ann Foglio, Daniela Maria do Amaral Ferraz Navarro, Márcia Vanusa da Silva & Maria Tereza dos Santos Correia (2015) Chemical Composition and Larvicidal Activity of the Essential Oil from Leaves of Eugenia brejoensis Mazine (Myrtaceae), Journal of Essential Oil Bearing Plants, 18:6, 1441-1447, DOI: 10.1080/0972060X.2014.1000390

To link to this article: http://dx.doi.org/10.1080/0972060X.2014.1000390

Published online: 17 Nov 2015.

APÊNDICE G - ARTIGO PUBLICADO NO PERIÓDICO INTERNATIONAL JOURNAL OF PHARMA MEDICINE AND BIOLOGICAL SCIENCES

Bezerra Filho, C. M; FRANCA, C. T.; OLIVEIRA, M. B. M. Compliance tobiosafety standards of the principal laboratories generating chemical waste of the pharmaceutical department of UFPE. International Journal of Pharma Medicine and Biological Sciences, v. 3, p. 57-63, 2014.

Int. J. Pharm. Med. & Bio. Sc. 2014

International Journal of Pharma Medicine and Biological Sciences

ISSN 2278 – 5221 www.ijpmbs.com
Vol. 3, No. 1, January 2014

Research Paper

COMPLIANCE TO BIOSAFETY STANDARDS OF THE PRINCIPAL LABORATORIES GENERATING CHEMICAL WASTE OF THE PHARMACEUTICAL DEPARTMENT OF UFPE

Clovis Macêdo Bezerra Filho[1], Camila Tenório Franca[1] and Maria Betania Melo de Oliveira[1]*

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Waste management has been an increasingly common practice in industries and laboratories for teaching and research across the country. Although some of these units have demonstrated interest in meeting the standards of biosafety, much still needs to be done to address the environmental issues and develope renewable technologies. The Pharmaceutical Department of UFPE has several laboratories, including research and extension groups whose activities, while of a heterogeneous nature, are directly linked to the use of chemical substances, production of medicines and other pharmaceuticals. The present work aimed to implement measures comprising a Chemical Waste Management Plan in the major chemical waste producing laboratories of that department, suggesting adjustments in physical structure, internal procedures, management of substances and educational activities. There are indications that these units will soon submit plans to conform to a Chemical Waste Management Program, still non-existent in the institution.

Keywords: Biosafety, Medicines, Management, Production

INTRODUCTION

Chemical waste is defined as anysubstance of a chemical nature that has physical and chemical characteristics, such as corrosivity, toxicity, melting and boiling points, density, reactivity, among others (Tomazini *et al.*, 2011). For the management of waste, a set of actions is necessary that involve biosafety, rational reduction of production and actions that minimize environmental impacts. In recent decades, waste management programs have been gaining strength within pharmaceutical-chemical industries, whether of a small or large scale (Azevedo, 2008). Brazil, in its position as an industrialized country in a state of accelerated economic development, has raised significantly its production of wastes, exponentially increasing the risk to the health of workers and the

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APÊNDICE H - ARTIGO PUBLICADO NO PERIÓDICO CURRENT BIOACTIVE COMPOUNDS

SILVA, L. C. N.; **Bezerra Filho, CM**; PAULA, R. A.; COELHO, L. C. B. B.; SILVA, M. V.; CORREIA, M. T. S. *Cratylia mollis* Lectin: A Versatile Tool for Biomedical Studies. Current Bioactive Compounds, v. 10, p. 44-54, 2014.

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44 *Current Bioactive Compounds* 2014, *10*, 44-54

Cratylia mollis Lectin: A Versatile Tool for Biomedical Studies

Luís Cláudio Nascimento da Silva[1], Clovis Macêdo Bezerra Filho[1], Raiana Apolinário de Paula[1], Luana Cassandra Breitenbach Barroso Coelho[1], Márcia Vanusa da Silva[2] and Maria Tereza dos Santos Correia[1,*]

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Abstract: Lectins are a heterogeneous group of proteins that specifically and reversibly bind to carbohydrates without altering their covalent structure. Advances in the plant lectin research have provided many insights into the role of carbohydrate-protein interactions, which have contributed to the development of various others (for example: oncology, immunology, microbiology, molecular biology and diagnostic). Isolectins isolated from *Cratylia mollis*, a native plant of Brazilian Caatinga biome, have demonstrated a remarkable biotechnological aptitude, especially the isoform called Cramoll 1,4 (pCramoll). This work aims to present a comprehensive review of the biomedical applications of pCramoll, which has been used as immunomodulatory, antitumoral, mitogenic, antiparasitic, and healing agent. Other biotechnological applications also involve the characterization of human cancerous tissues, and the development of af nity matrices and biosensors. The present review comprises of four main themes: (1) an overview of plant lectins and their biological effects; (2) general characteristics of the pCramoll; (3) biotechnological applications of this lectin; and (4) recent progress in production of pCramoll in heterologous system. The process of isolation, structural characterization and biotechnological applications of pCramoll is one of the greatest success stories among Brazilian lectins. Recently, the expression of functional recombinant Cramoll 1 (rCramoll) in *Escherichia coli* was reported by our group. This review will be a valuable resource for more studies in the lectin field, which has a great potential to reveal new targets for therapeutic strategies, molecular diagnosis and biotechnology process.

Keywords: Anti-parasitic, biosensor, immunomodulation, lectin, oncology, wound healing.

INTRODUCTION

Lectins are known as a large class of carbohydrate-binding proteins which play pivotal role in a range of cellular processes [1, 2]. Lectins are usually oligomeric proteins which exhibit a large structural and size diversity. Although each lectin polypeptide may contain different domains, at least one of them is a non-catalytic carbohydrate recognition site, responsible for their ability to recognize and interact with speci c sugar, without altering their structure [3, 4]. Even though the carbohydrate-binding domains are best characterized in plants, lectins are found in all forms of life [5- 7].

Plant lectins represent a group of proteins with obvious differences in their biochemical properties, molecular structure, carbohydrate-binding specificity and biological activities [3, 8]. In the past few years, hundreds of plant lectins have been purified and characterized in detail with respect to their biochemical properties [4, 9-11]. Based on their structural folds, mature lectins can be classified into 'merolectins', 'hololectins', 'chimerolectins', and 'superlectins' (Fig. **1**).

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E-mail: mtscorreia@gmail.com

There exist 12 plant lectin families based on their different carbohydrate-binding domains [7], which include: (1) *Agaricus bisporus* agglutinin homologs, (2) Amaranthins, (3) Class V chitinase homologs with lectin activity, (4) Cucurbitaceae phloem lectins (or Nictaba family), (5) Cyanovirin family, (6) EEA family, (7) GNA family, (8) Jacalin-related lectins, (9) LysM domain, (10) proteins with hevein domains, (11) proteins with legume lectin domains, and (12) Ricin-B family (Table **1**).

Though the discovery of lectins dates back to the nineteenth century, many questions about the biological role of these molecules still remain obscure. Lectins may be involved in sugar transport and carbohydrate storage and also function as molecular chaperones [9, 12, 13]. The ability of adhesion and agglutination of lectins has been associated with the interaction of both symbiotic and pathogenic microorganisms [14, 15].

OVERVIEW OF BIOMEDICAL APPLICATIONS OF PLANT LECTINS

Plant lectins have extensively been used as valuable tools in biomedical researches [16, 17]. The versatility of these biomolecules lies in their interactions with receptor-linked glycans on cell surfaces, which may trigger cell signaling and physiological responses [18] (Table **2**).

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RESUMOS EM CONGRESSOS PUBLICADOS DURANTE O CURSO DE DOUTORADO

1 - Bezerra Filho, CM; ZAGMIGNAN, A. ; CORREIA, M. T. S. ; SILVA, L. C. N. . CAATINGA PLANTS AS INHIBITORS OF *staphylococcus aureus* VIRULENCE: IN VITRO AND IN VIVO INSIGHTS. 2017. (Apresentação de Trabalho/Congresso).

2 - NASCIMENTO, L. M. G.; SANTANA, P. C.; LINS, A. M. P. S.; REIS, J. A.; ARAUJO, E. K. S. M.; Bezerra Filho, CM. O USO DE ANTIDEPRESSIVOS EM PACIENTES COM CÂNCER DE MAMA. 2017. (Apresentação de Trabalho/Outra).

3 - SILVA, M. M.; CAHU, T.; SILVA, R. P.; Bezerra Filho, CM; SOARES, K.; AZEVEDO, R.; NASCIMENTO, L.; BEZERRA, R. Production, characterization and antioxidant activity of Chitoligosaccharides obtained from Chitosan of Marine shrimp *Litopenaeus vannamei* processing waste. 2015. (Apresentação de Trabalho/Congresso).

4 - BARBOSA, A. V.; MOURA, P.A.D; Bezerra Filho, CM; SILVA, M. V.; CORREIA, M. T. S. Total phenols and antioxidant activities of *Buchenavia tetraphylla*, a Caatinga plant. 2015. (Apresentação de Trabalho/Congresso).

5 - MOURA, P.A.D.; Macêdo, C.B.F.; BRITO, M. V.; OLIVA, M. L. V.; SILVA, M. V.; CORREIA, M. T. S. Evaluation of the anticoagulant activity of species of Caatinga Plants. 2015. (Apresentação de Trabalho/Congresso).

6 - Macêdo, C.B.F.; MELO, A. C.; NASCIMENTO JUNIOR, J. A. A.; OLIVA, M. L. V.; SILVA, M. V.; CORREIA, M. T. S. Antioxidant activity and hemagglutination of leaf extracts from *Eugenia brejoensis*. 2014. (Apresentação de Trabalho/Congresso).

7 - MOURA, P.A.D.; Macêdo, C.B.F.; SILVA, M. V.; CORREIA, M. T. S. Antioxidant activyand Polyphenolscontets of *Bowdichia virgilioides* leaves, a plant from caatinga Biome. 2014. (Apresentação de Trabalho/Congresso).

ANEXO A - NORMAS DE SUBMISSÃO DE MANUSCRITOS PARA A REVISTA INTERNACIONAL PEPTIDES

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- **Abstracting and Indexing** p.2
- **Editorial Board** p.2
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Preparation and Submission of Manuscripts

(Revised Feb 2018)

Contents (click on the topic)

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When physical and spectroscopic data are presented in the body of the manuscript, the following general style must be used (with the various commonly used techniques presented in this same order):

> **Romucosine (1):** colorless needles ($CHCl_3$); mp 152–153 °C; $[\alpha]^{25}_{D}$ −110 (*c* 0.4, $CHCl_3$); UV (EtOH) λ_{max}(log ε) 235 (4.23), 275 (4.18), 292 (sh) (3.52), 325 (3.41) nm; IR (Nujol) ν_{max} 1680, 1040, 920 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.11 (1H, d, J = 7.6 Hz, H-11), 7.54–7.28 (2H, m, H-9, H-10), 7.27 (1H, m, H-8), 6.59 (1H, s, H-3), 6.10, 5.97 (each 1H, d, J = 1.5 Hz, OCH_2O), 4.86 (1H, dd, J = 13.7, 4.4 Hz, H-6a), 4.44 (1H, m, H-5a), 3.77 (3H, s, $NCOOCH_3$), 3.06 (1H, m, H-7a), 2.99 (1H, m, H-5b), 2.91 (1H, m, H-7b), 2.82 (1H, m, H-4a), 2.61 (1H, m, H-4b); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.8 (C, $NCOOCH_3$), 146.8 (C, C-2), 143.0 (C, C-1), 135.8 (C, C-7a), 130.7 (C, C-11a), 128.7 (CH, C-8), 127.79 (C, C-3a), 127.78 (CH, C-9), 127.2 (CH, C-10), 127.0 (CH, C-11), 125.6 (C, C-3b), 117.3 (C, C-1a), 107.6 (CH, C-3), 100.9 (CH_2, OCH_2O), 52.7 (CH_3, $NCOOCH_3$), 51.7 (CH, C-6a), 39.2 (CH_2, C-5), 34.5 (CH_2, C-7), 30.4 (CH_2, C-4); EIMS *m/z* 323 $[M]^+$ (98), 308 (28), 292 (5), 262 (20), 248 (21), 236 (81), 235 (100), 206 (17), 178 (27), 88 (17); HREIMS *m/z* 323.1152 (calcd for $C_{19}H_{17}NO_4$, 323.1158).

The correct presentation of NMR spectroscopic data is shown in the table below.

Table 1. NMR Spectroscopic Data (400 MHz, C_6D_6) for Aurilides B (**1**) and C (**2**)

	aurilide B (**1**)			aurilide C (**2**)	
position	δ_C, type	δ_H (J in Hz)	HMBC[a]	δ_C	δ_H (J in Hz)
1	170.0, C			170.2	
2	58.9, CH	3.23, m	1, 3, 4, 5	59.8	3.08, m
3	13.8, CH_3	1.21, d (7.1)	1, 2	14.0	1.25, d (7.1)
4	36.1, CH_2	2.63, s	2, 5	36.8	2.55, s
5	172.1, C			172.1	
6	54.3, CH	5.12, dd (9.0, 7.4)	5, 7, 9	54.4	5.15, dd (9.0, 5.6)
7	31.0, CH	1.97, m		32.0	1.98, m
8	20.1, CH_3	1.15, d (7.0)	6, 7, 9	20.4	1.17, d (7.0)
9	17.3, CH_3	1.25, d (7.0)	6, 7, 8	17.5	1.28, d (7.0)
10	169.9, C			170.11	
11	51.8, CH_2	4.40, d (18.0) 3.80, d (18.0)	10, 12, 13	51.8	4.39, d (18.0) 3.80, d (18.0)
12	56.8, CH_3	3.23, s	11, 13	57.1	3.22, s
13	170.0, C			170.14	
14	58.6, CH	5.24, d (10.0)	13, 18, 19, 20	58.7	5.26, d (10.0)
15	33.9, CH	2.48, m	14, 16, 18	34.1	2.49, m
16	27.4, CH_2	1.86, 1.30, m	14, 15, 17	27.6	1.89, 1.30, m
17	12.1, CH_3	1.03, t (7.1)		12.2	1.03, t (6.9)
18	14.8, CH_3	0.85, d (7.0)	15, 16	15.1	0.86, d (7.0)
19	30.7, CH_3	2.88, s	20	30.6	2.85, s
20	173.1, C			173.2	
21	54.7, CH	4.78, dd (8.8, 5.8)	20, 22	54.9	4.75, dd (8.6, 7.5)
22	31.7, CH	1.98, m		31.0	1.95, m
23	18.1, CH_3	0.88, d (6.0)	21, 22, 24	18.9	0.88, d (6.0)
24	20.2, CH_3	0.90, d (6.0)	23	20.3	0.90, d (6.0)
25	170.1, C			170.3	
26	78.5, CH	4.90, d (6.1)	25, 27, 31	80.4	4.54, d (7.5)
27	37.2, CH	2.17, m	26, 28	30.5	2.36, m
28	26.1, CH_2	1.50, 1.14, m	29	18.7	1.00, d (7.0)
29	11.8, CH_3	0.83, t (7.7)	27, 28	18.4	0.88, d (7.0)
30	14.9, CH_3	1.03, d (6.0)	26, 27, 28	169.7	
31	169.3, C			128.3	
32	128.0, C			146.0	7.75, t (9.0)
33	145.3, CH	7.74, t (9.8)	31, 42	30.9	2.74, m
34	30.9, CH_2	2.19, m	32, 33, 43	71.2	3.98, m
35	71.0, CH	3.97, m	34	41.2	2.02, m
36	41.1, CH	2.07, m	43	82.6	5.17, d (11.2)
37	82.5, CH	5.18, d (11.2)	1, 36, 38, 44	132.1	
38	131.4, C			134.6	5.62, t (7.7)
39	134.2, CH	5.61, t (7.7)	37, 44	21.4	1.95, 1.92, m
40	21.4, CH_2	1.95, 1.92, m	38, 39, 41	14.3	0.89, t[b]
41	14.1, CH_3	0.89, t[b]	39, 40	12.8	1.95, s
42	12.7, CH_3	1.95, s	31, 32, 33	10.1	0.66, (7.0)
43	10.2, CH_3	0.64, d (7.0)	35, 36, 37	11.4	1.54, s
44	11.3, CH_3	1.54, s	37, 38, 39		
NH (1)		7.69 brd (9.1)	10		7.66 brd (9.1)
NH (2)		6.75 brd (8.8)	25		6.70 brd (8.8)

[a]HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon.
[b]Signal partially obscured.

The correct format to present elemental analysis data is: anal. C 72.87, H 11.13%, calcd for $C_{37}H_{68}O_6$, C 73.02, H 11.18%. The structures of compounds are expected to be supported by high-resolution mass spectrometry (error limit 5 ppm or 0.003 *m/z* units) or elemental analysis. Melting point determinations should not be provided for compounds described as "amorphous solids". The unit of concentration to be used for optical rotation measurements is grams per 100 mL. UV extinction coefficient data should be provided as log ϵ values, to two places of decimals. In reporting ^{1}H NMR data of diastereotopic methylene protons, the one at lower field should be listed as the "a" proton and that at the higher field as the "b" proton, as in "H-10a" and "H-10b", respectively. If two proton or carbon signals in an NMR spectrum appear at the same chemical shift but are still distinguishable, an additional decimal place (three for ^{1}H NMR data and two for ^{13}C NMR data) may be used to designate the resonance in question. Carbon-13 NMR data should be reported to the nearest 0.1 ppm with the number of attached protons designated using the C, CH, CH_2, and CH_3 notation.

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(1) Pettit, G. R.; Searcy, J. D.; Tan, R.; Cragg, G. M.; Melody, N.; Knight, J. C.; Chapuis, J.-C. *J. Nat. Prod.* **2016**, *79*, 507–518.

(2) Linington, R. G.; Williams, P. G.; MacMillan, J. B. *Problems in Organic Structure Determination. A Practical Approach to NMR Spectroscopy*; CRC Press/Taylor and Francis Group: Boca Raton, FL, 2016.

(3) Harada, N.; Nakanishi, K.; Berova, N. In *Comprehensive Chiroptical Spectroscopy, Vol. 2; Applications in Stereochemical Analysis of Synthetic Compounds, Natural Products, and Biomolecules*; Berova, N., Polavarapu, P. L., Nakanishi, K., Woody, R. W., Eds.; John Wiley & Sons: New York, 2012; pp 115–166.

(4) Zheng, G.; Kakisawa, H. *Chin. Sci. Bull.* **1990**, *35*, 1406–1407; *Chem. Abstr.* **1991**, *114*, 43213*m*.

(5) Imai, A. Pharmacognosy of the Aerial Parts of Black Cohosh (*Cimicifuga racemosa*). Ph.D. Dissertation, University of Illinois at Chicago, Chicago, IL, 2013.

(6) Davis, R. U.S. Patent 5,708,591, 1998.

(7) Partial data for plakinic acid M were reported in the Supporting Information of ref 5a, but a more complete listing is given here for comparative purposes.

(8) World Health Organization. Fact Sheet No. 94, 2015. http://www.who.int/mediacentre/factsheets/fs094/en/ (accessed October 1, 2015).

For additional information on the reference and note format to use, see *The ACS Style Guide*, 3rd ed. (2006) (http://pubs.acs.org/books), available from Oxford University Press, Order Department, 2001 Evans Road, Cary, NC 27513 (http://www.oup.com).

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For IUPAC rules, see:

- *Nomenclature of Inorganic Chemistry, Recommendations, 1990*; Blackwell Scientific Publications: Oxford, England, 1990.
- *A Guide to IUPAC Nomenclature of Organic Compounds, Recommendations, 1993*; Blackwell Scientific Publications: Oxford, England, 1993.
- *Nomenclature of Organic Chemistry, Sections A–F and H*; Pergamon Press: Elmsford, NY, 1979.
- *Compendium of Macromolecular Nomenclature*; Blackwell Scientific Publications: Oxford, England, 1991.
- *Biochemical Nomenclature and Related Documents*, 2nd ed.; Portland Press, Ltd.: London, England, 1992.
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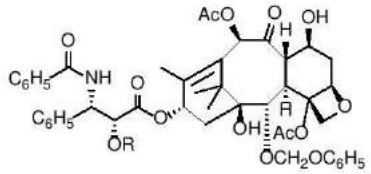

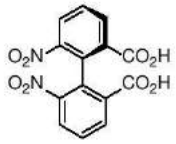

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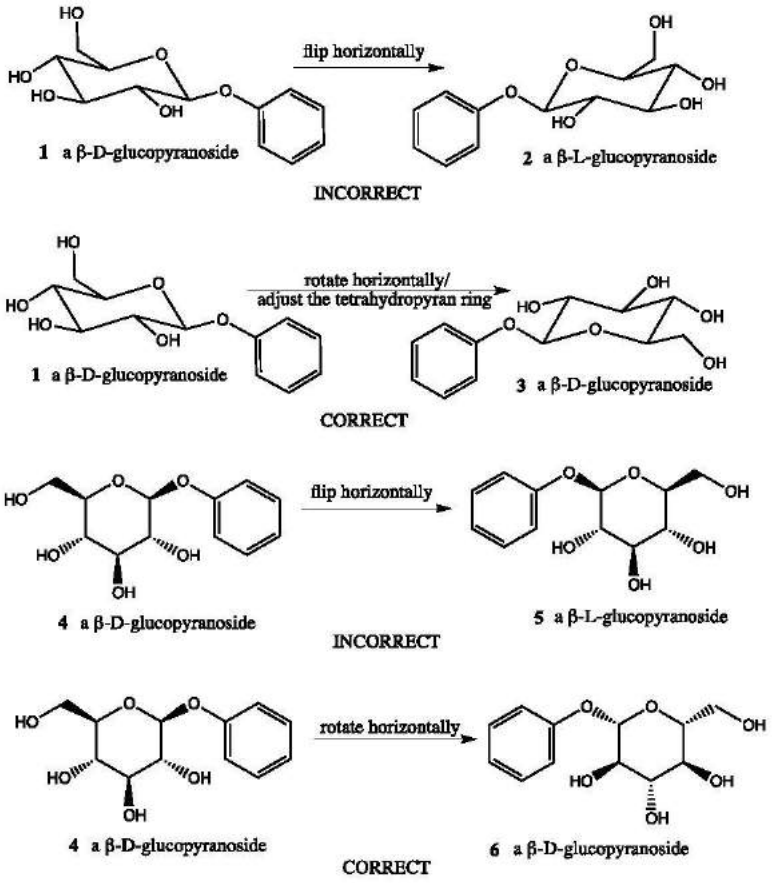

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

AUTHOR INFORMATION PACK

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GUIDE FOR AUTHORS

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