UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Biociências – Campus de Botucatu

AÇÃO ANTIBACTERIANA DA PRÓPOLIS VERMELHA SOBRE *Staphylococcus aureus* RESISTENTE À METICILINA (MRSA) E IMUNOMODULADORA SOBRE MONÓCITOS HUMANOS *IN VITRO*

Nicolas Ripari

Tese apresentada ao Programa de Pós-graduação em Biologia Geral e Aplicada, Área de concentração: Biomoléculas – Estrutura e Função, para obtenção de título de Doutor.

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Orientador: Prof. Titular José Maurício Sforcin

Co-orientadores: Prof. Adj. Ary Fernandes Júnior
Profa. Adj. Vera Lúcia Mores Rall

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“- Tudo isso é muito fácil de entender. O medo é o primeiro inimigo natural que o homem precisa vencer em seu caminho para o conhecimento. Além disso, você é curioso. Isso acertas as coisas. E há de aprender a despeito de você mesmo, é esta a regra.”

Dom Juan em: A erva do diabo. Carlos Castaneda.

Sumário

Resumo

O objetivo desta tese de doutoramento foi analisar a atividade antibacteriana da própolis vermelha em associação ou não com antimicrobianos sobre *Staphylococcus aureus* resistente à meticilina (MRSA), a ação imunomoduladora em monócitos humanos ($CD14^+$) isolados por *beads* magnéticas, e a capacidade de *killing* bacteriano dos monócitos sobre MRSA. Inicialmente, foi realizada uma revisão de literatura sobre a possibilidade de a própolis e seus constituintes exercerem ação contra MRSA. Em seguida, foi investigada a ação da própolis vermelha em combinação ou não com β-lactâmicos sobre MRSA e mecanismos envolvidos. Finalmente, as concentrações de própolis vermelha e de antimicrobiano que melhor exerceram ação antibacteriana in vitro foram avaliadas em monócitos, para observar um possível efeito imunomodulador. A revisão inicial demonstrou que os diversos tipos de própolis encontrados no globo e seus principais constituintes podem agir de forma inibitória contra diversas cepas e isolados de MRSA bem como em suas principais moléculas relacionadas à sua resistência à drogas. No capítulo 2, apresentamos o manuscrito proveniente da primeira fase da realização deste projeto, onde demonstramos que a própolis vermelha apresentou baixas concentrações inibitórias (MIC) para diversos tipos de isolados MRSA (MIC_{90} = 125 μg/mL). As combinações de própolis com imipenem apresentaram sinergismo pelo método de *Ckeckerboard* (FICI = 0,5) e de curva de sobrevivência bacteriana com diferença estatística entre o grupo controle ($p \leq 0,0001$). Esta mesma diferença estatística foi observada quando própolis e imipenem foram combinados com 25% de suas MICs nos testes de formação de biofilme, apresentando porcentagem de inibição do crescimento de biofilme menor que somente própolis ou imipenem à 75% de suas MICs sobre a maioria dos isolados (~ 47% - 64%). A porcentagem de condutividade de membrana dos isolados chegou a 100% também para esta combinação. Predições de molecular docking indicaram que o índice de energia livre de ligação dos compostos da própolis vermelha sobre a proteína ligante de penicilina 2a (PBP2a) foi parecido com ceftarolina (kcal/ mol = -7,1 a 7,8). A última fase foi avaliar a ação imunomoduladora da própolis e imipenem nas mesmas concentrações da fase I, em monócitos humanos. Observamos uma *down*-regulação de TLR-2 e exercida pela própolis vermelha e aumento na produção de IL-6 exercido pela combinação nos monócitos tratados. A capacidade de *killing* bacteriano dos monócitos, após tratamento com própolis, chegou próximo do controle LPS (~90%). A própolis vermelha em combinação com imipenem pode ser uma alternativa para o tratamento de MRSA por impedir sua multiplicação, formação de biofilme e modular ação bactericida de monócitos, provavelmente por aumento na produção de IL-6.

Palavras-chave: Própolis, Própolis vermelha, flavonoides, *molecular docking*, monócitos, imunomodulação, *Methicillin Resistant Staphylococcus aureus* (MRSA).

Abstract

The goal of this doctoral thesis was to analyze the red propolis antibacterial activity in association or not with antimicrobials on methicillin-resistant Staphylococcus aureus (MRSA), the immunomodulatory action on human monocytes (CD14+) isolated by magnetic beads, and the ability to kill bacteria of monocytes on MRSA. Initially, a literature review was carried out to search the possibility that propolis and its main constituents exert action against MRSA. Next, the action of red propolis in combination or not with β-lactams on MRSA and this mode-action was investigated. Finally, the concentrations of red propolis and antimicrobial that best exerted an in vitro antibacterial were evaluated on monocytes, to observe a possible immunomodulatory effect. The initial review demonstrated that the different types of propolis found on the globe and its main constituents can act in an inhibitory way against several MRSA strains and isolates as well as its main resistant-related molecules. In chapter 2, we present the manuscript from the first phase of this project. We demonstrate that red propolis had low inhibitory concentrations (MIC) for several types of MRSA isolates (MIC90 = 125 μg/mL). The combinations of propolis with imipenem showed synergism by the Ckeckerboard method (FICI = 0.5) and bacterial survival curve with statistical difference between the control group ($p \leq 0.0001$). This same statistical difference was observed when propolis and imipenem were combined with 25% of their MICs in the biofilm formation tests, showing a lower percentage of biofilm growth inhibition than only propolis or imipenem at 75% of their MICs (~47% - 64%). The membrane conductivity percentage of the isolates reached 100% also for this combination. Molecular docking predictions indicated that the free binding energy index of red propolis compounds to penicillin-binding protein 2a (PBP2a) was similar to ceftaroline (kcal/mol = -7.1 to 7.8). The last phase was to evaluate the immunomodulatory action of propolis and imipenem at the same concentrations as in phase I, in human monocytes on MRSA. We observed a down-regulation of TLR-2 excerced by red propolis and an increase in IL-6 production excerced by the combination in treated monocytes. The killling ability of monocytes after treatment with propolis or combination was close to the LPS (~90%). Red propolis in combination with imipenem may be an alternative for the treatment of MRSA by preventing its multiplication, biofilm formation and modulating the killing action of monocytes, probably due to IL-6 up-regulation.

Keywords: Propolis, red propolis, flavonoids, molecular docking, monocytes, immunomodulation, Methicillin Resistant *Staphylococcus aureus* (MRSA).

Introdução e revisão de literatura

Na década de 1920, Alexander Fleming descobriu, no laboratório em que trabalhava, um mofo que secretava um agente antiestafilocócico, com ação inibitória também contra outras bactérias Gram-positivas em placas de Petri (FLEMING, 1929). Foi descoberta então a primeira molécula antibiótica da classe dos β-lactâmicos, a penicilina G (benzilpenicilina), a qual, décadas depois, seria utilizada durante a segunda guerra mundial a fim de evitar e tratar infecções em soldados aliados (MACFARLANE, 1979). Um artigo clássico ilustrou o mecanismo de ação antibacteriana da penicilina, evidenciando seu mecanismo de ação sobre a porção DAla D-Ala no dipeptídeo da célula nascente (TIPPER & STROMINGER 1965). Mais adiante, a proteína ligante de penicilina (PBP – na sigla em inglês) foi evidenciada como o principal alvo de ação da penicilina (GEORGOPAPADAKOU & LIU, 1980). As PBPs são um grupo de enzimas essenciais no *cross-linking* da rede molecular do peptidoglicano da parede bacteriana. A peptidoglicana é uma rede macromolecular que forma a parede bacteriana presente também em combinação com outros componentes. Sendo assim, a atividade bactericida da penicilina se dá através de lise por pressão osmótica (TOMASZ, 1979), uma vez que a bactéria não consegue sintetizar a parede de forma eficiente durante sua divisão celular (FOSTER, 2019).

Após o extensivo uso da penicilina, surgiram os primeiros relatos de *Staphylococcus aureus* resistente à mesma (GOULD, 1958), resistência que se deu através da produção de β-lactamases do tipo penicilinases, que clivam penicilinas e prejudicam seu mecanismo de ação (BUSH, 1989). Iniciaram-se, então, os esforços por penicilinas estáveis à β-lactamases, sendo desenvolvida, como exemplo, a meticilina (KIRBY, 1944).

Após alguns anos, o surgimento do *S. aureus* resistente à meticilina (MRSA – na sigla em inglês) (SAROGLOU *et al*., 1980) veio acompanhado da descoberta de um novo mecanismo de resistência desta espécie: uma PBP modificada em seu sítio ativo para ação do β-lactâmico, a PBP2a (LIM & STRYNADKA, 2002). O β-lactâmico que se liga à PBP2a não consegue exercer seu mecanismo de ação e as PBPs conseguem continuar o *cross-linking* do peptidoglicano normalmente (SHALABY *et al*., 2020). A partir de então, diversas outras classes de antimicrobianos β-lactâmicos têm sido lançadas para o tratamento de *S. aureus* multirresistentes, bem como para outros gêneros de bactérias que possuam β-lactamases e PBP2a (BUSH & BRADFORD, 2016; FISHER & MOBASHERY, 2016).

MRSA são cepas multirresistentes, sensíveis à vancomicina, importantes em infecções hospitalares e que possuem o gene *mecA*, localizado no cassete *SCCmec* (FOSTER, 2019). Este gene codifica a enzima PBP2a (OKWU *et al*., 2019) e é um marcador importante para detecção

desta cepa através de sistemas automatizados como VITEK[(a)] ou métodos laboratoriais padrão ouro (JUNKINS *et al*., 2019). Cepas de MRSA podem ser tanto adquiridas de ambiente hospitalar (HA-MRSA) (CHAVEZ & DECKER, 2008) como adquiridas da comunidade (CA-MRSA) (MILLAR *et al*., 2007), desenvolvendo resistência periódica a novos fármacos que são lançados para seu tratamento (SHALABY *et al*., 2020).

MRSA pode causar endocardite piogênica, pneumonia supurativa, otite-média, infecções de pele, sepse, dentre outros problemas em seres humanos (OTTO, 2013; LAKHUNDI & ZHANG, 2018), além de causar doenças em animais de criação e *pets* (ALGAMMAL *et al*., 2020). Como exemplo, pacientes que passam por cirurgias cardíacas apresentam infecções principalmente por MRSA (ZHOU *et al*., 2020). Além disso, uma cepa CA-MRSA (USA300) foi detectada como principal cepa invasiva em pacientes portadores de diabetes no Irã (TAYEBI *et al*., 2021). Esta cepa é a mais comum em infecções de pele (LAUDERDALE *et al*., 2010), o que pode explicar a sepse nestes pacientes por apresentarem dificuldade na cicatrização (VARGAS *et al*., 2023). Nos últimos anos, foi descoberta uma molécula que pode agir no sítio ativo de PBP2a – a ceftarolina (β-lactâmico cefalosporina) (PARISH & SCHEINFELD, 2008), podendo agir também em um sítio alostérico de PBP2a, abrindo perspectivas para mais um mecanismo de ação na procura de novos agentes antimicrobianos (OTERO *et al*., 2013; GONZALES *et al*., 2015). No entanto, resistência por MRSA foi detectada alguns anos após seu uso (LONG *et al*., 2014), o que evidencia a urgência na procura por novas formulações antimicrobianas.

Produtos naturais são fontes importantes de novos fármacos, como antimicrobianos e outros de interesse na química médica (HARVEY *et al*., 2015). Neste contexto, a própolis é um produto apícola com extenso potencial para desenvolvimento de novos fármacos e formulações (SFORCIN, 2016; EL-GUENDOUZ *et al*., 2019).

A palavra própolis tem origem do grego e significa “em defesa da cidade” (*pro* – em prol de, *polis* – cidade). Trata-se de uma mistura resinosa elaborada por abelhas a partir de matéria prima localizada em partes aéreas de fontes botânicas que estão ao redor da colmeia. Além do material vegetal, as abelhas adicionam cera e secreções de glândulas para amalgamar o material e produzir este produto apícola. A própolis é utilizada na colmeia para vedar frestas, manter de forma indireta a temperatura interna constante através da vedação; e embalsamar invasores mortos que são grandes demais para serem transportados para fora, impedindo sua putrefação e consequente disseminação de infecções no interior do conjunto (TORETI *et al*.,

2013). A ação antimicrobiana da própolis já foi constatada pelo ser humano há milênios e é considerada até hoje como a ação farmacológica mais estudada, apresentando extensa atividade antibacteriana (PRZYBYŁEK & KARPINSKI, 2019), antifúngica (CERQUEIRA *et al*., 2022) e antiviral (RIPARI *et al*., 2021). Nos últimos anos, algumas revisões organizaram a atividade antibacteriana da própolis, demonstrando seu maior efeito em bactérias Gram-positivas do que em Gram-negativas (PRZYBYŁEK & KARPINSKI, 2019; ALMUHAYAWI, 2020), com concentrações de inibição bem variadas, provavelmente devido à sua rica composição química.

Como as abelhas utilizam matéria prima das plantas que estão ao redor das colmeias, a composição química da própolis pode variar entre as diferentes regiões do globo, devido aos diferentes ecossistemas e vegetação característica de cada bioma (SALATINO *et al*., 2011; BANKOVA *et al*., 2014). Assim, há diferentes tipos de própolis encontradas no Brasil, provenientes de diferentes fontes botânicas e normalmente caracterizadas de acordo com sua cor, variando entre marrom, verde e vermelho (SALATINO *et al*., 2005).

A própolis vermelha é típica do manguezal nordestino brasileiro (ALDANA-MEJIA *et al*., 2021), podendo também ser encontrada com composição química e coloração semelhantes em Cuba (RUFATTO *et al*., 2017). Suas principais fontes botânicas são as espécies *Dalbergia ecastaphyllum* e *Syngonium podophyllum*, e apresenta composição química com alguns compostos que não são encontrados com frequência em extratos de outras amostras de própolis como os flavonoides liquiritigenina, isoliquiritigenina e benzofenonas (CCANACCAPATINTA *et al*., 2020; ALDANA-MEJIA *et al*., 2021; MAROOF *et al.*, 2023). Sua cor vermelha deve-se à presença dos compostos retusapurpurinas A e B (CCANA-CCAPATINTA *et al*., 2020).

Tanto o extrato de própolis vermelha como alguns de seus compostos isolados apresentam ações antioxidante, anti-inflamatória e antibacteriana *in vitro* e *in vivo* (BUENOSILVA *et al*., 2013; FREITAS *et al*., 2017; REIS *et al*., 2019; SHI *et al*., 2020), além de ação inibitória *in vitro* contra MRSA e outras cepas de *S. aureus* (SILVA *et al*., 2021). Recentemente, trabalho de nosso grupo demonstrou que a própolis vermelha exerceu ação imunomoduladora sobre monócitos humanos (SANTIAGO *et al*., 2023). Este trabalho demonstrou também que, mesmo em altas concentrações, a própolis vermelha parece não intervir na viabilidade celular, e esta toxicidade seletiva torna-a uma importante fonte para procura de novos agentes antimicrobianos. Além do mais, os componentes que são

encontrados na própolis vermelha, como liquiritigenina, isoliquiritigenina e oblongifolina B já foram avaliados isoladamente, apresentando potencial inibitório contra MRSA (GAUR *et al*., 2016; SILVA *et al*., 2021).

Em 2021, nosso grupo publicou uma revisão sobre a atividade antiviral e imunomoduladora da própolis, compilando resultados que evidenciaram sua ação em células envolvidas na resposta imune, a atividade sobre diversos tipos de vírus, bem como a ausência de efeitos adversos após administração concomitante com medicamentos (RIPARI *et al*., 2021). A revisão publicada por PRZYBYŁEK & KARPINSKI (2019) mencionou que a ação antibacteriana da própolis é mais acentuada em espécies Gram-positivas, sendo o provável mecanismo de ação a mudança do potencial de membrana das bactérias. A mudança neste potencial de membrana indica que a gama de componentes que existem no extrato de própolis podem estar agindo em um ou mais alvos da membrana bacteriana, como as enzimas PBPs e βlactamases. Neste sentido, esta tese de doutorado se inicia com a primeira revisão desenvolvida e publicada por nosso grupo sobre a atividade anti-MRSA da própolis de diversos continentes, onde também comentamos sua atividade imunomoduladora sobre fagócitos e o possível mecanismo de ação antiestafilocócico dos componentes encontrados com maior frequência nas diferentes amostras de própolis (RIPARI *et al*., 2022 – Capítulo 1).

Antimicrobianos β-lactâmicos da classe das cefalosporinas são atualmente as prescrições injetáveis mais utilizadas nos EUA, além de serem extensivamente pesquisados como novos agentes para infecções bacterianas (BUSH & BRADFORD, 2016). Cefalosporinas como ceftarolina e ceftobiprole são efetivas contra infecções por MRSA (LEVENTOGIANNIS *et al*., 2023) que já apresenta resistência a outras cefalosporinas devido às enzimas β-lactamases tipo cefalosporinases produzidas por estas cepas (NUCLEO *et al*., 2018). A procura de antimicrobianos que tenham ação anti-cefalosporinases é uma justificativa válida para estudar o sinergismo de novas fontes naturais com cefalosporinas contra MRSA. Cefoxitina é um exemplo de cefalosporina onde MRSA já é resistente (TAREEN & ZAHRA, 2023), sendo um possível alvo para estudos de novas fontes antimicrobianas em conjunto com esta e outras cefalosporinas.

Outra classe de β-lactâmicos, os carbapenemas, são utilizados frequentemente em infecções nosocomiais (DAVANI *et al*., 2021). Imipenem foi o primeiro carbapenema utilizado clinicamente, a partir da modificação do antibiótico tienamicina (KAHAN *et al*., 1979; BUSH & BRADFORD, 2016). Outro dos poucos membros da classe dos carbapenemas, o ertapenem,

também é uma escolha para infecções bacterianas devido à sua alta estabilidade (CIELECKAPIONTEK *et al*., 2008). Cepas de MRSA não apresentam enzimas β-lactamases do tipo carbapenemases (que clivam carbapenemas) (BUSH & BRADFORD, 2016), o que torna esta classe de β-lactâmicos importante no estudo de novas intervenções anti-MRSA. De acordo com FOSTER (2019), agentes antimicrobianos que agem em componentes da membrana celular em conjunto com β-lactâmicos podem desestabilizar PBP2a, reestabelecendo a sensibilidade de MRSA aos β-lactâmicos, uma vez que expõe outras PBPs sensíveis à ação destes antimicrobianos, tornando a PBP2a o seu "calcanhar de Aquiles". Assim, o estudo do sinergismo da própolis (que apresenta mudança no potencial de membrana bacteriana) juntamente com carbapenemas pode fornecer pistas sobre o mecanismo de ação anti-MRSA de seus componentes bem como propor novas intervenções terapêuticas. Diante destas observações, elaboramos o Capítulo 2 versando sobre a ação anti-MRSA da própolis vermelha isoladamente ou em combinação com antimicrobianos.

Embora o uso de animais em experimentação seja considerado um fator chave no desenvolvimento da Biomedicina, existem barreiras éticas amplamente discutidas para estudos *in vivo* (DOMÍNGUEZ-OLIVA *et al*., 2023). Assim, estudos *in vitro* são importantes para detectar possíveis novos fármacos e minimizar gastos, propondo intervenções mais assertivas nos ensaios pré-clínicos e clínicos (SINENKO *et al*., 2023). Como a antibioticoterapia é sistemicamente distribuída, o estudo da própolis em monócitos humanos é importante para determinar sua possível ação citotóxica e imunomoduladora. Nosso grupo tem estudado a ação imunomoduladora da própolis por décadas, verificando a ausência de citotoxicidade *in vitro*, *in vivo* e em ensaios cínicos (ORSATTI & SFORCIN, 2012; CONTI *et al*., 2016; SANTIAGO *et al*., 2016; SILVA *et al*., 2017; CONTE *et al*., 2021; CARDOSO *et al*., 2022; SANTIAGO *et al*., 2023; TASCA *et al*., 2023). Estes e diversos outros estudos reforçam a toxicidade seletiva da própolis, a qual seguramente pode ser uma fonte de novas moléculas com atividade antimicrobiana e imunomoduladora. Tais atividades podem favorecer a regulação da resposta imune e evitar inflamações exacerbadas, mitigando danos teciduais além de promover a resolução da infecção. Em geral, a própolis verde brasileira foi mais estudada quanto à sua ação imunomoduladora e antibacteriana. Mais recentemente, nosso grupo investigou a ação de diferentes amostras de própolis em monócitos humanos, demonstrando sua ação antiinflamatória e citotóxica contra células tumorais (CONTE *et al*., 2021; PARDO-MORA *et al*., 2021; CARDOSO *et al*., 2022; SANTIAGO *et al*., 2023). Em conjunto, estes dados abrem

perspectivas para o estudo da ação antibacteriana e imunomoduladora da própolis vermelha e seus componentes, investigando se células envolvidas na resposta imune sobreviveriam às concentrações inibitórias para MRSA e se haveria modulação da capacidade de *killing* bacteriano realizado por monócitos, o que está apresentado no Capítulo 3.

Uma vez que extratos de própolis são comercializados mundialmente, ensaios clínicos já foram realizados para avaliar sua ação cicatrizante em infecção bacteriana de pele (ROCHA *et al*., 2022), ação antiviral em pacientes infectados por vírus da herpes (VYNOGRAD *et al*., 2000) e em pacientes HIV positivos (CONTE *et al*., 2021; TASCA *et al*., 2023), dentre outros *clinical trials* revisados por nosso grupo (RIPARI *et al*., 2021). Neste sentido, esta tese de doutoramento foi concebida para analisar a atividade antibacteriana da própolis vermelha em associação ou não com antimicrobianos sobre MRSA, a ação imunomoduladora em monócitos humanos ($CD14^+$) isolados por *beads* magnéticas, e a capacidade de *killing* bacteriano dos monócitos sobre MRSA. Os resultados aqui obtidos podem trazer perspectivas para novas intervenções utilizando própolis vermelha ou seus principais constituintes em ensaios préclínicos e clínicos, no intuito de contribuir com os diversos problemas causados por MRSA e originar patentes com novos agentes antimicrobianos e/ou imunomoduladores.

Objetivos

Objetivo geral

O objetivo deste trabalho foi investigar a ação anti-MRSA da própolis vermelha, sua ação sinérgica com os antimicrobianos β-lactâmicos cefoxitina, imipenem e ertapenem, e sua ação imunomoduladora em monócitos humanos, bem como os possíveis mecanismos de ação envolvidos nestas atividades biológicas.

Objetivos específicos

- Determinar a concentração inibitória (MIC – na sigla em inglês) e bactericida (MBC), do extrato de própolis vermelha (RPE) contra cepas HA-MRSA e CA-MRSA por microdiluição em caldo;
- Determinar MICs e MBCs de cefoxitina (CEFO), imipenem (IMI) e ertapenem (ERTA) contra estas cepas MRSA por microdiluição em caldo;
- Determinar o efeito sinérgico entre RPE e CEFO, IMI ou ERTA pelo método de *checkerboard*;
- Determinar o efeito sinérgico entre RPE e CEFO, IMI ou ERTA por método de curva de sobrevivência bacteriana;
- Determinar a ação antibiofilme de MRSA e combinações, durante a formação do biofilme ou sobre o biofilme já formado;
- Investigar a ação da própolis vermelha e combinações no potencial de condutividade de membrana de cepas MRSA;
- Investigar *in silico* os compostos primordiais da própolis vermelha sobre o sítio ativo de PBP2a por *molecular docking*;
- Analisar o possível efeito citotóxico dos tratamentos em monócitos humanos isolados por *beads* magnéticas pelo método do MTT;
- Investigar o perfil de citocinas pró- e anti-inflamatórias, pelo método de ELISA, produzido por monócitos após tratamento com RPE ou com a combinação que exercer sinergismo contra MRSA;

- Investigar a ação do RPE em monócitos sobre marcadores de membrana responsáveis pelo reconhecimento de antígenos (TLR-2 e TLR-4), pela apresentação de antígenos (HLA-DR) e ativação de linfócitos T (CD80), após tratamento com RPE em combinação ou não contra MRSA;
- Determinar a capacidade de *killing* dos monócitos sobre cepa padrão de MRSA após tratamento com RPE ou com combinação efetiva.

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Capitulo 1 - Can bee propolis help us fight against methicillin-resistant *Staphylococcus aureus* (MRSA)?

Can bee propolis help us fight against methicillin-resistant *Staphylococcus aureus* (MRSA)?

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Abstract

Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common multidrug-resistant pathogen in nosocomial infections. Since the 1950's, MRSA has acquired several resistance factors including efflux pumps and drug target modifications. Some studies have investigated the anti-MRSA capacity of propolis samples collected in different regions and their immunomodulatory action. The aim of this review is to gather the main data published up to August 2022 about propolis action on MRSA strains as well as its modulatory action on phagocytes. **Methods**: The PubMed database was used looking for full articles containing the keywords "propolis", "immunomodulation", "MRSA" and the name of each compound. As propolis contains a variety of compounds making it impossible to isolate the major bioactive components, we also reviewed the main compounds found in several samples of propolis and their possible mechanisms towards the resistance factors displayed by MRSA. Some perspectives for using propolis-based medications and the formulation of new antimicrobial/immunomodulatory agents are discussed. **Key findings/Conclusions:** Propolis extracts and their active compounds exert antibacterial action over diverse MRSA strains acting on resistance factors. Moreover, propolis modulates pro-inflammatory markers in phagocytes. Because propolis compounds may act synergistically, it's crucial to understand how these components interact in order to synthesize standardized formulations and enhance their bioavailability for clinical applications to combat MRSA.

Keywords: MDR-bacteria, phenolic compounds, antibiotic resistance, natural products

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multidrug-resistant (MDR) pathogen commonly associated with nosocomial infections and community infections[1,2].

The emergence of MRSA came shortly after reports of penicillin resistance, when methicillin was extensively used to treat *S. aureus* infections in the 1950's[2]. Ever since, this specie has acquired resistance to several classes of antimicrobials, especially β-lactams, due to a variety of reasons: efflux pumps, wall thickening, changes in colonization status, and antimicrobial modification or modification of antimicrobial targets[3,4]. In fact, the latter is one of the main causes of MRSA resistance to β-lactams, as these drugs act on penicillin binding proteins (PBPs) enzymes, responsible for the cross-linking in the bacterial cell wall's peptidoglycan[1] .

MRSA can express a modified PBP at the β-lactams binding site: the PBP2a, encoded by the *mecA* gene[5]. Antimicrobials that bind to the PBP2a are not effective but the sensitive PBPs continue the cross-link normally, preventing bacterial lysis by osmotic pressure[6]. Besides, MRSA is a bacterial biofilm producer that can prevent the action of antimicrobials[7].

Thus, new antimicrobials are urgently needed to control MDR bacteria and propolis may contribute in this scenario, since some studies have shown its action directly on the pathogen, both in standard strains and in clinical isolates.

Propolis is a resinous product made by *Apis mellifera* bees. Among its numerous functions in the hive, propolis has been used to embalm invading intruders preventing their putrefaction and infections inside the hive due to several natural antimicrobials in its composition, originated from the raw material that bees collect from nearby plants, resulting in a product highly loaded with phenolic compounds[8,9].

Propolis exhibits plenty of pharmacological properties and its antibacterial activity has been studied for decades. Data from literature indicates its potent inhibitory action mainly

against Gram-positive bacteria than Gram-negative ones[10]. Moreover, there are some studies addressing propolis extracts against Gram-positive MDR bacteria[11, 12].

The aim of this mini review is to gather the main data published up to August 2022 about propolis action on MRSA strains. In addition, key-compounds found in the ethanolic extracts of propolis acting on MRSA were also revised. Propolis' modulatory action on phagocytes enhancing their killing status is also reported.

Methodology

The PubMed database was used looking for full articles containing the keywords "propolis", "immunomodulation", "MRSA" and the name of each compound. As propolis contains a variety of compounds making it impossible to isolate the major bioactive components, we also reviewed the isolated compounds found in several samples of propolis and their possible mechanisms towards the resistance factors displayed by MRSA.

Propolis anti-MRSA activity

As the chemical composition of propolis is very complex, extracts are commonly classified by color and raw propolis origin site[32]. However, as bees look for botanical sources closer to the hive, the chemical profile of propolis samples can vary between the regions[33]. This fact explains why the minimum inhibitory concentration (MIC) or minimum bactericidal concentrations (MBC) values against MRSA, change even using the same standard strains (eg. ATCC 43300), indicating that propolis components may exert a greater or lesser activity against MRSA (Table 1).

Sforcin[34] reported that propolis compounds may act synergistically to exert their therapeutic potential. Few years later, Przybyłek and Karpiński[10] proposed some mechanisms related to propolis antibacterial action, reinforcing the idea that its constituents may act together and more efficiently against Gram-positive bacteria. However, further investigation is still needed to understand which molecules exert an anti-MRSA activity. As an example, Silva et al.[12] observed that Brazilian red propolis acted on MRSA catalase *in vivo*. The interaction of propolis compounds and catalase was analyzed *in silico*, verifying that oblongifolin E and xanthochymol presented a strong binding with catalase's active site. Such analisis were feasible because the chemical composition of their propolis extract was well defined.

Many studies have reported the synergistic interaction of antimicrobials and propolis extracts against MRSA, such as gentamicin[13], topical mupirocin[14], vancomycin[15], among others[16]. This synergism occurs mainly with vancomycin and some β-lactams, preventing the resistance of *S. aureus* to vancomycin and restoring MRSA sensitivity to β-lactams. Foster (2019)[1] reported that antimicrobials acting on the wall and membrane of MRSA can destabilize PBP2a, exposing susceptible PBPs to the β-lactams.

Our group has been studying the anti-MRSA activity of propolis alone or in combination with antimicrobials. Performing *in silico* analysis, we observed that the components of red propolis extract (mainly isoliquiritigenin, medicarpin and oblongifolin) interact with the PBP2a active site in an equal or superior manner comparing to ceftaroline - an antimicrobial that can interact with the same site of PBP2a. Furthermore, some β-lactams commonly used in intensive care units may have a higher antimicrobial and antibiofilm capacity against MRSA when combined with the red propolis extract[17].

Besides the action of propolis directly on MRSA, it displays an immunomodulatory action, which may help the host to resolve infections. The next session details the action of the main compounds found in propolis acting directly on MRSA strains and propolis activity on immune cells.

Table 1 shows the anti-MRSA activity of propolis from different geographic regions.

Table 1. Anti-MRSA activity of propolis extracts obtained from different geographic regions ordered chronologically.

Propolis type	**MRSA strain**	**MIC (µg/mL)**	**MBC (µg/mL)**	**Reference**
Turkish propolis	ATCC 33951	1024	ND	14
Brazilian green propolis	ATCC 43300	40	ND	18
Pacific propolis	15 clinical isolates	640 – 1280	ND	19
Argentinian propolis	ND	65	ND	20
South Brazilian propolis	ATCC 33511 and 48 clinical isolates	1.4	ND	21
Four samples from Portugal	ATCC 6538 and 5 isolates,	240 – 1090	ND	22
Special extract GH 2002	ATCC 25923, ATCC 10442 and 10 clinical isolates	130 – 500	500 - 1000	23
Polish propolis	ATCC 43300 and 5 clinical isolates	390 – 780	780 - 3130	16
French poplar propolis	6 clinical isolates	30 – 97	ND	24
Brazilian green propolis	ATCC 43300 and 10 clinical isolates	243.3 - 295.5 (MIC_{90})	ND	25
Three European propolis samples	NCTC 10442	300 – 1600	600 - 1600	26
Three Moroccan propolis samples	ATCC 6538 and 3 isolates	360	980 - 1220	27
Taiwanese green propolis	ATCC 43300	<2	4	28
Southeast Brazilian Brown propolis	ATCC 1708	178.9 (IC50)	ND	29
Australian propolis	ATCC 43300	900	900	30
Brazilian Red propolis	ATCC 43300	31.25	62.5	12
Hungarian propolis	ATCC 700699 and 2 clinical isolates	400	ND	15
Chinese red propolis	ATCC 43300	50	200	31

Brazilian red propolis	ATCC 33951. USA300 and isolates	15.6 – 125	500 - 2000	17

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; ND: not described.

Anti-MRSA action of propolis compounds

Some isolated compounds from propolis extracts have been tested against MRSA (table 2). Usually, ethanolic extracts of propolis contain mainly flavonoids and terpenes among phenolic and acidic compounds[35]. Caffeic acid phenethyl ester (CAPE), apigenin and artepillin C are found in several samples of propolis, especially in the so-called "green" propolis[36]; however, their anti-MRSA mechanisms have not been elucidated yet.

Caffeic and ferulic acids are key compounds found in most of the characterized propolis extracts[37; 38; 39]. Santos et al.[40] reported that caffeic acid may inhibit MRSA efflux pumps and Sundaramoorthy et al.[41] verified that ferulic acid also inhibited the efflux pump in MRSA isolates, more specifically the NorA efflux pump. Kaempferol and rutin also inhibited the efflux pumps [42; 43].

In addition to pumps, standard strains of MRSA exhibit an upregulated *mecA* gene[5]. The *mecA* gene encodes PBP2a, and ferulic acid seems to reduce PBP2a mRNA expression[44]. Amin et al. (2016)[45] observed that luteolin + quercetin exerted a functional variation in *mecA* gene and damage in bacterial cell membrane. Interestingly, quercetin alone increased bacterial membrane permeability and altered membrane potential and membrane fluidity[46].

The production of toxins is another virulence factor displayed by MRSA and susceptible *S. aureus*[47]. Some propolis compounds such as kaempferol and luteolin have been described for blocking α-hemolysin and δ-hemolysin[48; 49] and luteolin and naringenin acted only in α-toxin production[50; 51].

MRSA USA300 strain is the main cause of soft tissue infection[52]. The flavonoid quercetin was efficient against this strain, reducing the thermal stability of casein hydrolase P (ClpP) [53; 54].

Recently, a new type of propolis has been investigated against MRSA: the red one. Brazilian red propolis exerted an efficient action on MRSA strains and isolates. Oblongifolin E and xanthochymol probably acted on catalase[12], and oblongifolin B, guttiferone E and isoliquiritigenin acted on PBP2a[17].

Figure 1 shows the possible mechanisms of action involved in the anti-MRSA capacity of propolis compounds.

Table 2. The anti-MRSA action of the main phenolic compounds found in propolis extracts.

Compound	Strain	Mechanism of action	Reference
Apigenin + lysine	USA300	ND	55
Apigenin	USA300	ND	56
Artepillin C	Isolates	ND	24
CAPE	Isolate	ND	57
CAPE	ATCC 43300	ND	58
Caffeic acid	Isolates	ND	59
Caffeic acid	RN4220	↓ efflux pumps	40

Caffeic acid	ATCC 43300	ND	60
Ferulic acid	ATCC 33951 and 33593	↓ PBP2a mRNA expression	44
Ferulic acid	Isolate	↓ NorA efflux	41
Galangin	ATCC 6538	ND	61
Galangin	Isolates	ND	62
Hydroxycinnamic acids	isolates and KCCM 40510/ KCCM 40511	ND	63
Kaempferol	Clinical isolates	↓ β-lactamase	42
3-O-alpha-L-(2',3'-di-p-coumaroyl)rhamnoside (KCR)	Clinical isolates	ND	64
Kaempferol-derived	N315 and NCTC 10442	ND	65
Kaempferol	ATCC 29213	ND	66
Kaempferol	USA300	↓ α-hemolysin	49
Luteolin or apigenin	Isolates	ND	67
Luteolin	USA300 and USA400	↓ α-toxin	50
Polyluteolin	ATCC 43300	ND	68
Luteolin	ATCC 33591 and clinical isolates	ND	69
Luteolin + quercetin	ATCC 43300	Variation in *mecA* gene and Damage in cell membrane	45
Luteolin	MRSA N315	↓ α-hemolysin, δ-hemolysin, and hlaA levels	48
Liquitirigenin and isoliquitirigenin	Isolates	ND	70
Naringenin	ATCC 10832	↓ α-toxin	51
Naringenin-derivatives	Isolates	ND	71
Oblongifolin E and xantochymol	ATCC 43300 and isolates	↓ catalase	12
Oblongifolin, guttiferone E and isoliquiritigenin	ATCC 33951, USA300 and isolates	↓ PBP2a binding	17

Pinobanksin-3-cinnamate	ATCC 43300	ND	72
Quercetin	USA300	↓ central carbon metabolism and glutamine biosynthesis	53
Quercetin	MRSA T144	↑ bacterial membrane permeability and changes in membrane potential and fluidity	46
Quercetin	USA300	Binding to casein hydrolase P (ClpP) and reducing theClpP thermal stability	54
Quercetin	ATCC 43300	ND	73
Rutin	MRSA-274819	↓ efflux pumps	43
Rutin	IBRS MRSA 011	ND	74

ND: not described. ↓ : inhibition or reduction; ↑ : increase.

Immunomodulatory action of propolis

The direct action of propolis on MRSA has already been studied and described in the previous section. One may speculate that propolis may exert an indirect effect on MRSA strains by stimulating the immune cells to fight against them.

Propolis action on immune cells isolated from rats, mice and humans has been well documented *in vivo*[75; 34]; however, studies assessing MRSA coculture with immune cells are rare.

Most of the articles dealing with the immunomodulatory action of propolis *in vivo* employed murine peritoneal macrophages[76], RAW 264.7 cells[78; 79; 80], and J774A.1 macrophages [81; 82]. Other studies have adopted macrophages derived from the mouse's bone marrow[83], human dendritic cells[84] and rabbit neutrophils[85]. *In vivo* studies using human peripheral blood mononuclear cells (PBMC) have also been performed [86; 87; 88; 89]. *In vivo*, the main models employed male Wistar rats[80] and BALB/c mice[76].

A key point of the immunomodulatory action of propolis is the stimulation of the Toll-like receptors (TLR) expression and, consequently, the production of proinflammatory cytokines[76]. In an experimental model incubating murine macrophages and splenic cells with Brazilian propolis, an increased expression of TLR-2 and TLR-4 was observed, what may lead to the production of pro-inflammatory cytokines IL-1β and IL-6[77], which in turn may enhance the processing and presentation of antigens to T lymphocytes, connecting innate and adaptive immunity.

In contrast to the pro-inflammatory action, an anti-inflammatory activity may also be observed using a propolis sample from the temperate zone. Macrophages from the bone marrow of rats were isolated and the anti-inflammatory action of UK propolis was characterized by two mechanisms: suppressing the secretion of IL1β and IL-6 release while it was less effective in TNF-alpha and metabolic reprogramming of macrophage activity stimulated with LPS[83].

Besides analyzing propolis immunomodulatory action, no toxicity was observed after incubating human or murine cells with propolis from Brazil. No toxicity was seen in propolis-treated mice, rats or rabbits as well[85], demonstrating the safety of propolis during the assays[90].

These findings corroborate the statement that propolis has a remarkable immunomodulatory action which occurs by different pathways; however, there is still a lack of studies seeking the individualized action of compounds in the immune cells.

Table 3 shows propolis effects on different immune cells.

Table 3. Propolis effects on immune cells.

Propolis Type	Animal and cell line	Mechanism of action	Reference
Brazilian green propolis	Rabbit neutrophils (PMNs)	↓ ROS production	85
Southeast Brazilian propolis	Male BALB/c mice	↓ IFN-γ production	76
Propolis	Male BALB/c mice (macrophages and spleen cells).	↑ TLRs expression and the production of pro-inflammatory cytokines	77
Brazilian green propolis	Human peripheral blood mononuclear cells	↑ CD14+ and ↑ mitochondrial activity	86
Brazilian green propolis	J774A.1 macrophages	↓ ROS, RNS, cytokine IL-1 α , IL-1 β , IL-4, IL-6, IL-12p40, IL-13, TNF- α , G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1 β, and RANTES production	81
Brazilian green propolis	MG6	↓ NF-κB activation in microglia	91
Artepillin C (Brazilian green propolis)	RAW264.7 macrophages	↓ ROS, RNS, IL-1β, IL-3, IL-4, IL-5, IL-9, IL-12p40, IL-13, IL-17, TNF-α, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1 β , RANTES, and KC production , and blocking the expression of NF-κB	78
Brazilian green propolis	Human peripheral blood mononuclear cells	↑ TLR-4 and CD80 expression. TNF-α and IL-10 production was affected depending on propolis concentration	87
Propolis from Sonora State-Mexico	Human peripheral blood mononuclear cells	↓ TNF-α and ↑ IL-10	88
Brazilian green propolis	Human Dendritic cells	↑ NF-KB, TNF-α, IL-6 and IL-10	84
Polur ethanol extract of propolis	RAW 264.7	↓ NOX and ROS; ↓ COX-2, IL-1$\boldsymbol{\beta}$ and IL-6 expression.	92

Chinese propolis (EECP)	RAW 264.7	Accelerating nucleus translocation to activate p38/p-p38 and Erk/p-Erk kinase.	79
Eucalyptus propolis (EEEP) and Baccharis propolis (EEBGP)	RAW 264.7	↑ p38/p-p38 gene expression via Erk/p-Erk kinase-Nrf2 signaling pathway	79
Poplar propolis	Human PBMC	↑ IL-6 and IL-1β	89
Brazilian red propolis	Human embryonic kidney cells	↑ Nrf2 that regulates the antioxidant gene	90
UK propolis	Mouse bone marrow-derived macrophages	↓ IL-6 and IL-1β and metabolic reprogramming of the LPS's activity in macrophages	83
Brazilian red propolis	Mice liver cells	Activation of Nrf2- ARE pathway	90
Self-nano emulsifying drug delivery system (SNEDDS) of propolis extract	RAW 264.7 macrophage-like transformed cell line	↑ NO	80
Self-nano emulsifying drug delivery system (SNEDDS) of propolis extract	Adults male Wistar rats	Better absorbing of the compost	80
Senegalese propolis	Murine J774.1 macrophage cells	↓ LPS-induced expression of inducible iNOS	82

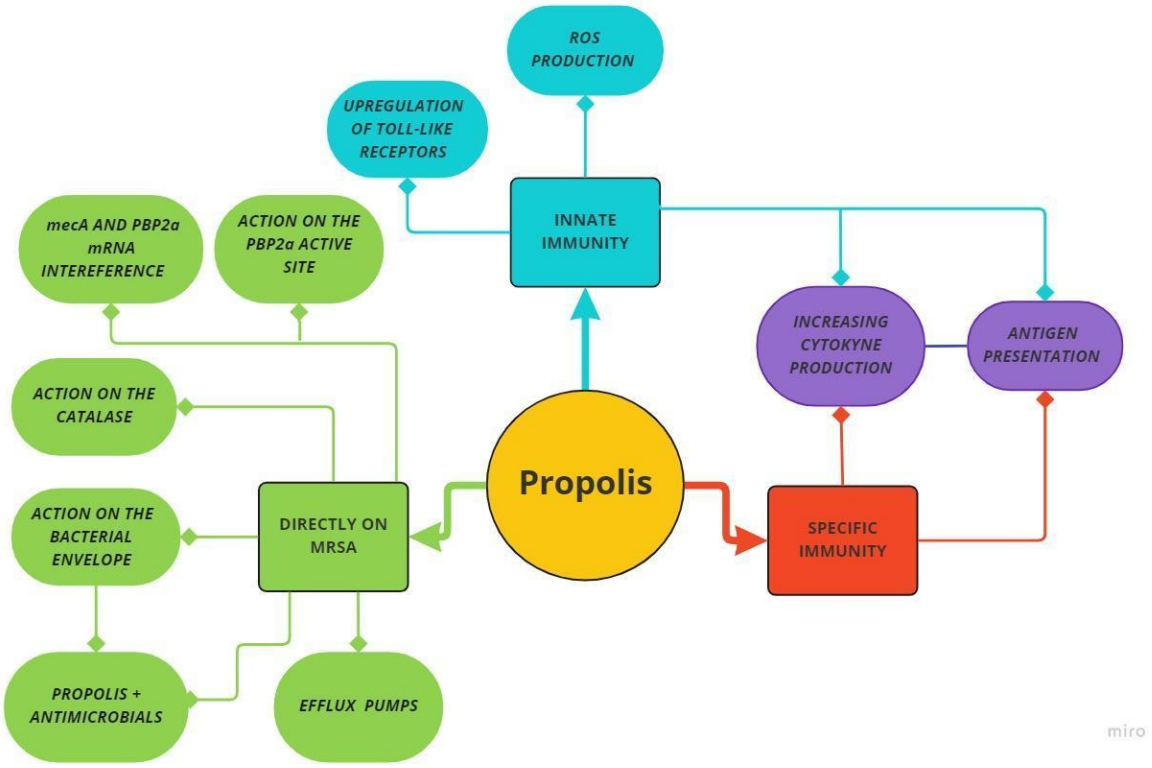

Figure 1. Main anti-MRSA mechanisms exerted by propolis.

Conclusions and future perspectives

The anti-MRSA activity of propolis and its components indicates that this bee-product can help us fight against this pathogen, expanding the current possibility of therapeutic approaches.

In hospital daily situations, propolis administration to MRSA-infected patients can be difficult, although a clinical trial administering green propolis (5% - twice a day) associated with the conventional therapy for diabetic patients with foot ulcers revealed an improvement of wound healing[96]. In diabetics patients, MRSA is one of the main pathogens that impairs wound care[97]. In addition, other clinical trials showed the effect of propolis on wound healing[98], which indicates that propolis has a huge potential against MRSA in clinical/practical situations. Besides, a clinical trial has revealed that the intake of dry propolis capsules to hospitalized COVID-19 patients reduced their hospital stay[93]. However, the major issue with natural compounds isolated from propolis is their polarity and solubility and a possible solution to overcome this problem is the development of microemulsions or coupling phenolic compounds in lipid-based encapsulations[94] and other modifications to improve the absorption and bioavailability of these compounds at the site of infection. Formulating propolis-based products is a feasible approach to increase the safety and standardization of these compounds.

A major challenge to understanding how propolis exerts an antibacterial activity is its rich chemical composition with no major bioactive component. Thus, the *in silico* molecular docking analysis can be a useful tool to advance in understanding propolis effects. It is worth mentioning the promising tools to achieve this goal such as proteomic analysis[95]. Understanding the different targets of its distinct compounds can lead to new pharmaceutical formulations such as

ointments and antimicrobials/immunomodulators. New propolis-based medications may work alone or in combination with antimicrobial drugs to combat MRSA or other bacteria with

resistance factors and biofilm production, being the latter a great challenge to treat infections[4]. The combination of antimicrobials and the ability of propolis to prevent MRSA biofilm formation may help their action on bacterial cells.

Further studies are needed to identify bioactive propolis compounds and propose the main mechanisms involved in the antibacterial action, to understand by which ways propolis extracts may act on MRSA. Up to now, extracts from different continents act on the bacterial envelope and alter the membrane potential of the bacteria. Regarding propolis compounds, the phenolics, found in most extracts, inhibit efflux pumps, β-lactamases, and alter toxins, *mecA* gene and mRNA of PBP2a. In addition, some compounds found mostly in Brazilian red propolis interact with catalase and PBP2a enzymes and this may be the first clue to produce formulations that mimic the interaction between propolis compounds, in an attempt to standardize the concentration of their phenolic compounds in a given medicine.

In addition to the direct action over MRSA cells, propolis immunomodulatory action can also help the host's immune response; however, further investigation is still needed to assess which components may act on the bacteria and on the immune cells.

Humanity has benefited from antimicrobials easily soluble and absorbed by the human body. Bacterial resistance has emerged and now we have to focus on phenolic compounds, which are promising new antimicrobials. It's up to us to understand the natural synergism of propolis constituents to obtain standardized formulations that can help fighting against MRSA.

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Capitulo 2 - Brazilian red propolis and β-lactams exerts an efficient antibacterial action over methicillin-resistant *Staphylococcus aureus* (MRSA) strains

Brazilian red propolis and β-lactams exerts an efficient antibacterial action over methicillin-resistant *Staphylococcus aureus* (MRSA) strains

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Abstract

Aims: The antibacterial activity of red propolis extract (RPE) and brown propolis extracts (BPE) and its synergistic effect with cefoxitin (CEFO), imipenem (IMI) and ertapenem (ERTA) was evaluated *in vitro* against methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

Methods: MRSA ATCC 33591, community-associated (CA-MRSA) USA300, and four clinical isolates were used. Broth microdilution assay was performed to obtain inhibitory and bactericidal concentrations of propolis, CEFO, IMI and ERTA. Propolis in combination with antimicrobials was evaluated on the formation or eradication of biofilm. The bacterial relative membrane conductivity of the strains was assessed after RPE and combinations exposition. Surface/binding computational analyses between RPE compounds and PBP2a were performed.

Results: BPE samples had low activity against MRSA (MICs 3.2 to 5 $g.l^{-1}$; MBCs 10 to 15 $g.l^{-1}$), so the subsequent assays were carried out only with RPE and antimicrobials. RPE exerted a bacteriostatic action (MICs 0.0156 to 0.125 $g.l^{-1}$; MBCs 0.5 to 2 $g.l^{-1}$) but the combinations with IMI and ERTA showed the highest inhibitions, observing in the time-kill curve. However, the FICI index showed synergism (≥ 0.5) only to the RPE+IMI combination. The RPE+IMI was the most effective combination in the inhibition of the biofilm, showing the highest values of membrane conductivity, too. Computational predictions indicated that RPE constituents may interact with PBP2a.

Conclusions: RPE and RPE+IMI exerted an antibacterial and antibiofilm activity on MRSA strains probably due to membrane/wall damage and interactions with PBP2a.

KEYWORDS:

Propolis; MDR-bacteria; β-lactam antibiotics; biofilm; PBP2a

Introduction

Multidrug resistant (MDR) bacteria are resistant to several antimicrobials by diverse mechanisms and included in the so-called "ESKAPE" pathogens – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*. (Kang et al. 2020).

Some MDR *S. aureus* strains are called methicillin-resistant *S. aureus* (MRSA) due to the intense use of methicillin in the 1950s, leading to resistant strains to this antimicrobial agent (Lakhundi and Zhang 2018). MRSA is involved in hospital infections, causing a high mortality rate, extended stay, and higher public health costs (Morrisette et al. 2020). In addition, MRSA is one of the most common infections in the first year after heart transplantation (Zhou et al. 2020).

MRSA presents the *mecA* gene, which is related to resistance to most β-lactam antimicrobials (Okwu et al. 2019) for encoding a penicillin-binding protein (PBP), the PBP2a (Foster, 2019) that confers broad clinical resistance to the β-lactams (Shalaby et al. 2020). In addition, this gene is found in MRSA biofilm-producer clones (Vergara et al. 2017), which is considered an important virulence factor for the ongoing infection and failure in antibiotic therapy (Kranjec et al. 2021). In Brazil, some "pandemic clones" of MRSA can transfer *mecA* among other resistance genes and have become endemic in the population (Andrade et al. 2020).

MRSA can cause pyogenic endocarditis in humans as well as suppurative pneumonia, otitis, and sepsis. In addition to humans, MRSA can cause disease in pets and livestock (Algammal et al. 2020), increasing the risk of acquiring community infections.

Thus, new approaches to combat and control MRSA have been urgently researched. A recent review has reported that ethanolic extracts of several plants have been shown to be effective against MRSA *in vitro* (Okwu et al. 2019), revealing the sensitivity of MRSA to phenolic compounds. Such compounds are abundant in propolis extract, which is a bee product marketed for the prevention/treatment of many diseases.

Propolis is a resinous product made by bees from the material collected from plants around the hive. Propolis color varies from brown, green, and red, depending on its botanical origin (Salatino et al. 2005). Bees use it to protect the hive from rain and humidity, keeping the internal temperature constant, embalming dead intruders that cannot be removed, and maintaining an antiseptic environment within the hive (Toreti et al. 2013). However, propolis chemical composition depends on the geographic region and botanical source (Bankova et al. 2014).

Dalbergia ecastophyllum (L.) Taub. (Fabaceae) has been reported as the primary botanical source of red propolis produced in northeast Brazil. The compounds present in this plant and red propolis extract (e.g. isoliquiritigenin and vestitol) have been described for their antioxidant, anti-inflammatory, and antibacterial activity (Bueno-Silva et al. 2013; Shi et al. 2020). Its red color is due to the presence of isoflavans retusapurpurins A and B (Freires et al. 2016; Ccana-Ccapatinta et al. 2020).

The antibacterial action of several propolis samples produced worldwide has been well documented (Sforcin et al. 2000; Nazeri et al. 2019; Przybyłek and Karpiński 2019; Almuhayawi, 2020). In general, propolis is more efficient against Gram-positives than Gram-negative ones, probably due to bacterial wall damage (Przybyłek and Karpiński 2019). Notably, antimicrobials interfering in Gram-positive bacteria wall components are promising to be combined with β-lactams against MRSA, because they interfere in PBP2a stability by inhibiting functional membrane microdomains (Foster 2019) and may reverse the resistance. In this sense, many propolis extracts (not characterized as "red propolis") in combination with *β*-lactams and other antimicrobials presents synergistic or additives effects against MRSA (Fernandes Júnior et al., 2005; Grecka et al., 2019) mainly with antimicrobials that act in cell wall synthesis (Al-Ani et al., 2018). Currently, *β*-lactams are used in nosocomial infections, such as those of the cephalosporin class - used in MRSA infections - and carbapenems, used against several MDR bacteria. Cefoxitin (CEFO) is a cephalosporin used for the treatment of MRSA with immunomodulatory effect (Eld et al., 2021). Carbapenems such as imipenem (IMI) and ertapenem (ERTA) are broad-spectrum antimicrobials and are used against a variety of

nosocomial infections and synergize with other drug classes in infections by MRSA (Totsuka et al., 1999; Anderson, 2006; Davani et al., 2021).

Studies of anti-MRSA activity of red propolis alone or in combination with β-lactams are scarce. Recently, Zhang et al. (2022) showed an activity of a “Chinese red propolis” over MRSA. Therefore, our study aimed to evaluate the antibacterial activity of Brazilian red propolis extract (RPE) and brown propolis extracts (BPE). The most promising extract (RPE) was evaluated in combination with lower concentrations of CEFO, IMI and ERTA over different MRSA strains *in vitro*. The antibacterial, bacterial membrane permeability and antibiofilm activity of these combinations was assessed as well. Additionally, surface/binding computational analysis were performed between PBP2a enzyme active site and RPE compounds.

Material and methods

MRSA strains

Four MRSA strains were provided by the Clinical Hospital, Botucatu Medical School, UNESP (H44, H55, H97, and H119). These strains were obtained from patients under intensive care and their identification was confirmed by the VITEK systems and PCR for the *mecA* gene. In addition, two standard strains were used: community-associated strain, CA-MRSA USA300 (Lauderdale et al., 2010) and MRSA of the American Type Culture Collection (ATCC 33951 – *Staphylococcus aureus* subsp. *aureus* Rosenbach) provided by the Microbiology/Immunology Sector, UNESP. *S. aureus* ATCC 29215 was used as a control.

Cefoxitin (CEFO), Imipenem (IMI) and ertapenem (ERTA) preparation

Cefoxitin, Imipenem (seccondary pharmaceutical standard) and ertapenem (97% HPLC) were obtained from Sigma-Adrich Company and stock solutions aliquots of 10 $g.l^{-1}$ (CEFO), 2.5 $g.l^{-1}$ (IMI) and 1 $g.l^{-1}$ (ERTA) in sterile distilled water were stored (4°C, -20 °C and -80 °C,

respectively), according to the manufacturer's recommendation. Before the tests, these stock solutions were diluted 1:10 in BHI broth or MHB culture medium. BHI broth was chosen because all MRSA strains grew in the assay control-well what didn't in MHB medium.

Brown and red propolis origin and extraction

Brown propolis samples were collected in tree Brazilian cities: Luiz Antônio (São Paulo State-southeast Brazil), where *Pinus* spp. is the predominant plant source; Cabo Verde (Minas Gerais State – southeast) whose main vegetal source is *Morus alba*, and brown propolis from União da Vitória (Paraná State – south of Brazil), produced from *Araucaria*. Each sample of brown propolis provided a different extract (BPE-pi, BPE-ma, and BPE-ar, respectively). Red propolis was collected in an apiary located in Canavieiras city, Bahia State, northeast Brazil, produced by *D. ecastophyllum*.

Red and brown propolis extracts were obtained in hydroalcoholic solution (7:3), followed by percolation. Extracts were filtered and evaporated in a rotary evaporator and lyophilized furnishing the crude hydroalcoholic extracts. These crude hydroalcoholic extract were used in antimicrobial tests.

For chemical analyses, hydroalcoholic RPE were partitioned in organic solvents, providing five fractions (hexane, dichloromethane, ethyl acetate, butanol, and methanol/water). The organic and aqueous solvents fractions were analyzed by comparative thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Then, the organic and aqueous fractions were subjected to different chromatographic modalities: classic column chromatography, liquid vacuum chromatography, circular chromatography (Cromatotron®), and column flash chromatography, using different adsorbents (Silica Gel, Sephadex LH 20, C18) and various mobile phases: hexane, ethyl acetate, dichloromethane, butanol, methanol, water, and acetic acid. The obtained sub-fractions were monitored by TLC, analytical HPLC, and gas-phase chromatography. The isolation and purification of the metabolites were achieved

by more refined chromatographic techniques, such as counter-current chromatography (HSCC) using different solvent systems and HPLC on an analytical or semi-prepared scale, considering the polarity of the components in the samples.

Lyophilized hydroalcoholic RPE (40 $g.l^{-1}$), BPE-pi (40 $g.l^{-1}$) and BPE-ar (60 $g.l^{-1}$) were resuspended in DMSO. BPE-ma was resuspended in Tween 20 at 50 $g.l^{-1}$. Before the microdilution tests (see section below), BPE-pi and BPE-ar stock solutions were diluted 1:1 in broth. No dilution was done before the tests with BPE-ma. DMSO cytotoxicity concentrations for MRSA strains ranged from 25% to 50%.

Kyrbi Bauer antibiogram for Gram-positive bacteria

Petri dishes (150 × 15 mm) were prepared with Mueller Hinton agar. Each MRSA strain was adjusted to the 0.5 MacFarland scale (1.5×10^8 colony-forming units, CFU/mL) and spread on the plates with a sterile swab in six directions. After, 12 antimicrobial disks (CEFAR - MULTIFAR) were inserted in the plate and incubated for 24 h at 35 °C for further measuring the inhibition diameters. The twelve antimicrobials used were penicillin (PEN, 10 IU), oxacillin (OXA, 1 µg), levofloxacin (LVX 5 µg), rifampicin (RIF, 5 µg), chloramphenicol (CLO, 30 µg), vancomycin (VAN, 30 µg), clindamycin (CLI, 2 µg), erythromycin (ERI, 15 µg), sulfazotrin (SUT, 25 µg), tetracycline (TET, 30 µg), gentamicin (GEN, 10 µg) and ciprofloxacin (CIP, 5 µg). Interpretation was done according to the CEFAR's manual (CLSI, 2019) for establishing resistance (R) intermediary resistance (I) and sensitivity (S).

Minimum bactericidal and inhibitory concentrations of RPE, IMI and ERTA (MBC and MIC)

The broth microdilution method was performed according to the CLSI (2015) recommendations using the MHB and BHI broth medium at 200 µL of final volume/well. Initially, each strain was spread on MHA and stored at 37 °C/20 h. After, RPE and antimicrobials were diluted (1:10),

preparing two-fold serial dilutions in a sterile 96-well plate (volume/well = 100 µL). Colonies of each strain were mixed in sterile saline solution (0.9%) until reaching the turbidity of the 0.5 MacFarland scale, making 1:100 dilutions in broth (~10^6 colony-forming units - CFU/mL). Then, 100 µL was inoculated in each well (final volume/well = 200 µL). The number of colonies (~ 1 to 5 10^5 CFU/mL) was confirmed by counting the CFU after inoculating bacteria in the control well using the Pour Plate method.

After 20 h / 37°C, 10 µL of each well were spread on MHA or BHI agar to observe the colony growth and determine the minimum bactericidal concentration (MBC). Immediately after this inoculation, 50 µL of resazurin aqueous solution (0.001%) were added to the wells and the minimum inhibitory concentration (MIC) was obtained.

Checkerboard assay

In order to obtain quantitative synergism results, strains ATCC 33951 and USA300 were chosen for checkerboard testing according to Pillai et al. (2005). CEFO, IMI or ERTA antimicrobials underwent serial dilution (1:1) in 96-well plates at the horizontal end H of well 8 (MIC x 4) to well number 1. Likewise, RPE underwent the same serial dilution at the vertical end of the plate, from top to bottom. Thus, serial cross dilution was performed in the other wells. After inoculation (0.5 Mc Farland 1:100) and culture (24 h/37 °C), 50 µL of aqueous rezasurin standard solution were introduced to calculate the fractional inhibitory concentration index (FICI) = A / MIC_A + B / MICB.

The MICA and MICB refer to RPE or antimicrobial MICs separately. “A” and “B” are MICs observed when antimicrobials are together. FICI ≤ 0.5 reveals synergism, while FICI between 0.51 and 1.5 was considered as an additive effect. FICI ≥ 4 indicates antagonism.

Time-kill curve (survival curve)

Four tubes were inoculated with the MIC of RPE (Tube 1), CEFO, IMI, or ERTA (Tube 2), a combination of CEFO, IMI or ERTA plus RPE using 25% of their MICs (Tube 3), and a growth control tube (Tube 4). Immediately after inoculation, a 96-well plate was prepared with the tube's content and incubated at 37 °C for 24 h in an EPOCH 2-plate reader (Biotek, USA). Automatic measurements were taken every 2 h at 600 nm for 24 h.

Antibiofilm assays

RPE antibiofilm activity was assessed according to Stepanovic et al. (2007) and Goodwine et al. (2019) with few modifications. The 96-well plates containing 100 µL TSB (1% glucose) were used in the presence or not of RPE, CEFO, IMI, ERTA (MIC 25%, MIC 50%, MIC 75%) or combinations using the MIC 25% of RPE plus MIC 25% of antimicrobials. The 0.5 MacFarland scale was obtained using a calibrated spectrophotometer with TSB 1% glucose (O.D. 625nm = 0.08 to 0.1). Each well was inoculated with these cultures (100 µL of 0.5 MacFarland) in static incubation (37 ºC/48 h). After, wells were washed three times with 200 µL of sterile phosphate-buffered saline (PBS – pH 7.4) and the plate was dried at room temperature/4 h. The formed biofilm at the wells' bottom was fixed with 150 µL of methanol for 20 min. After overnight drying at room temperature, the biofilm was stained with 150 µL of violet crystal (1%) for 15 min. The plate was washed under running water (recommended for Staphylococci) to remove the dye excess. The stained biofilms were suspended in 33% acetic acid (150 µL) before 3x3 well-reading at 570 nm in the EPOCH-2 plate reader. The biofilm formation was determined using the formula: % inhibition of biofilm formation = 100 – [(mean of the treated culture / mean of control) × 100].

Two hundred microliters of the inoculum (0.5 MacFarland TSB 1% glucose) were dispensed in each well of a flat-bottom 96-well plate to evaluate the effects of RPE on the biofilm already formed. After static incubation (37 ºC/24 h), planktonic cells were gently removed, and the plate was washed three times with PBS. Then, each well received 200 µL of BHI-containing concentrations of RPE or combinations (ranging from the value of MIC × 1 to MIC × 20) and

200 µL of BHI for the control wells. Plates were read at 600 nm (3 x 3 read), incubated for 48 h/37 ºC, and then performed a new reading at the same wavelength.

Samples of the biofilms were collected from the bottom with a loop and further spread on BHI agar plates. After incubation for 48 h/37 ºC, the bactericidal biofilm concentration was determined as the lowest concentration where no growth was seen on the BHI agar plates.

Cell membrane permeability

According to Diao et al. (2014), the bacterial membrane permeability was determined and expressed as relative electrical conductivity (%). After incubation (37ºC / 20h), the strains were centrifuged at 5000 rpm / 10 minutes. The supernatant was discarded, and the cells were suspended with aqueous glucose solution (5%) until their electrical conductivity was near the electrical conductivity of glucose solution without bacteria. RPE, IMI, and combinations were added to 5% glucose solution containing bacteria, using the MICs or combinations at 25% of their MICs. The MBC was also tested for RPE. The electrical conductivity values of these solutions were assumed as L1. The same procedure was done with the MRSA-washed glucose solution without RPE or antimicrobials, and these values were assumed as L2. All the electric conductivity measurements from L1 and L2 were performed at 0 h and 4 h-37°C. The control was obtained by the electrical conductivity of MRSA glucose solution without antibiotics boiled in a water bath for 5 min at 100°C (considered as L0).

The percentage (%) of relative electrical conductivity was obtained using the formula:

$$\text{Conductivity (\%)} = [(L2 - L1) / L0] \times 100.$$

Surface/binding computational analyzes

In order to analyze possible interactions of RPE compounds and PBP2a, docking calculations between the active site of PBP2a and each RPE compound alone were performed using the AutoDock Vina software. The three-dimensional structure of PBP2a was obtained from the Protein Data Bank (PDB-1MWT) and the chemical structures of RPE constituents were obtained from the PubChem database (pubchem.ncbi.nlm.nih.gov): liquitirigenin (CID 114829),

isoliquiritigenin (CID 638278), vestitol (CID 92503), neovestitol (CID 44257510), medicarpine (CID 336327), *o*-methylvestitol (CID 44446856), gutiferone A (CID 5352090) and oblongifolin A (CID 11570735). Controls were made with ceftaroline (CID 9852981) and methicillin (101320272) antimicrobials. The active site of PBP2a was defined by protein visualization using the UCSF Chimera software (University of California, San Francisco, CA, USA) delimiting a geometric cube around the active site of PBP2a. The x, y, and z coordinates for the center (19.1502, 21.6499, and 86.4755, respectively) and size (44.1608, 55.816, and 35.8297, respectively) of PBP2a were delimited to determine the lowest free binding energy (Kcal/mol).

Statistical analysis

Multiple analyses of variance (one way-ANOVA) followed by Tukey's test were performed using GraphPad Prism 9.0 software ($p < 0.05$).

Results

RPE chemical composition

The main phenolic compounds liquiritegenin (1) and isoliquiritigenin (2) flavones are usually found in plants of the Fabaceae family (Figure 1). Vestitol (3), neovestitol (4), medicarpine (5), and *0*-methylvestitol (6) were flavonoids usually extracted from the Brazilian red propolis. Guttiferone E (7) is a benzophenone and oblongifolin B (8) belongs to the polycyclic polyprenylated acylphloroglucinols. These constituents are also found in plant species from the Fabaceae family. All compounds shown in Figure 1 were found in *D. ecastophyllum*, the primary botanical source of red propolis.

Antibiogram for MRSA strains

Results of sensitivity (S), intermediated resistance (I) and resistance (R) profile of MRSA strains according to the CEFAR manual for interpreting disk antibiogram techniques (CLSI, 2019) are shown in Table 1.

MIC and MBC of RPE, IMI and ERTA

All strains were subjected to broth microdilution assays to determine the minimum inhibitory and bactericidal concentration (MIC and MBC). We used two culture media to determine MICs: Mueller Hinton Broth (MHB) and brain heart infusion (BHI). CLSI recommendations call for determine MICs using MHB. However, our strain ATCC 33951 and isolates H44 and H119 did not show bacterial growth in the growth control well (GC). When BHI were used, all strains were grown in GC wells. Therefore, we decided to follow up the study with BHI. USA300 strain and isolates that grew on MHB showed an inhibition range similar to that observed using BHI.

BPE extracts showed MICs and MBCs ranging from 3.2 to 15 $g.l^{-1}$ for ATCC strain (supplementary Tables 1 and 2). Thus, BPE extracts were not used in the subsequent assays.

IMI showed a slight variation in MICs values for the strains H55, H97, H119, and USA300. On the other hand, the MICs for the strains H44 and ATCC 33951 varied from 0.0312 $g.l^{-1}$ to 0.250 $g.l^{-1}$ (Table 2).

MIC and MBC values were obtained in three independent assays. The lowest MICs obtained were considered to calculate the concentrations (MIC 25%, 50% and 75%) of the subsequent assays.

Synergistic action of RPE with IMI or ERTA

As shown in Table 4, RPE showed a synergistic effect with IMI only, although it showed an additive effect (FICI = 0.6) with ERTA. The combination of RPE + CEFO showed an indifferent effect (FICI = 3).

These results were confirmed by survival curve, where the ATCC 33951 strain was significantly inhibited at 24h at the RPE + IMI combination (Figure 2). The combination RPE + ERTA inhibited only a fraction of the bacterial growth / 24h, although there is a statistical difference in relation to the GC (Figure 3). Finally, the combination RPE + CEFO was not able to inhibit MRSA growth / 24h (Figure 4).

Thus, USA300 strain and isolates were combined with IMI or ERTA to obtain survival curve results and graphs are shown in the supplemental material (Supplementary Figure 1).

Antibiofilm activity

All strains exhibited a decreased biofilm formation by optical density (575 nm) using MIC 75% or MIC 50% for RPE (Fig. 5A), IMI (Fig. 5B) or ERTA (Fig. 5C), but not using CEFO (Fig. 4D). Lower growth was seen after incubating the strains with the combinations using 25% of each MIC compared to control (Fig. 6).

Although the best antibiofilm results for antimicrobials alone were obtained using IMI and ERTA concentrations (Fig. 5B and C), the most prominent inhibitory action on the biofilm was obtained with the combination RPE + IMI (Fig. 6).

The combination RPE + IMI was as effective as RPE or IMI only, especially for the strains USA300 and ATCC 33951.

RPE + ERTA were also effective against biofilm formation for the strains H97, H119, and USA300 compared to RPE or ERTA alone. This combination exerted no effect on biofilm growth for H55 and ATCC strains.

Regarding the biofilm already formed, higher concentrations of RPE and antimicrobials were used, starting from MIC × 1 until MIC × 20. The concentration of RPE that killed the bacteria in this assay was 20 × MIC for H44 and 5 × for the ATCC strain, with no growth observed on the agar. The other concentrations exerted no bactericidal effect over the formed biofilm, and other strains were not affected by the highest concentrations.

For IMI and ERTA, these bactericidal concentrations ranged from MIC × 15 and MIC × 20 for H97 and H119 strains, respectively. Other strains were not affected by the highest concentrations of IMI, ERTA and CEFO. Thus, we considered MIC × 20 to show the inhibition of the biofilm already formed for RPE, CEFO, IMI and ERTA.

Figure 7 shows the growth inhibition of biofilm already formed in optical density (O.D.). RPE at MIC × 20 concentration was not able to break the already formed biofilm, but it was able to inhibit the continuous growth of the biofilm / 48h observed in the control. Antimicrobials were able to eradicate the biofilm in most strains, but their combinations with RPE (at MIC × 5) just inhibited biofilm-formed growth, with remarkable activity for the combinations RPE + IMI and

RPE + ERTA. For strain ATCC 33951, IMI concentrations were MIC × 5 (alone) and MIC × 1.25 (combinations) due to its high MIC observed and its stock solution concentration (2.5 g.l-1). Importantly, O.D. values for tests containing RPE start higher than the control due to the red color of the RPE.

Bacterial membrane permeability

Bacterial membrane permeability can be expressed by the relative percentage of conductivity in the 5% glucose bacterial solution. When the relative conductivity calculation resulted in > 100%, it was considered 100%. Figure 8 shows that RPE MICs were not able to increase the conductivity. However, when the bacteria were exposed to the RPE bactericidal concentration (MBC), a time-dependent increase in permeability was observed for the most strains (Fig. 8). The MIC of IMI reached 100% permeability in MRSA within the first time of exposure. The combination of RPE (25%) and IMI (25%) showed similar results in both periods (Fig. 9).

Interactions between PBP2a active site and propolis key-compounds

Each molecule was tested separately to observe a possible interaction with the active site portion of PBP2a (1MWT). Ceftaroline (CID 9852981) , a fifth-generation cephalosporin, and methicillin (CID101320272) were used as reference molecules for the same site. The free binding energy (Kcal/mol) was considered only without active torsional binding (Table 6). Oblongifolin A, Gutiferone A and isoliquiritigenin have free binding energy equal (Oblongifolin = -7.6) to or minor (Gutiferone, isoliquiritigenin = - 7.8) than ceftaroline. Interaction between isoliquiritigenin and the active site of PBP2a is shown in figure 10.

Discussion

Strategies for controlling the resistance of MRSA strains and for treating related diseases have been extensively investigated (Nataraj and Mallappa 2021), such as the use of drug-carrying nanomaterials, beta-lactamase blockers, and antimicrobial synergism (Gonzales et al. 2015; Bal et al. 2017; Gao et al. 2020). Notably, the combination of many classes of antimicrobials with β-lactams deserves attention since this strategy aims to interfere in the anchoring of a penicillin-binding protein (PBP), the PBP2a, which is resistant to β-lactams and is the primary mechanism by which MRSA exhibits its resistance (Shalaby et al. 2020). Interfering in PBP2a may expose other PBPs favoring the action of β-lactams (Foster 2019). Several propolis samples have already been shown to be effective against Gram-positive bacteria, and their mode-action seems to induce specifically damage to the membrane/bacterial wall (Przybyłek and Karpiński 2019). Furthermore, we first evaluated the action of different Brazilian propolis samples – the so-called brown or red propolis – on the MRSA ATCC 33951 strain (supplementary Table 1). However, the brown propolis extracts exerted a mild effect and were much less efficient than RPE, so that we just analyzed RPE in the subsequent assays with all strains.

As reviewed by Sforcin (2016), the antibacterial activity of different propolis samples may be a consequence of the synergistic effect of its constituents. The chemical composition of our RPE showed some differences compared to the chemical composition of other propolis samples, such as the compounds oblongifolin, guttiferone, vestitol, neovestitol, and medicarpin. Our sample also presented liquiritigenin and isoliquiritigenin as crucial compounds, which have already been tested *in vitro* against MRSA and exhibited MICs ranging from 0.05-0.1 $g.L^{-1}$ (Gaur et al. 2016). Silva et al. (2021) conducted a study with red and green propolis. In addition to the red propolis showing better results against several Gram-positive MDRs, the compound oblongifolin (also found in our sample) showed good inhibitory action (0.0312 gl^{-1}) and might interact with catalase's amino acids, showed by docking calculations, too.

Regarding in vivo studies, Saddiq and Mohamed (2016) evaluated the action of an aqueous Saudi red propolis extract in rats with bacteremia and lung damage caused by MRSA (ATCC 6538). These authors observed that propolis attenuated lung damage due to its antioxidant, immunomodulatory, and anti-inflammatory action. Indeed, the antioxidant action of red propolis has been reported in vitro and in vivo (Freitas et al. 2017; Reis et al. 2019).

Synergistic action of antimicrobials interfering in the cell wall (Foster 2019) or teichoic acid synthesis (Hill et al. 2019) with β-lactams has been demonstrated against MRSA strains, as well as using a carbapenem-penicillin-anti-β-lactamase combination (meropenem-piperacillin-tazobactam) (Gonzales et al. 2015). Carbapenem ME1036 was also investigated against MRSA in vitro (Kurazono et al. 2004) as well as in clinical trials (Shalaby et al. 2020). Antimicrobials CEFO, IMI and ERTA are broad-spectrum β-lactams whose primary mode-action is binding to PBPs. Furthermore, we tested our hypothesis using these antimicrobials simultaneously with RPE to evaluate a possible synergistic action. Separately, RPE, CEFO, IMI, and ERTA displayed a bacteriostatic effect on MRSA, once their MBCs were much higher than the inhibitory concentration (MIC).

Survival curve assays revealed that the combination RPE + IMI or RPE + ERTA exerted a synergistic action for all strains, with inhibition of bacterial growth. Other studies have also reported the inhibitory action of IMI (0.0711 $g.l^{-1}$) and ERTA (0.008 – 0.256 $g.l^{-1}$) on MRSA strains (Kurazono et al. 2004; Jacqueline et al. 2006).

Increased relative membrane conductivity was observed after exposing MRSA strains to RPE MBCs and RPE + IMI (25% of each MICs). These data showed that RPE is probably acting on the MRSA membrane and facilitating the action of IMI and ERTA. Likewise, other propolis samples in combination with other antimicrobials have also been shown to be effective over MRSA in vitro (penicillin, erythromycin, clindamycin, cefoxitin, ciprofloxacin, tobramycin, chloramphenicol, linezolid, tetracycline, trimethoprim, and sulfamethoxazole) (Wojtyczka et al. 2013) and *in vivo* (topical mupirocin) (Onlen et al. 2007).

Bryan et al. (2016) reported that Russian propolis – but not a red one - exhibited an inhibitory action on the formation of MRSA biofilm. Indeed, despite the β-lactam resistance by PBP2a, another significant challenge in the treatment of MRSA infections is the destruction of the biofilm, often requiring the use of more than one antibiotic in hospitalized subjects (Bi et al. 2021). Here, RPE + IMI exerted an effective antibiofilm activity against all strains, with the concentration of 25% of their MICs on the formation of biofilm, or MIC × 5 for the biofilm already formed. The combination RPE + ERTA also exhibited a positive effect on the biofilm already formed.

The computational analysis revealed that RPE compounds showed a similar binding affinity to PBP2a active site, compared to ceftaroline. Ceftaroline antimicrobial is widely used in hospital infections by MRSA. Ceftaroline can bind to the active site of PBP2a, a capacity that most β-lactams no longer have (Shalaby et al., 2020). Our docking calculations were performed with each component of propolis alone on the active site of PBP2a. All compounds showed similar binding, equal or superior to the ceftaroline. Methicillin was used to validate these results and, as expected, has a lower interaction with this PBP2a site when compared to ceftaroline and RPE compounds. This reinforces that, as we had hypothesized, the red propolis compounds are acting on PBP2a and exposing other PBPs not resistant to the action of imipenem or ertapenem. A more refined molecular docking assessment is recommended to look for a possible interference on other bacterial components such as teichoic acid and chaperones.

Our findings suggested the potential of RPE presented a synergistic effect in combination with IMI for all MRSA strains, besides RPE's inhibitory potential alone. Other strains like Methicillin-susceptible *S. aureus*, and Gram-positive *Staphylococcus epidermidis* and *Listeria monocytogenes* were also susceptible to RPE alone, with low MBCs (Supplementary table 2).

Conclusion

Red propolis extract has good inhibitory capacity against MRSA and synergistic effect with imipenem, probably because its compounds act directly on the active site of PBP2a.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

NR and JMS: conceptualization. NR, AFMP, JAAM, JKB, AFJ and VLMR: methodology and formal analysis. NR, JKB and JMS: original draft preparation. All authors have read and agreed to the published version of the manuscript.

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Tables

Strain	Inhibition zone (mm)											
	PEN_{10}	**OXA_{1}**	**LVX_{5}**	**RIF_{5}**	**CLO_{30}**	**VAN_{30}**	**CLI_{2}**	**ERI_{15}**	**SUT_{25}**	**TET_{30}**	**GEN_{10}**	**CIP_{5}**
H44	25 ± 15 (R)	0 ± 0 (R)	18 ± 0.4 (R)	20 ± 0.14 (R)	18.5 ± 2.1(R)	10 ± 0 (R)	22 ± 2,8 (R)	18.5 ± 0.7 (R)	17 ± 2.8 (R)	6 ± 1.4 (R)	11.5 ± 7.7 (R)	9.5 ± 0.7 (R)
H55	15 ± 0 (R)	5 ± 0 (R)	20 ± 0.7 (R)	18 ± 0.35 (R)	18 ± 2.8 (R) (I)	10.5 ± 0.7 (R)	17,5 ± 3,5 (R)	11 ± 7.07 (R)	14 ± 1.4 (R)	17,5 ± 0.7 (R)	15.5 ± 4.9 (R)	17 ± 2.8 (R)
H97	0 ± 0 (R)	0 ± 0 (R)	19 ± 0.7 (R)	19 ± 4.0 (R)	17 ± 1.8 (R)	10 ± 0 (R)	16,5 ± 0,7(R)	18.5 ± 4.9 (R)	14 ± 0 (R)	6 ± 1.4 (R)	13.5 ± 9.1 (R)	17 ± 2.8 (I)
H119	0 ± 0 (R)	0 ± 0 (R)	11 ± 0.8 (R)	21 ± 0 (R)	0 ± 0 (R)	9 ±1.4 (R)	16,5 ± 0,7 (R)	15.5 ± 0.7 (R)	13.5 ± 0.7 (R)	5 ± 0 (R)	3.5 ± 1.9 (R)	13.5 ± 7.7 (R)
USA300	0 ± 0 (R)	0 ± 0 (R)	14 ± 0.3 (R)	18 ± 0.35 (R)	15.5 ± 3.5 (R)	10 ± 0 (R)	15,5 ± 0,7 (R)	4.5 ± 0.7 (R)	15.5 ± 0 (R)	4,5 ± 0.7 (R)	11 ± 1.4 (R)	11.5 ± 2.1 (R)
ATCC 33951	0 ± 0 (R)	0 ± 0 (R)	12 ± 0 (R)	11 ± 8.0 (R)	13 ± 3.6 (R)	10 ± 0 (R)	5 ± 0.7 (R)	13 ± 4,9 (R)	13.5 ± 0.7 (R)	14 ± 1.4 (R)	6 ± 3.9 (R)	12 ± 3.1 (R)

Table 1 Table 1 Antimicrobial susceptibility of Methicillin-resistant Staphylococcus aureus (MRSA) strains.
Data represent results of two independent assays. Penicillin (PEN, 10 IU), oxacillin (OXA, 1 µg), levofloxacin (LVX 5 µg), rifampicin (RIF, 5 µg), chloramphenicol (CLO, 30 µg), vancomycin (VAN, 30 µg), clindamycin (CLI, 2 µg), erythromycin (ERI, 15 µg), sulfazotrin (SUT, 25 µg), tetracycline (TET, 30 µg), gentamicin (GEN, 10 µg), ciprofloxacin (CIP, 5µg). R – resistance.

Table 2 Minimum inhibitory concentrations (MIC) of red propolis extract (RPE), imipenem (IMI) and ertapenem (ERTA) on MRSA strains by the broth microdilution method with subsequent disclosure of bacterial multiplication by resazurin. MICs were obtained from two similar assays. Vancomycin and MSSA 29215 were used as controls.

Strains	**MIC ($g.l^{-1}$)**				
	RPE	CEFO	IMI	ERTA	VANC
H44	0.0156 – 0.0625	0.0156 – 0.0312	0.0312 – 0.0625	0.025	0.0039
H55	0.0312 – 0.0625	≤0.0019 – 0.0019	0.0039 – 0.0078	≤0.00156 – 0.00156	0.0019
H97	0.0156 – 0.0312	0.0078	0.0078	0.0125 – 0.025	0.0039
H119	0.0625 – 0.125	0.0039 – 0.0078	0.0039 – 0.0078	0.0125 – 0.025	0.0078
USA300	0.0625 – 0.125	0.0039 – 0.0078	0.0039 – 0.0078	≤0.00156 – 0.00156	0.0039
ATCC 33951	0.062.5 – 0.125	0.500	0.250 – >0.250	0.025	0.00097
ATCC 29215	0.0312	0.0625	0.00097	0.00625	0.0039

Table 3 Minimum bactericidal concentration (MBC) of red propolis extract (RPE), imipenem (IMI) and ertapenem (ERTA) on MRSA strains by the broth microdilution method with subsequent incubation in BHI agar. MBCs were obtained from two similar assays. Vancomycin and MSSA 29215 were used as controls

Strains	**MBC ($g.l^{-1}$)**				
	RPE	CEFO	IMI	ERTA	VANC
H44	2 – >2	>1	0.250 – >0.250	>0.1	>0.1
H55	1	0.0625	>0.250	0.0625 – 0.1	>0.1
H97	1 – 2	0.0156	0.250 – >0.250	0.1 – >0.1	>0.1
H119	1 – 2	0.250	>0.250	0.1	>0.1
USA300	2	>1	>0.250	>0.1	>0.1
ATCC 33951	0.50 – 1	1	0.250 – >0.250	>0.1	>0.1
ATCC 29215	1	0.250	0.250 - >0.250	0.250 – 0. 5	>0.1

Table 4 Fractional inhibition concentration index (FICI) of red propolis extract (RPE) in combination with cefoxitin (CEFO), imipenem (IMI) and ertapenem (ERTA) against MRSA strain ATCC 33951 obtained by checkerboard technique. I - indifference, S - synergism, AE - additive effect. These results were seen in duplicates.

ATCC 33951						
Combination	A ($g.l^{-1}$)	B ($g.l^{-1}$)	MICA ($g.l^{-1}$)	MICB ($g.l^{-1}$)	FICI	Interpretation
RPE + CEFO	0.078	0.150	0.0375	0.150	3	I
RPE + IMI	0.0023	0.0375	0.150	0.75	0.5	S
RPE + ERTA	0.01815	0.00312	0.0375	0.025	0.6	AE

Table 5 Percentage (%) of inhibition of biofilm formation by MRSA strains after 48 h. Values presented for propolis extract and antimicrobials separately represent their MIC 75%. Combinations were obtained with the MIC 25%. RPE – red propolis extract, IMI – imipenem, ERTA – ertapenem.

Strains	RPE (MIC 75%)	IMI (MIC 75%)	ERTA (MIC 75%)	RPE + IMI (MIC 25%)	RPE + ERTA (MIC 25%)
H44	37.78	10.53	30.4	56.50	22
H55	61.46	74.10	52.86	19.80	0
H97	22.23	0	26.29	28.60	25.71
H119	0	23.95	18.44	46.90	31.7
USA300	40.67	48.50	34.65	60.20	57.50
ATCC 33951	38.65	67.84	70.97	64.40	0

Table 6 Free binding energy (Kcal/mol) of RPE compounds with the active site of PBP2a (PDB-1MWT), without compounds active torsional binding. Constants were obtained by surface/binding analyzes using Autodock Vina software. Ceftaroline and methicillin were used as controls.

Compound/ CID (PubChem)	Free binding energy (Kcal/mol)
Liquiritigenin / 114829	-7.2
Isoliquiritigenin / 638278	-7.8
Vestitol / 92503	-7.2
Neovestitol / 44257510	-7.1
Medicarpin / 336327	-7.4
o-Methylvestitol / 44446856	-7.2
Gutiferone A / 5352090	-7.8
Oblongifolin A / 11570735	-7.6
Ceftaroline / 9852981	-7.6
Methicillin/ 101320272	-5.6

Figures

Figure 1 Key-compounds found in the red propolis extract (RPE) by HPLC and TLC. 1 – liquiritigenin, 2 – isoliquiritigenin, 3 – vestitol, 4 – neovestitol, 5 – medicarpin, 6 – O-methilvestitol, 7 – guttiferone E, 8 – oblongifolin.

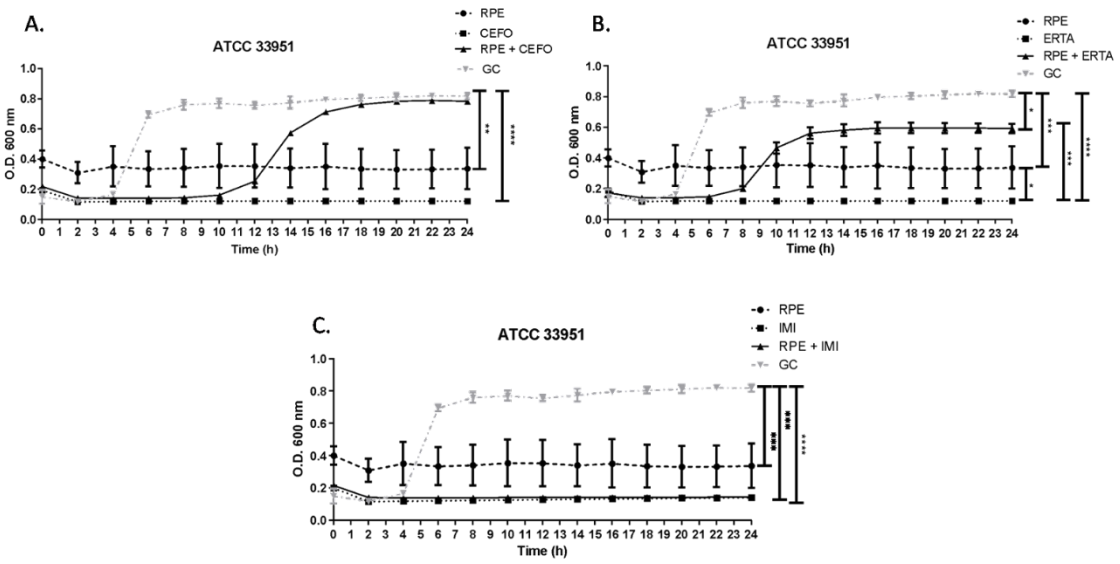


Figure 2 Survival curve of MRSA ATCC 33951 after incubation with MIC of red propolis extract (RPE ●), cefoxitin (CEFO ■) (A), ertapenem (ERTA ■) (B) or imipenem (IMI ■) (C) and the combination RPE + CEFO (A), ERTA (B) or IMI (C) (25% MIC ▲). Data represent mean and standard deviation of two similar assays. * (P ≤0.05), ** (P ≤ 0.01), *** (P ≤ 0.001) **** (P ≤ 0.0001) significantly different. (GC▼) - growth control .

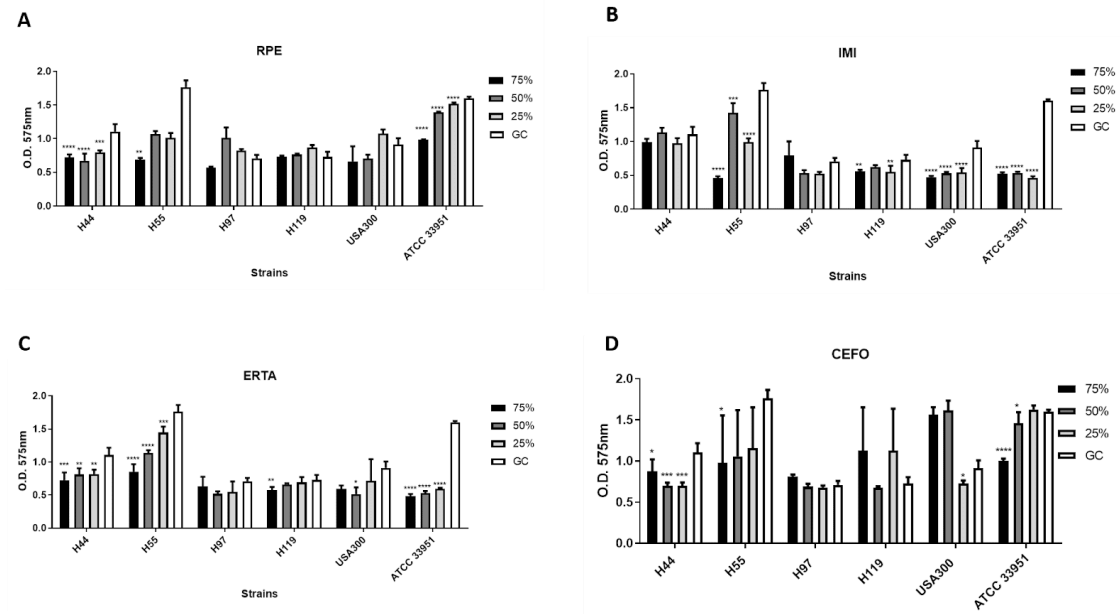


Figure 3 MRSA biofilm formation after incubation with broth (growth control – GC), red propolis extract (RPE – A), imipenem (IMI – B), ertapenem (ERTA – C) and cefoxitin (CEFO - D) using concentrations ranging from 25% to 75% of their MICs. Bars represent mean and standard deviation of triplicates. * (P ≤0.05), ** (P ≤ 0.01), *** (P ≤ 0.001) **** (P ≤ 0.0001) significantly different from growth control (GC).

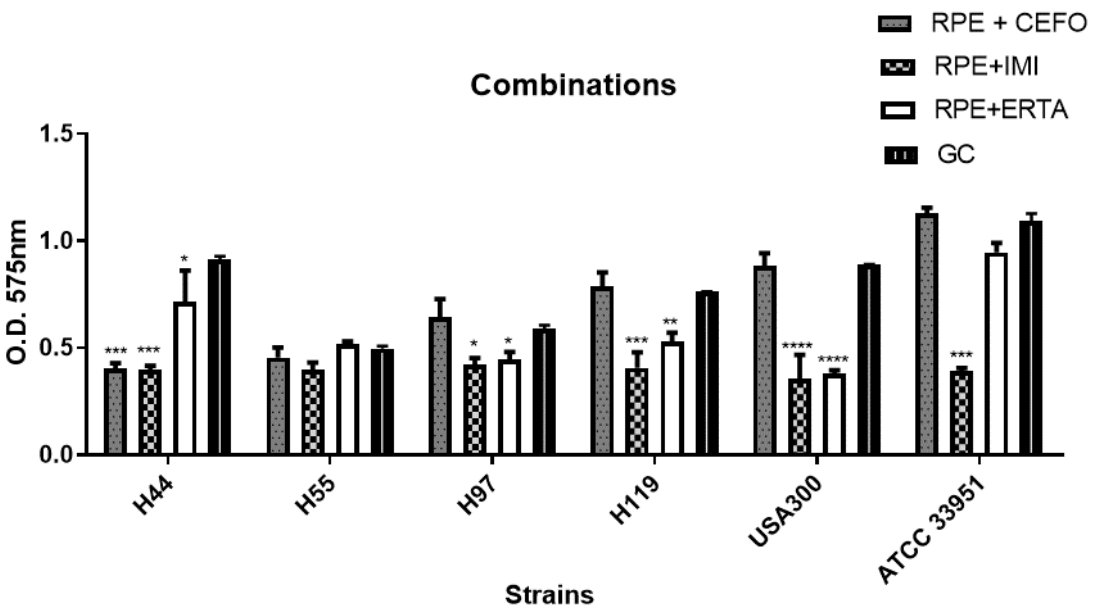


Figure 4 MRSA biofilm formation after incubation with red propolis extract (RPE) in combination with cefoxitin (CEFO), imipenem (IMI) and ertapenem (ERTA), using the MIC 25% of RPE and MIC 25% of each antimicrobial. Bars represent mean and standard deviation of the triplicates.* (P ≤0.05), ** (P ≤ 0.01), *** (P ≤ 0.001) **** (P ≤ 0.0001) significantly different from growth control (GC).

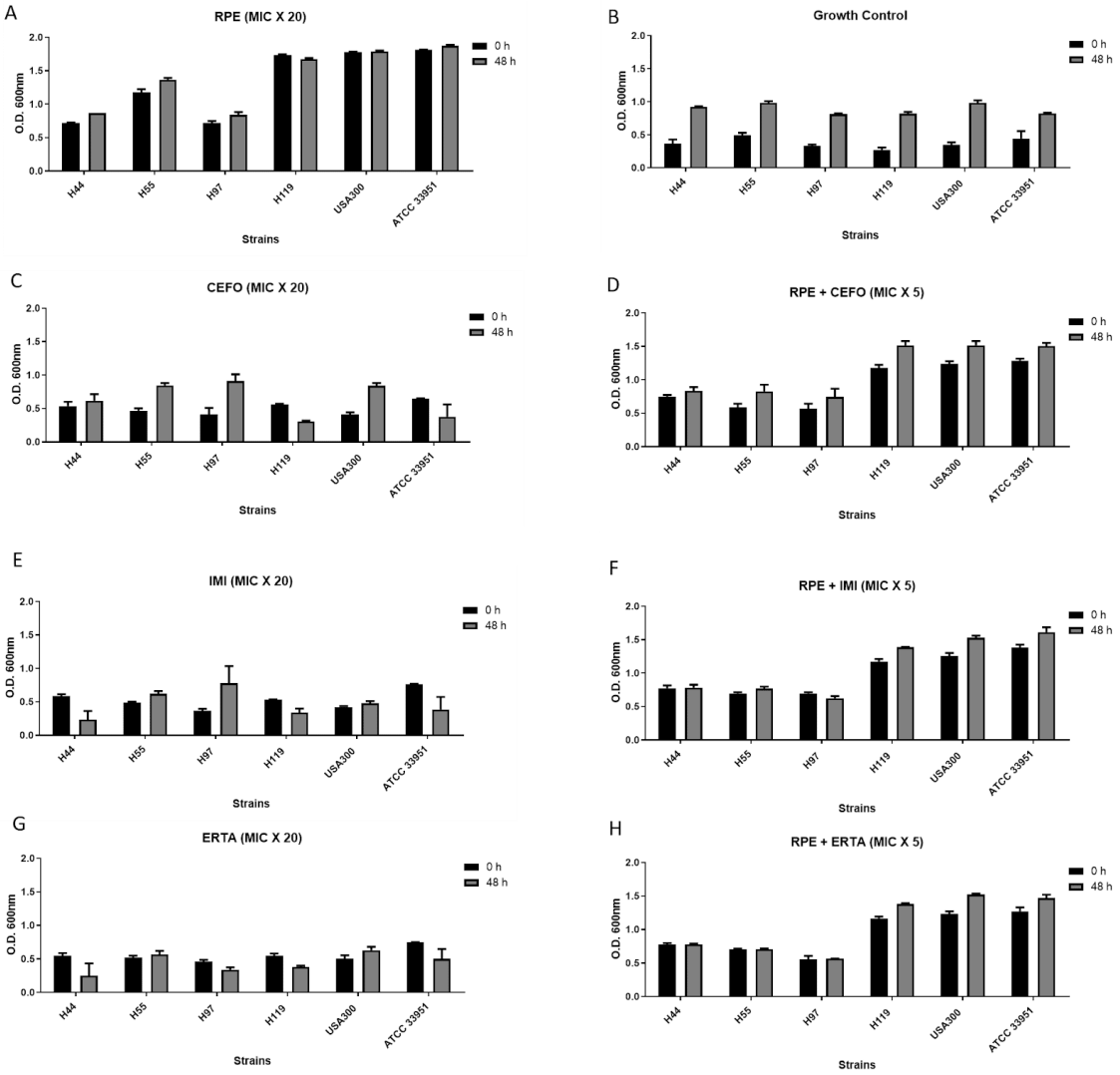


Figure 5 Biofilm eradication assay. Bars represent the optical density (O.D.) values of the biofilm already formed after 24h (dark bars) and the same biofilm treated with RPE or antimicrobials after 48h (gray bars). (A) RPE at MIC x 20, (B) growth control, (C) CEFO at MIC x 20, (D) RPE + CEFO at MIC x 5, (E) IMI at MIC x 20, (F) RPE + IMI at MIC x 5, (G) ERTA at MIC x 20, (H) RPE + ERTA at MIC x 5. Data represent mean and standard deviation of triplicates of two independent assays.

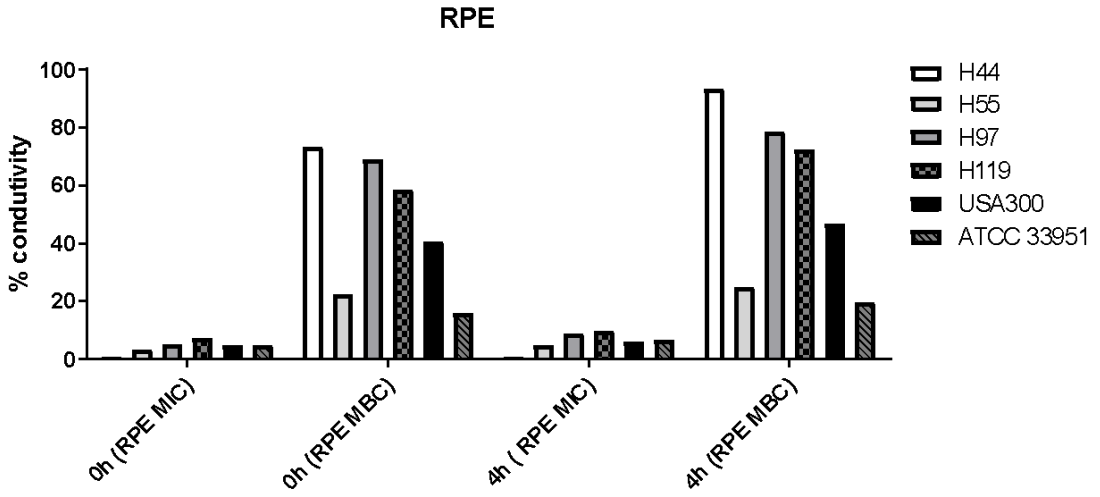


Figure 6 Percentage (%) of membrane electrical conductivity of MRSA strains after incubation with MIC or MBC of RPE at 0 h and 4 h incubation.

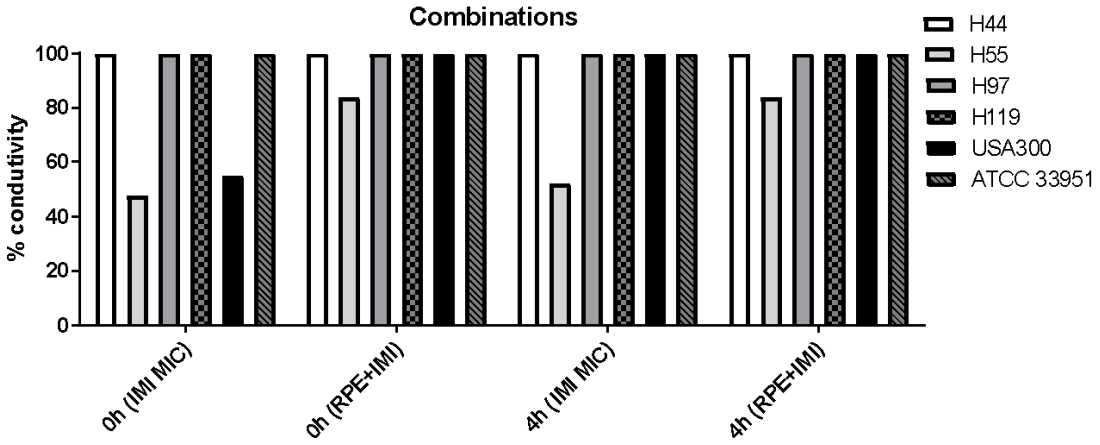


Figure 7 Percentage (%) of membrane electrical conductivity after incubation with MIC of IMI and combinations of RPE+IMI (25% of their MICs) at 0 h and 4 h incubation.

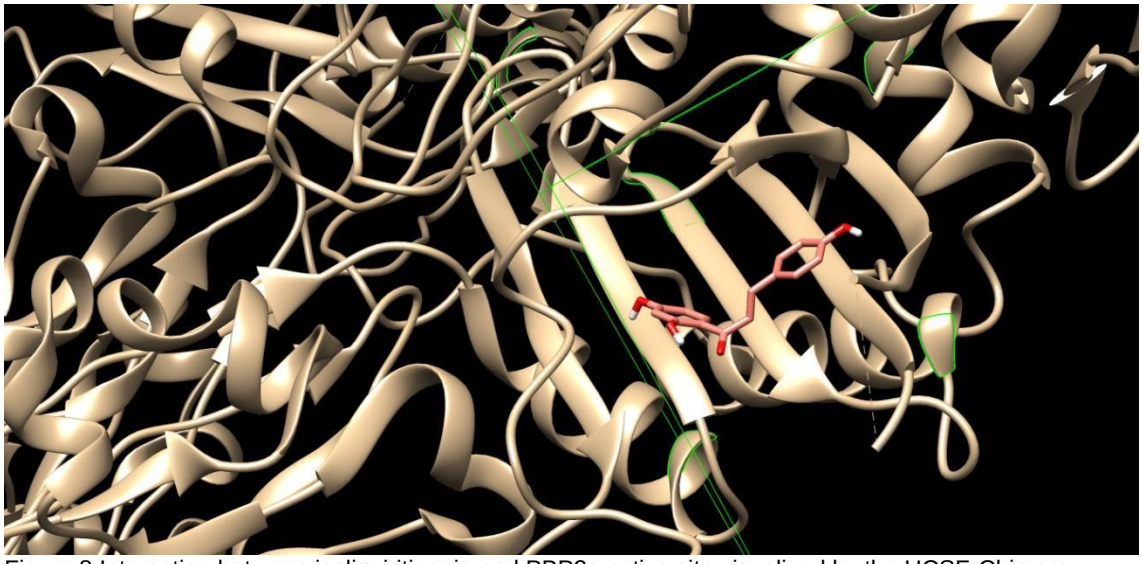
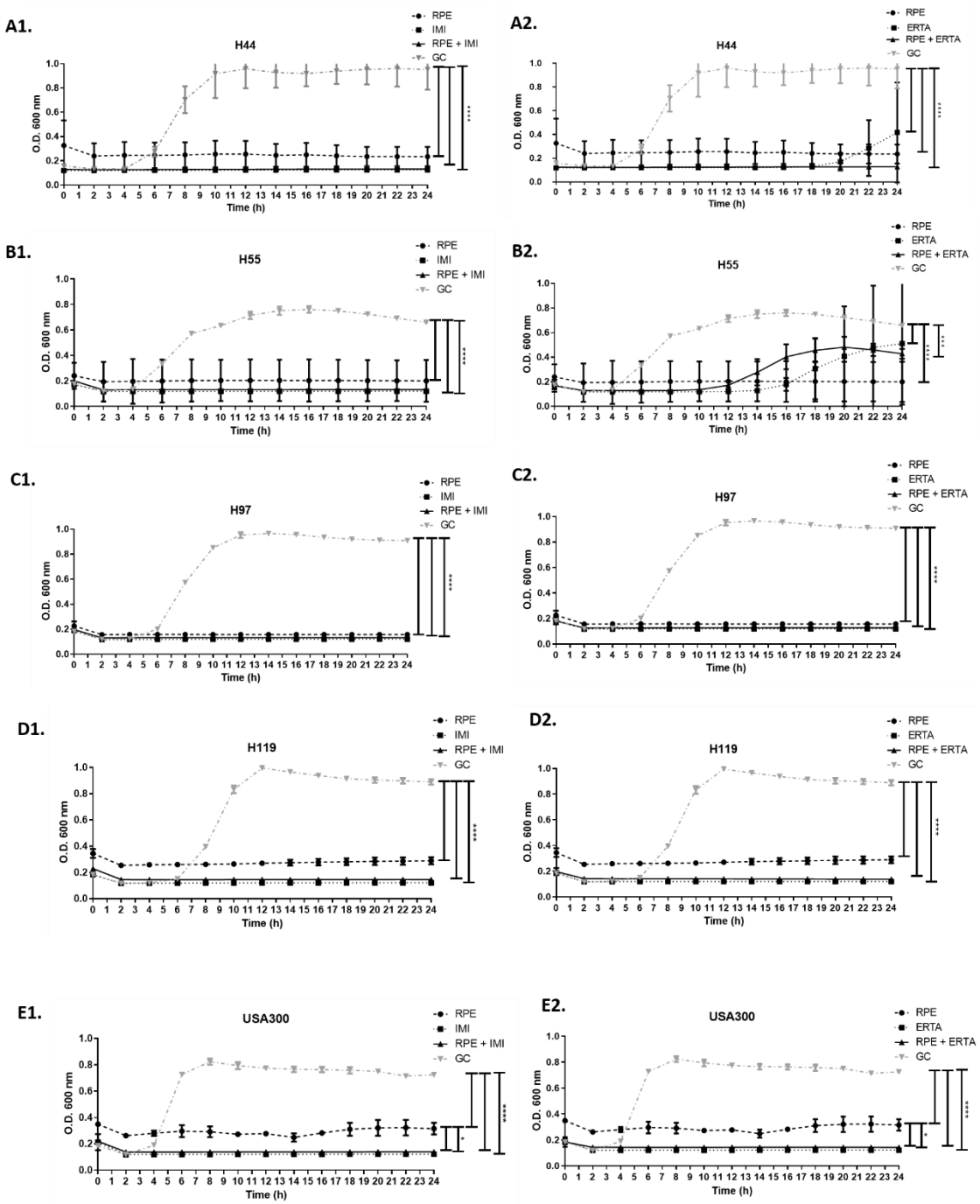


Figure 8 Interaction between isoliquiritigenin and PBP2a active site visualized by the UCSF-Chimera.

Supplementary Material

Sample	**MIC ($g.l^{-1}$)**	**MBC ($g.l^{-1}$)**
BPE-ar	3,2 – 7,5	15
BPE-ma	>22,5	>22,5
BPE-Pi	5 – 10	10

Supplementary table 1. Minimum inhibitory concentration (MIC) and minimum Bactericidal concentration (MBC) of brown propolis. extract from Araucaria (BPE-ar), Morus alba (BPE-ma) and Pinus (BPE-pi) on MRSA ATCC 33951. Representative values were obtained in three separate experiments.



Supplementary Figure 1. Survival curve of MRSA strains after incubation with MIC of red propolis extract (RPE ●), imipenem (IMI ■ – right side), and ertapenem (ERTA ■ – left side) alone or in the combination of RPE with IMI or ERTA (25% of their MICs ▲) over MRSA strains H44 (A1, A2), H55 (B1, B2), H97 (C1, C2), H119 (D1, D2) and USA300 (E1, E2). Data represent the mean and standard deviation of 2 similar assays. * $p<0.05$; **** $p<0.001$. GC – growth control (▼).

Strain/ATCC	MIC ($g.l^{-1}$)	MBC ($g.l^{-1}$)
MSSA/25923	0.0321 – 0.0625	0.250
MSSA/29213	0.0321	0.125
S. epidermidis/35984	0.0321- 0.0625	0.0625 – 0.125
L. monocytogenes/15313	0.0312	0.0312 – 0.062.5

Supplementary Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were obtained for red propolis extract (RPE) on MSSA ATCC 25923 and 29213, *Staphylococcus epidermidis* ATCC 35984, and *Listeria monocytogenes* ATCC 15313. Representative values were obtained in two separate experiments.

Capitulo 3 - Brazilian red propolis in combination with imipenem modulates cytokine production, surface markers expression and monocyte bactericidal activity against methicillin-resistant *Staphylococcus aureus* (MRSA)

Brazilian red propolis in combination with imipenem modulates cytokine production, TLR-2 expression and monocyte bactericidal activity against methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Propolis is a bee product found all over the globe with a well-known antibacterial activity. Previous findings of our group revealed that the combination of Brazilian red propolis (BRP) with lower concentration of imipenem (IPM) exerted a bactericidal action against methicillin-resistant *Staphylococcus aureus* (MRSA). Here, we aimed at investigating BRP in combination or not with IPM on human monocytes to assess a possible immunomodulatory action. Monocyte metabolic activity was analyzed by MTT assay, cytokine production (TNF-α, IL-1β, IL-6, IL-8, IL-10) by ELISA, the expression of cell markers (TLR-2, TLR-4, HLA-DR, CD80) by flow cytometry, and the bactericidal activity over MRSA. BRP alone or in combination with IPM did not affect the metabolic activity of monocytes. BRP downregulated TLR-2 expression and

stimulate IL-6 production as well BRP + IPM-treated cells. No differences copared with the control group were seen on the others parameters. Bctericidal activity of BPR alone and combination was near to the LPS-activated monocytes. Data indicated the potential of BRP or BRP + IPM to enhance killing activity of monocytes over MRSA infection, probabily because immunomodulaton TLR-2 and IL-6 citokyne.

KEYWORDS

propolis, monocyte, Toll-like receptor, cytokine, methicillin-resistant *Staphylococcus aureus*

1 INTRODUCTION

Propolis has aroused the interest of researchers and consumers for its pharmacological properties and the potential for the development of new drugs, although it has been used by several civilizations since antiquity (Sforcin and Bankova, 2011; Watanabe et al., 2011; Sforcin, 2016; Weis et al., 2022).

Propolis is a resinous material produced by bees depending on phytogeographic conditions and used in the beehive as a defensive and constructive material (Bankova, 2005). In Brazil, several types of propolis can be found including the red one, found mainly in the northeast region. The red color is attributed to retusapurpurins A and B. *Dalbergia ecastaphyllum* is the main vegetal source of Brazilian red propolis (BRP), which has been increasingly investigated in recent years (Piccinelli et al., 2011; Rufatto et al., 2018; Alencar et al., 2023; Justino et al., 2023).

Our group has endeavored to elucidate the mode-actions of Brazilian green propolis, revealing that it may affect macrophages, monocytes, dendritic cells, and lymphocyte's function. Consequently, it modulate innate and adaptive immunity (Orsatti et al., 2010; Búfalo et al., 2014; Conti et al., 2016; Conte et al., 2022; Conti et al., 2023; Santiago et al., 2023a).

In continuity, we demonstrated that BRP alone or in combination with imipenem (IPM) exerted an antibacterial action on methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Ripari et al., 2023). MRSA is involved in hospital infections, causing a high mortality rate, prolonged hospitalization, and high public health costs (Morrisette et al. 2020). Therefore, new therapeutic approaches are crucial to kill multidrug-resistant bacteria directly and/or by improving immune cells activity.

Since antimicrobial drugs are systemically distributed and may affect circulating immune cells, we were interested in evaluating the effects of BRP alone or in combination with IPM (using the same concentrations reported by Ripari et al., 2023) on human monocytes to observe a possible cytotoxic action, cell's surface markers expression (TLR-2, TLR-4, HLA-DR and CD80), the production of pro- and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10), and the bactericidal activity against MRSA. A possible synergistic action of BRP + PM was also investigated, reducing both BRP as well the antimicrobial concentration.

2 MATERIAL AND METHODS

2.1 Brazilian red propolis extract

BRP was collected in the beekeeper's association of Canavieiras (COAPER), Bahia, Brazil, and its chemical composition was analyzed by HPLC-DAD, revealing that its main constituents were calycosin, formononetin, guttiferone E, isoliquiritigenin, liquiritigenin, medicarpin, neovestitol, 7-*O*-methylvestitol, oblongifolin B, and vestitol (Ripari et al., 2023; Santiago et al., 2023b).

BRP extract was dissolved in dimethyl sulfoxide (DMSO) and then diluted in RPMI 1640 culture medium (Cultilab, Brazil) to obtain 62.5 µg/ml (Ripari et al., 2023).

2.2 MRSA strain and imipenem

MRSA (ATCC 33591 – *S. aureus* subsp. *aureus* Rosenbach) was provided by the Microbiology/Immunology Sector, UNESP.

The antimicrobial IPM (pharmaceutical secondary standard) was obtained from Sigma–Aldrich Company (USA) and aliquots of 2.5 mg/ml in sterile distilled water were stored at −20°C according to the manufacturer's recommendation. IPM was diluted in RPMI 1640 to obtain desired MIC concentration (250 µg/mL) found in Ripari et al. (2023).

2.3 Subjects and monocyte isolation

Five healthy volunteers over 18 years old, non-smokers, neither ill nor using any kind of medication were randomly accepted after signing a consent form to participate in this study. This study was approved by the Ethics Committee of the Botucatu Medical School, UNESP (CAAE 35248620.0.0000.5411).

Blood samples were collected and placed in sterile tubes containing 1% heparin. Then, peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Paque Plus gradient separation density = 1.077 (GE Healthcare Bio-Sciences AB, Sweden) by centrifugation. The mononuclear cell ring was collected and washed twice in RPMI medium for 10 min at 200 × g. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of complete RPMI.

Monocyte ($CD14^+$ cells) isolation was performed by the negative magnetic separation technique. Cells were incubated with magnetic beads coupled with monoclonal antibodies contained in the commercial kit: MACS® Monocyte Isolation Kit II Human (Miltenyi Biotec Inc, USA), obtaining CD14+, CD3-, CD7-, CD16-, CD19-, CD56-, CD83- and CD235- cells.

Viable cells were counted by staining with 0.2% trypan blue solution and adjusted to 1 × 10^6 cells/ml. After, 500 µl were seeded in 24 well culture plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland).

Monocytes were incubated for 18 h with BRP (62.5 µg/ml) and IPM (250 µg/ml). To observe a possible synergism on monocyte functions, BRP and IPM were combined using 25% of their minimal inhibitory concentration (15.62 and 62.5 µg/ml, respectively), which exerted a synergistic effect against the same MRSA strain (Ripari et al., 2023). Cells were also incubated with lipopolysaccharide (LPS – 0.1 µg/ml) as a positive control and with 5% DMSO (propolis solvent). The supernatant was collected for cytokine determination and the cells were used to assess their expression of surface markers.

2.4 Monocyte metabolic activity

Cellular metabolic activity was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium – Sigma-Aldrich, USA) assay based on the reduction of MTT from yellow to purple formazan dye by mitochondrial succinate dehydrogenase (Bellamakondi et al., 2014). After incubation for 18 h, the cell culture supernatant was removed and 300 µl of MTT dissolved

in RPMI 1640 was added. After 3 h, MTT was removed and DMSO (200 µl) was added to solubilize the formazan crystals. Optical density was read at 540 nm on an ELISA reader and cell viability percentages were calculated. Results were expressed in relation to control cells incubated with medium only, considered as 100% viability.

2.5 TLR-2, TLR-4, HLA-DR, and CD80 expression by flow cytometry

To evaluate TLR-2, TLR-4, HLA-DR, and CD80 expression, 1×10^6 monocytes/ml (500 µl) were placed in 24 wells flat-bottomed microplates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and cells were incubated with BRP, IPM, their combination, LPS or DMSO. After 18 h, cells were washed, resuspended in 1 ml of electrolyte solution (ISOTON II) and incubated with specific fluoresceinated monoclonal antibodies. Cells were separated into two groups (A and B). In group A, the stimulated cells were incubated with FITC-conjugated anti-TLR-2 antibody (clone TLR2.1 – Biolegend, USA) and PE-anti-TLR-4 antibody (clone HTA125 – Biolegend). In group B, monocytes were incubated with FITC-anti-HLA DR (clone L243 – Biolegend) and PE-anti-CD80 (clone D210 – Biolegend) antibodies. Cells were labeled with PerCP-Cy5.5-conjugated anti-CD14 (clone M5E2 – Biolegend), which allowed selection of the CD14+ monocyte gate only. After incubation, cells were centrifuged for 10 minutes at 465 × g for washing, fixed in 500 µl ISOTON II containing 50 µl 5% *p*-formaldehyde and analyzed by flow cytometry using a FACSCanto II flow cytometer (Becton Dickinson, USA).

The acquisition was performed using the FACSDiva software (BD Biosciences), 30,000 events per sample was standardized and the population of interest was based on size (FSC) and granularity (SSC) parameters, doublets were discarded using single cell strategy and selected cells CD14+. The data were analyzed with FlowJo software (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Results were represented as the fluorescence intensity (MFI) and as the percentage of cells CD14+ cells expressing the surface markers.

2.6 Cytokine production by ELISA

To evaluate cytokine production, monocytes were distributed into 24-well plates and incubated with BRP, IPM, their combination, LPS (0.1 µg/ml), DMSO, or RPMI 1640 as control for 18 h at 37°C and 5% CO_2. After, the supernatants were harvested for TNF-α, IL-1β, IL-6, IL-8, and IL-10 measurement by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D Systems, USA).

2.7 Killing of MRSA by monocytes

After treatment with BRP, IPM, or BRP + IPM for 18 h, the supernatant of cell culture was discarded and monocytes were challenged with 2 × 10^6 MRSA (ATCC 33951), in a 1:4 monocyte:bacteria ratio, according to Dubois and Dubois (2019). After 2 h at 37°C, the supernatant of each cell culture was collected and the respective wells were washed with sterile distilled water to disrupt the monocyte membrane, releasing the phagocytized bacteria. After washing, a volume of 7 ml was obtained and 100 µl were pipetted and seeded in BHI agar in triplicate. Plates were incubated at 37°C and, after 24 h, colony-forming units (CFU) in the plates containing monocyte-MRSA co-cultures (experimental plates) were counted. Plates containing MRSA and nontreated monocytes were considered as control (100%). The percentage of bactericidal activity was determined according to the formula: [1 – (mean CFU of experimental plates/mean CFU of control plates)] ×100.

2.8 Statistical analysis

Data were analyzed using the Prism 5 (GraphPad San Diego, USA). Analysis of Variance (ANOVA) followed by Dunnet's or Tukey test ($p < .05$) were used. Data represent mean ± standard-deviation (SD) of 5 individuals.

3 RESULTS

3.1 Monocyte phenotyping

Monocyte ($CD14^+$ cells) isolation was properly performed by the negative magnetic separation technique, obtaining 85.4% of a cell purified population (Figure 1).

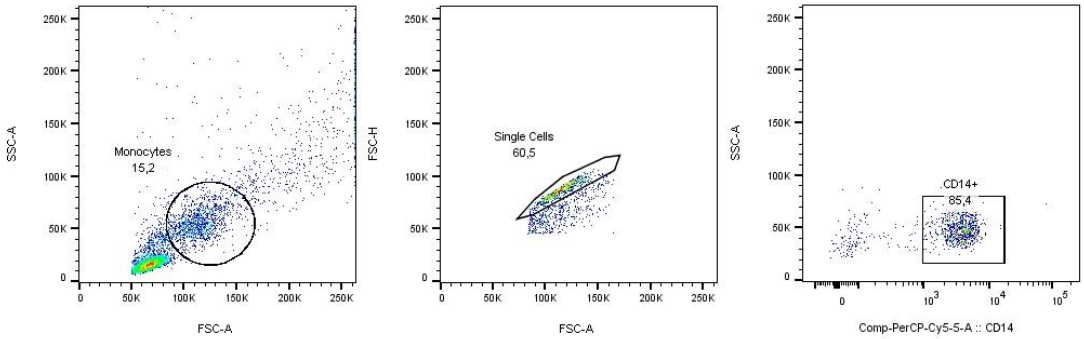


FIGURE 1. Strategy of gate: (A) Dot plot related to size (FSC-A) x granularity (SSC-A) expected for monocytes, (B) Single cells strategy (FSC-H X FSC-A) for discarded doublets and (C) Gate of monocytes CD14+.

3.2 BRP did not affect the metabolic activity of monocytes

Monocytes were incubated with the concentrations of BRP, IPM or BRP + IPM that inhibited MRSA strains in vitro (Ripari et al., 2023). Data revealed that such concentrations did not affect the metabolic activity of monocytes, which reflects cell viability. LPS and propolis solvent (5% DMSO) exerted no cytotoxic effects (Figure 2).

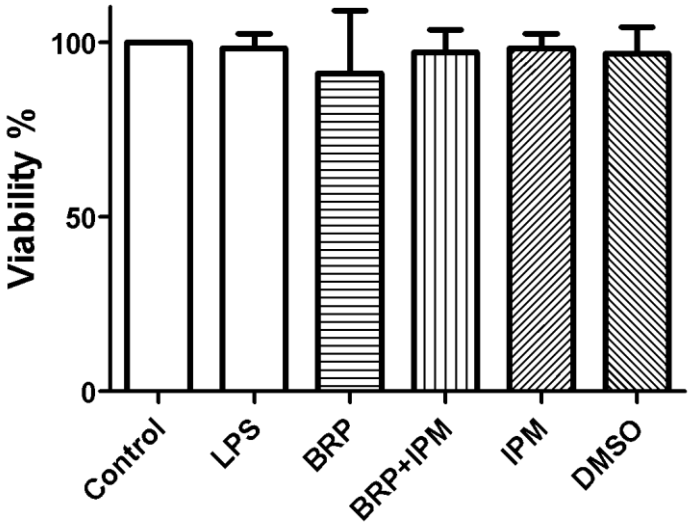


FIGURE 2 Monocyte metabolic activity (%) after incubation with RPMI 1640 (control – C), LPS (0.1 µg/ml), BRP (62.5 µg/ml), BRP + IPM (15.62 + 62.5 µg/ml), IMI (250 µg/ml), or 5% DMSO after 18 h. Data represent mean ± SD (n = 5). (*p* >.05).

3.3 BRP + IPM tends to upregulate TLR-2 and HLA-DR

BRP downregulated nonsignificantly TLR-2 expression and maintained the basal expression of TLR-4, CD80 and HLA-DR. BRP + IPM upregulated TLR-2 compared to propolis alone and showed a tendency to upregulate HLA-DR. IPM exerted no significant effects on cell markers (Figure 3).

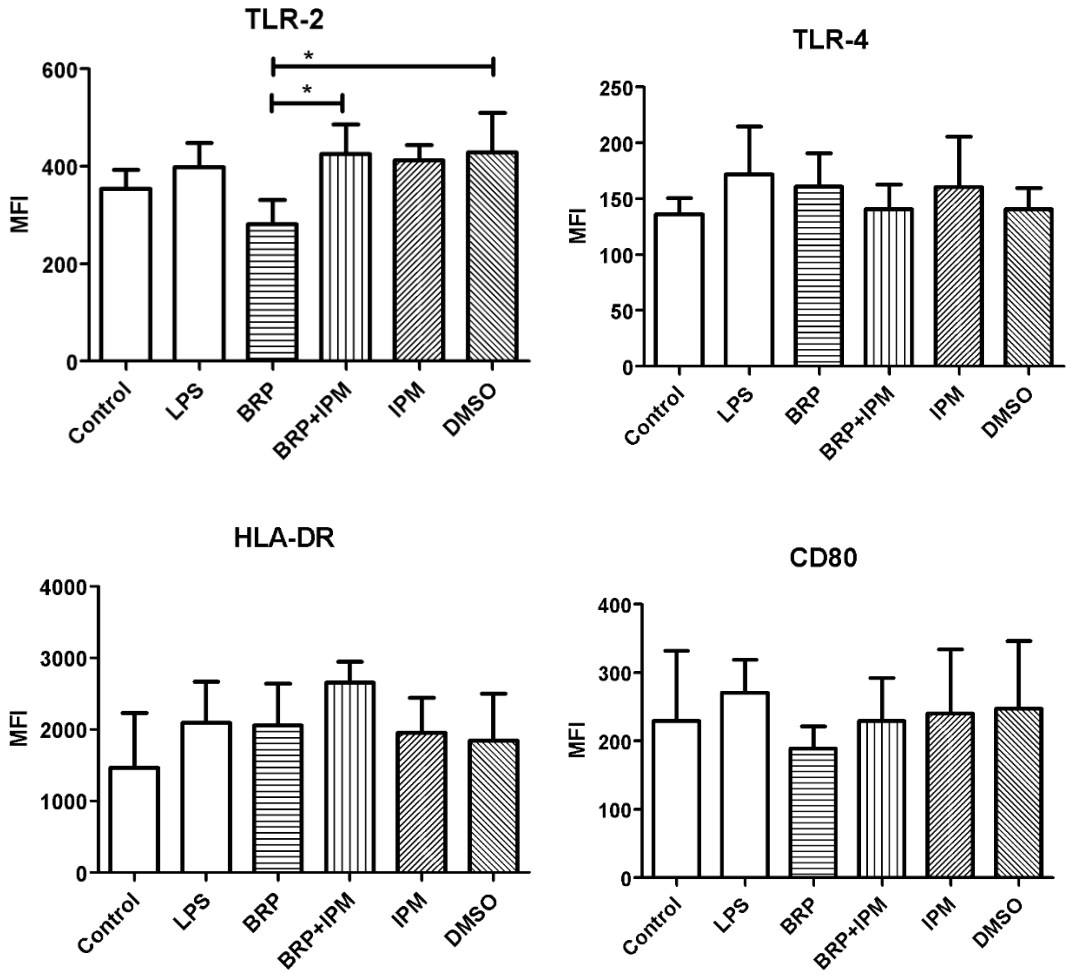
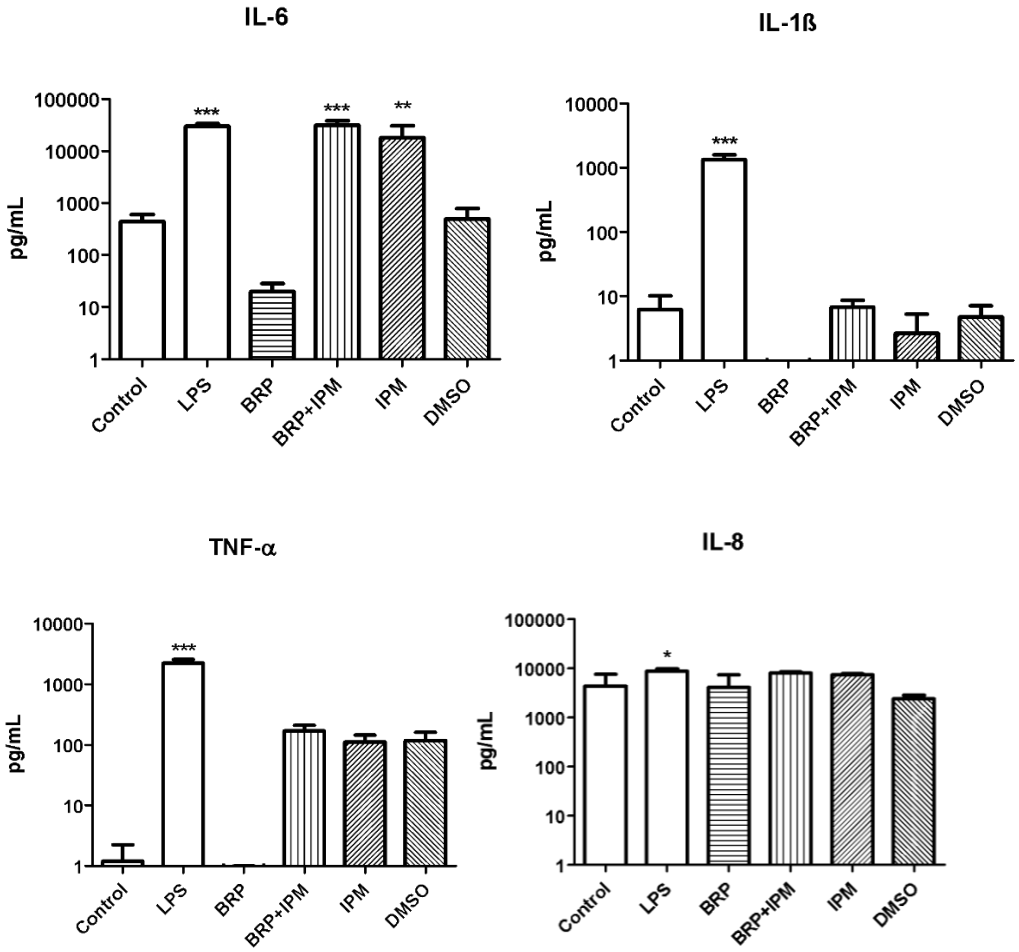


FIGURE 3 Median fluorescence intensity (MFI) of TLR-2, TLR-4, HLA-DR and CD80 by monocytes CD14+ incubated with RPMI 1640 (control – C), LPS (0.1 µg/ml), BRP (62.5 µg/ml), BRP + IMI (15.62 + 62.5 µg/ml), IMI (250 µg/ml), or 5% DMSO for 18 h. Mean ± SD; n = 5. Significantly different from propolis: *p<.05).

3.4 BRP displayed an anti-inflammatory action on cytokine production while BRP + IMI exhibited a pro-inflammatory profile

BRP + IPM significantly enhanced IL-6 ($p < .001$). IPM and the combination of BRP + IMI stimulated TNF-α nonsignificantly. BRP, IPM or their combination didn't affect IL-8 and IL-10 production. LPS, used as a positive control, was capable s to timulated cytokine production except IL-8 cytokine (Figure 4).



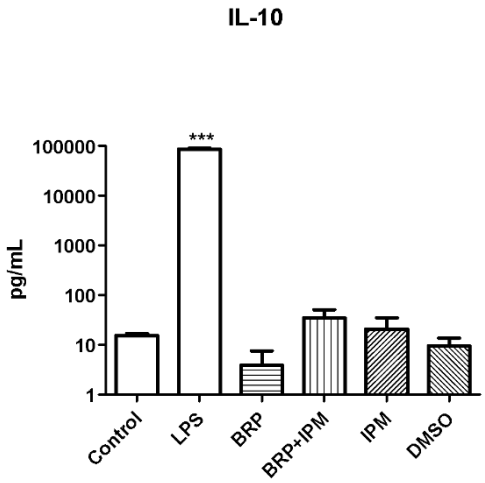


FIGURE 4 IL-1β, TNF-α, IL-6, IL-8 and IL-10 production (pg/ml) by monocytes after incubation with RPMI 1640 (control – C), LPS (0.1 µg/ml), BRP (62.5 µg/ml), BRP + IMI (15.62 + 62.5 µg/ml), IMI (250 µg/ml), or 5% DMSO for 18 h. Mean ± SD; n = 5. Significantly different from control: *** $p < .001$.

3.5 BRP induced a similar killing activity to LPS

Previous treatment with BRP, BRP + IPM and IPM stimulated the killing activity of monocytes against MRSA ATCC 33591 strain like LPS-treated cells, with significant difference when it's compared with the control group (Figure 5).

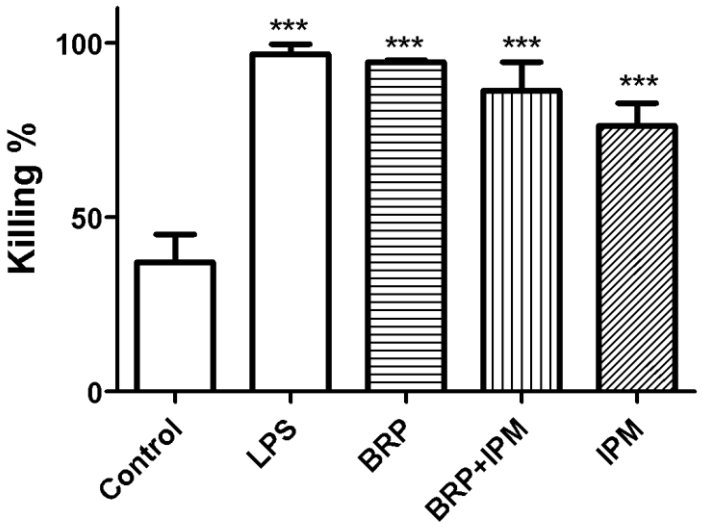


FIGURE 5 Percentage (%) of bactericidal activity of monocytes incubated with LPS (0.1 µg/ml), BRP (62.5 µg/ml), BRP + IPM (15.62 + 62.5 µg/ml), IPM (250 µg/ml), or 5% DMSO. Mean ± SD; n = 5 ($p > 0.5$).

4 DISCUSSION

Treating antimicrobial-resistant bacteria such as MRSA is still a challenge worldwide (Creech et al., 2023). Searching for new alternatives to overcome this issue, we demonstrated recently that BRP alone or in combination with IPM exerted antibacterial and antibiofilm effects on MRSA strains likely due to membrane/wall damage and interaction with PBP2a active site, which confers broad clinical resistance to *β*-lactams (Ripari et al., 2023). Here, we aimed at investigating the same concentrations of BRP and IPM used by the former authors on human monocytes, and a possible synergistic action of BRP + IPM was investigated, reducing the BRP and antimicrobial concentration.

The monocyte was adopted in this assay since antimicrobials circulate in the bloodstream and may affect the function of cells involved in the host immunity. Before evaluating the impact of BRP alone or in combination with IPM on monocyte functions, a possible cytotoxic action was investigated. None of the treatments affected the metabolic activity and viability of monocyte, what indicated that a selective toxicity against MRSA strains at the same concentrations previously reported by our group (Ripari et al., 2023), but not to human monocytes.

BRP + IPM enhanced IL-6 production by monocytes as well as TLR-2 expression, exhibiting a pro-inflammatory profile and enhancinh the anti-MRSA activity of these monocytes. Other parameters are not affected by BRP or combination. These findings reinforced previous data from literature pointing Brazilian red propolis immunomodulatory activity. Bueno-Silva et al. (2015) showing that Brazilian red propolis attenuates inflammatory signalling cascade in LPS-activated macrophages. Franchin et al. (2016) reported that vestitol isolated from red propolis inhibited neutrophils migration in the inflammatory process. Antimicrobial activity of propolis as well documented were BRP alone enhanced the fungicidal activity of monocytes against *Candida albicans* (Santiago et al., 2023b). Brazilian green propolis stimulated the bactericidal activity of dendritic cells and monocytes against *Streptococcus mutans* (Conti et al., 2016; Santiago et al., 2016).

IL-8 – a chemokine/cytokine involved in the recruitment of leukocytes to the inflammatory site (Liu et al., 2021) was not altered after incubating monocytes with the treatments. HLA-DR molecules are involved in antigen presentation. CD80 is expressed on antigen presenting cells (APCs providing costimulation to T cells (Soskic et al., 2021). HLA-DR and CD80 expression was not affected by the treatments as well.

In our study, IPM basically induced a higher IL-6 and TNF-α production than control. Antimicrobials may modulate cytokine production by monocytes, affecting the natural defence against bacteria (Ziegeler et al., 2006). These authors reported that IPM did not affect TNF-α and IL-10 production nor HLA-DR expression by monocytes. These data are relevant for the selection of antibiotic agents in immunocompromised and/or septic patients.

Last, it is worth mentioning that a high variability was seen on monocytes' response to the treatments, what clearly indicates that humans respond differently to therapeutic approaches.

Our findings are interesting because of their application depending on the context: BRP at inhibitory concentrations for MRSA did not exert cytotoxic effects on monocytes, indicating a selective toxicity. BRP keep the basal production of pro-inflammatory cytokines and downregulated TLR-2 expression, what indicates it's potential to mitigate the inflammation caused by the infection. On the other hand, the combination BRP + IMI led monocytes to a pro-inflammatory state by upregulating both TLR-2 and cytokines without affecting the killing activity over MRSA 33591 strain. Further investigation is needed to explore its potential to stimulate monocytes to fight against other multidrug-resistant infectious agents.

5 CONCLUSIONS

Our findings indicated the potential of Brazilian red propolis to mitigate the infection without affecting monocytes. On the other hand, the combination of propolis with imipenem may stimulate monocytes to fight against MRSA by upregulating cytokine production and TLR-2 expression, exhibiting a pro-inflammatory profile.

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CONFLICT OF INTEREST

All authors declared that they have no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Design of the study: Nicolas Ripari and José Maurício Sforcin. Data and statistical analysis: Nicolas Ripari, Mariana da Silva Honorio, Arthur Alves Sartori, and José Maurício Sforcin. Writing, proofreading, and editing: Nicolas Ripari, Jairo Kenupp Bastos and José Maurício Sforcin. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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Considerações Finais

Nossos trabalhos demonstraram que a própolis vermelha apresenta potencial contra isolados adquiridos de hospital e cepas padrões de MRSA. Os testes antimicrobianos revelaram que baixas concentrações de extrato de própolis vermelha podem ser bacteriostáticas e bactericidas contras MRSA.

Em relação à cepa padrão ATCC 33591, observamos que as concentrações bacteriostáticas de própolis vermelha mantiveram a produção basal de citocinas e baixa regulação de TLR-2. Em um contexto de infecções cutâneas, estes dados sugerem que própolis vermelha poderia mitigar a lesão tecidual provocada por inflamação crônica causada por infecção por MRSA pois pode agir de for bacteriostática sem regular positivamente citocinas pró-inflamatórias. Assim, pode-se especular que pacientes portadores de diabetes, obesidade ou outras comorbidades poderiam ter menor chance de infecção invasiva se forem tratados topicamente com própolis vermelha ou seus compostos primordiais.

Em um contexto de infecção invasiva, a combinação de própolis vermelha com imipenem poderia não só impedir a evolução da infecção bacteriana como também modular a função de monócitos para um estado pró-inflamatório, indicando que auxiliaria o sistema immune emu ma resolução mais rápida da infecção.

Dados *in silico* predisseram que os compostos primordiais da própolis vermelha podem agir no sítio ativo de PBP2a - enzima que confere resistência aos antimicrobianos β-lactâmicos. Quando desafiamos cepas de MRSA com própolis em combinação com carbapenemas, observamos que a capacidade antimicrobiana foi tão forte quanto própolis ou imipenem e ertapenem (carbapenemas) isolados. Por outro lado, a combinação de própolis e cefoxitina (cefalosporina) não apresentou sinergismo. Estes dados fornecem pistas de que os compostos da própolis estariam agindo em PBP2a e não em β-lactamases, uma vez que MRSA não possui carbapenemases, mas sim cefalosporinases.

Este conjunto de dados aqui obtidos permitiu a elaboração de um projeto de pós-doutoramento já aprovado pela FAPESP, visando dar continuidade a esses achados.