UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Fitocompostos capazes de inibir a adesão e outros fatores de virulência bacterianos

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS como requisito parcial para a obtenção do título de Mestre em Ciências.

Orientador: Prof. Dr. Alexandre José Macedo
Co-orientador: Dra. Danielle da Silva Trentin

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When you make the finding yourself - even if you're the last person on Earth to see the light — you'll never forget it.

(Carl Sagan)

A certeza absoluta não deixa espaço para fazer mais perguntas e, portanto, não permite que se aprenda mais.

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RESUMO

O surgimento de cepas bacterianas resistentes a múltiplos fármacos impulsiona a busca por agentes antimicrobianos que possuem novos mecanismos de ação, incluindo compostos antivirulência. Apesar da ampla variedade de moléculas derivadas de química combinatória produzidas pela indústria farmacêutica, produtos naturais continuam a desempenhar um papel chave no desenvolvimento de fármacos. A seleção de plantas como fonte de compostos antimicrobianos é adequada do ponto de vista ecológico, uma vez que elas naturalmente produzem uma grande variedade de metabólitos secundários que atuam como defesa química contra micro-organismos no ambiente. Neste estudo, nós relatamos que miricetina (Myr), um flavonoide comum derivado de vegetais, frutas, nozes, frutas e chá, pode diminuir a produção de vários fatores de virulência de *Staphylococcus aureus* utilizando diferentes ensaios fenotípicos. Para explorar o mecanismo pelo qual Myr inibe a virulência de *S. aureus*, enquanto a sua forma glicosilada não, verificamos os níveis de expressão de genes relacionados à virulência e empregamos simulações de dinâmica molecular com enzimas cruciais no processo de patogênese. Além disso, Myr conferiu um grau significativo de proteção contra a infecção estafilocócica em modelo *in vivo* de *Galleria mellonella*. Outro foco deste estudo e com base em dados anteriores, o extrato de *Harpochilus neesianus* foi selecionado para o fracionamento bioguiado, uma vez que não há estudos fitoquímicos e de atividade biológica relatados na literatura para esta espécie. Utilizando o ensaio de proteinase e análises por MALDI-TOF, peptídeos foram identificados como os compostos bioativos, sendo então isolados por cromatografia em Sephadex G-50 e RP-C18. Este estudo revela compostos derivados de plantas com um elevado potencial como protótipos antivirulência contra agentes bacterianos patogênicos e uma possível aplicação destes agentes na concepção de superfícies biomédicas anti-infectivas.

Palavras-chave: fatores de virulência; terapia antivirulência; biofilme; produtos naturais; *Staphylococcus aureus*; *Staphylococcus epidermidis*

ABSTRACT

Plant-derived compounds able to inhibit adhesion and other bacterial virulence factors

The emergence of drug-resistant bacterial strains drives the search for antimicrobials possessing new modes of action, including antivirulence compounds. Despite the wide variety of molecules derived from combinatorial chemistry by the pharmaceutical industry, natural products still play a key role in the development of pharmaceuticals. The selection of plants as source of antimicrobial compounds is appropriate from the ecological standpoint, since they naturally produce a wide range of secondary metabolites that act as a chemical defense against microorganisms in the environment. In this study, we report that myricetin (Myr), a common flavonol derived from vegetables, fruits, nuts, berries and tea, can remarkably decrease the production of several *Staphylococcus aureus* virulence factors using different phenotypic assays. To explore the mechanism by which Myr inhibits *S. aureus* virulence, while its glycosylated form does not, we verified the relative expression levels of virulence related genes and employed molecular dynamics simulations with pivotal enzymes in pathogenesis process. Furthermore, Myr conferred a significant degree of protection against staphylococcal infection in *Galleria mellonella in vivo* model. In addition to this study and based on previous data, *Harpochilus neesianus* extract was selected for the bioguided fractionation, since no phytochemical studies and biological activity is reported in the literature for this species. By using proteinase assay and MALDI-TOF analyses, peptides were identified as bioactive compounds which were isolated by Sephadex G-50 and RP-C18. This study reveals plant-derived compounds with high potential as antivirulence prototypes against bacterial pathogens and a possible application of these agents in the design of anti-infective biomedical surfaces.

Keywords: virulence factors; antivirulence therapy; biofilm; natural products; *Staphylococcus aureus*; *Staphylococcus epidermidis*

1.0 INTRODUÇÃO

Um número crescente de relatos sobre micro-organismos resistentes a múltiplos fármacos, associados a novos mecanismos de resistência, tem emergido globalmente, sugerindo que podemos enfrentar o início de uma era pós-antibiótica. A indústria farmacêutica tem negligenciado por muito tempo o campo de pesquisa de novas classes de drogas antimicrobianas, onde nenhuma nova classe foi introduzida no mercado há pelo menos 25 anos (SPELLBERG *et al.*, 2008). As abordagens tradicionais para combater as infecções microbianas baseiam-se na interrupção do crescimento, principalmente através da inibição ou regulação de enzimas envolvidas na biossíntese da parede celular, na síntese e reparação de ácido nucleicos (DNA e RNA), na síntese de proteínas e também na ruptura de estruturas de membrana (KOHANSKI *et al.*, 2010). Embora essas estratégias sejam ainda eficazes, as infecções causadas por micro-organismos resistentes muitas vezes não conseguem responder ao tratamento padrão, resultando em doença prolongada, despesas de saúde mais elevadas e um maior risco associado de morte.

Estima-se que 8% a 10% da população nos Estados Unidos e 5% a 6% das pessoas em outros países industrializados tiveram o uso recomendado de dispositivos médicos implantáveis para reconstituir as funções vitais do corpo, conseguindo uma melhor qualidade de vida ou expansão da longevidade (JOUNG, 2013). No entanto, os dispositivos médicos estão associados com um potencial risco de infecções bacterianas e fúngicas. Estas infecções geralmente envolvem a colonização e formação de biofilme no próprio biomaterial, dificultando o alcance e ação de agentes antimicrobianos e das defesas do hospedeiro. Uma das estratégias mais modernas na prevenção destas infecções está relacionada com a modificação da superfície e a incorporação de agentes

antiaderentes no biomaterial (VON EIFF *et al.*, 2005). Neste sentido, dispositivos médicos constituídos de um material antiadesivo ou pelo menos resistente à colonização seriam candidatos adequados para evitar a contaminação e diminuir o uso de antimicrobianos.

Dada a emergência e propagação acelerada da resistência antimicrobiana, existe um grande interesse na descoberta de agentes capazes de controlar infecções através da atenuação da virulência (CEGELSKI *et al.*, 2008). A inibição da virulência microbiana constitui uma abordagem alternativa para o desenvolvimento de novos agentes antimicrobianos, uma vez que fatores de virulência têm um importante papel na patogênese, incluindo as etapas de colonização e invasão de tecidos, além do fato de que não são essenciais para a sobrevivência microbiana. O uso de alvos de virulência que não ameaçam a viabilidade bacteriana pode oferecer uma pressão de seleção reduzida aos fármacos, diminuindo a pressão por mutações de resistência (RASKO e SPERANDIO, 2010). Além disso, agentes antivirulência podem potencialmente ser utilizados como novos fármacos, ou em combinação com antimicrobianos já estabelecidos, dificultando a interação altamente dinâmica entre patógeno-hospedeiro ou patógeno-patógeno ou patógeno-material.

Apesar da ampla variedade de moléculas derivadas de química combinatória apresentada pela indústria, produtos naturais continuam a desempenhar um papel chave no desenvolvimento de produtos farmacêuticos (NEWMAN e CRAGG, 2016). Notavelmente, os compostos naturais que apresentam propriedades anti-infectivas, não só no tratamento direto de doenças, mas também como compostos líderes no desenvolvimento de fármacos, são responsáveis por cerca de 80% das entidades químicas que têm sido reconhecidas pelo “Food and Drug Administration” (órgão governamental dos Estados Unidos da América responsável pelo controle de

medicamentos e alimentos) ao longo dos últimos 30 anos (NEWMAN e CRAGG, 2012). A seleção de plantas como fonte de compostos antimicrobianos é adequada do ponto de vista ecológico, uma vez que elas naturalmente produzem uma grande variedade de metabólitos secundários que atuam como defesa química contra micro-organismos no ambiente. Além disso, muitos produtos naturais são entidades dotadas de diversidade química única, incluindo estruturas complexas que não podem ser facilmente obtidas por via sintética, o que pode conferir mecanismos de ação diferentes dos atuais antimicrobianos e, por conseguinte, contribuir com novas alternativas contra bactérias resistentes.

Dessa forma, este trabalho pretende estudar o potencial antivirulência de compostos derivados de plantas contra diferentes patógenos de importância clínica e também a possível aplicação destes agentes na concepção de superfícies biomédicas anti-infectivas. Os resultados estão apresentados na forma de dois manuscritos. A fim de facilitar a leitura, as tabelas e figuras foram inseridas ao longo do texto. Há ainda um manuscrito dedicado a abranger uma revisão atualizada e completa de produtos naturais isolados a partir de plantas, com estruturas quimicamente caracterizadas, que apresentaram atividade contra alguns dos principais fatores de virulência bacterianos. Podemos afirmar que nenhuma revisão na literatura até então compilou diferentes fatores de virulência bacteriana como alvos de várias classes de produtos vegetais naturais, conectando suas vias de ação com o processo de patogênese bacteriana, e com tamanho número de estruturas químicas.

1.1 PATOGENICIDADE E VIRULÊNCIA

Micro-organismos habitam toda a superfície do corpo humano, vivendo no intestino, no nariz, nos dentes e especialmente sobre a pele e as membranas mucosas. A

maioria das bactérias presentes na microbiota vivem em harmonia com seus hospedeiros humanos, muitas são benéficas, sendo algumas necessárias. No entanto, diversas bactérias, classificadas como patógenos oportunistas, são capazes de colonizar, invadir, e danificar o hospedeiro e, portanto, causar doenças infecciosas. Patogenicidade é a capacidade de um agente infeccioso causar doença, e os micro-organismos podem produzir diversos fatores de virulência, os quais podem aumentar seu grau de patogenicidade. A maioria dos patógenos causa doença através da combinação de duas propriedades: (i) toxicidade, relacionada ao grau de danos provocados por um metabólito produzido pelo patógeno, e (ii) capacidade de invasão, a capacidade do patógeno de penetrar e se propagar no hospedeiro. A relação entre o hospedeiro e um agente patogênico é dinâmica, uma vez que cada um modifica sua defesa em funções do outro. A consequência de um processo infeccioso dependerá da virulência bacteriana, bem como do *status* do hospedeiro em relação a diferentes fatores de risco, tais como o estado imunológico, idade, dieta e estresse, que podem determinar sua susceptibilidade à infecção (MADIGAN *et al.*, 2010).

Virulência pode ser definida como o efeito de determinadas características microbianas expressas durante o processo infecioso que aumentam o *fitness* microbiano. Tais características, ou fatores de virulência, são moléculas produzidas por micro-organismos que contribuem para a patogenicidade do mesmo, mas que não são necessárias para a manutenção da sua sobrevivência (CASADEVALL e PIROFSKI, 1999). Estes fatores incluem, por exemplo, a cápsula de *Streptococcus pneumoniae*, as toxinas de *Clostridium difficile* e *Vibrio cholerae*, e a proteína M de estreptococos do grupo A. Fatores de virulência podem ter uma infinidade de papéis funcionais, incluindo a capacidade para facilitar a fixação microbiana, invasão, ou ambos, bem como a promoção do crescimento de um micro-organismo em um hospedeiro através da

inibição da fagocitose e regulação da capacidade de sobrevivência intracelular (CASADEVALL e PIROFSKI, 2001). Ainda, se um número suficiente de bactérias está presente em um sítio (*quorum*), elas podem produzir biofilmes e se tornam mais virulentas que quando na forma planctônica (vida livre). A seguir, no quadro 1, estão alguns tipos de fatores de virulência:

Quadro 1. Fatores de virulência que auxiliam as bactérias a invadir o hospedeiro e evadir suas defesas (adaptado de Murray et al., 2009).

Fatores de adesão: algumas bactérias patogênicas colonizam mucosas usando pili (fímbria) para aderir às células. Outras bactérias podem ainda secretar quantidades densas de substâncias extrapoliméricas a fim de formar biofilmes.
Fatores de invasão: mecanismos que permitem uma bactéria invadir as células eucarióticas e facilitam sua entrada nas superfícies mucosas. Algumas destas bactérias são patógenos intracelulares obrigatórios, mas a maioria são agentes intracelulares facultativos.
Cápsula e outros componentes de superfície: muitas bactérias são cercadas por cápsulas que as protegem de opsonização e fagocitose.
Endotoxinas: as endotoxinas são constituídas por componentes de lipopolissacarídeos tóxicos da membrana exterior de bactérias Gram-negativas. Endotoxinas exercem efeitos biológicos profundos sobre o hospedeiro e pode ser letais, sendo liberadas quando a bactéria encontra-se em processo de degeneração da parede celular.
Exotoxinas: as exotoxinas, ao contrário das endotoxinas, são proteínas secretadas a partir de bactérias viáveis. Exotoxinas podem ser agrupadas em várias categorias (por exemplo, neurotoxinas, citotoxinas e enterotoxinas) com base no seu efeito biológico em células hospedeiras.

Sideróforos: os sideróforos são fatores de ligação de ferro, que permitem às bactérias adquirirem este íon necessário para o crescimento bacteriano. Algumas bactérias produzem sideróforos para competir com o hospedeiro por ferro.

Portanto, cada patógeno possui uma variedade de fatores de virulência específicos. Alguns destes fatores são codificados cromossomicamente e são intrínsecos para as bactérias, por exemplo, cápsulas e endotoxina, ao passo que outros são obtidos a partir de elementos genéticos móveis como plasmídeos e bacteriófagos.

Devido à magnitude do problema das doenças infecciosas, os microbiologistas e imunologistas tem se esforçado para compreender os mecanismos do sistema imune do hospedeiro, bem como identificar e caracterizar os fatores de virulência bacterianos, com objetivo de inibir fatores de virulência como forma de controle de infecções.

1.2 TERAPIA ANTIVIRULÊNCIA

O aparecimento e prevalência crescente de cepas bacterianas que são resistentes aos antibacterianos disponíveis, impulsionam a descoberta de novas abordagens terapêuticas. Neste sentido, estratégias alternativas baseadas preferencialmente na atenuação da virulência bacteriana e não na inibição do crescimento estão sendo investigadas, visto que os fatores de virulência possuem um papel fundamental na colonização e invasão, mas não são essenciais para a sobrevivência das bactérias (CEGELSKI *et al.*, 2008). Ao impedir a expressão ou a atividade de fatores de virulência, as bactérias seriam menos capazes de colonizar ou causar danos ao hospedeiro e, portanto, estariam “desarmadas”.

Neste contexto, estas estratégias apresentam uma série de vantagens, pois potencialmente devem (i) oferecer uma pressão de seleção reduzida ao desenvolvimento

de mutações de resistência, (ii) proporcionar o aumento do repertório de alvos farmacológicos, (iii) gerar antimicrobianos com novos mecanismos de ação, (iv) evitar as alterações dramáticas e indesejáveis na microbiota do hospedeiro que estão associadas com os antibacterianos atuais e (v) atenuar a virulência de modo que o sistema imune do hospedeiro possa facilmente superar a infecção (RASKO e SPERANDIO, 2010).

A combinação de fármacos antivirulência com agentes antibacterianos já estabelecidos poderia levar ao desenvolvimento de terapias otimizadas, a fim de aumentar a eficácia e reduzir potencialmente o desenvolvimento de resistência bacteriana a estes agentes já existentes. Considerando a diversidade de alvos bacterianos de virulência que poderiam ser bloqueados, a combinação desses agentes com efeito sinérgico cria várias possibilidades para futuros tratamentos (ALLEN *et al.*, 2014).

A indústria farmacêutica despertou substancial interesse na terapia antivirulência como observado para os casos dos anticorpos capazes de neutralizar toxinas bacterianas, com pelo menos cinco candidatos em ensaios clínicos (MOHAMED *et al.*, 2005; SUBRAMANIAN *et al.*, 2005; VITALE *et al.*, 2006; LÓPEZ *et al.*, 2010; LOWY *et al.*, 2010). O sucesso inicial dessas antitoxinas parece fornecer evidência empírica para uma maior investigação sobre outras abordagens antivirulência.

No capítulo 1 desta dissertação, nós revisamos sete fatores de virulência que são considerados importantes durante o processo de infecção bacteriano, sendo eles: *quorum sensing*, biofilme, motilidade, toxinas, enzimas, pigmentos e surfactantes. Uma abordagem geral sobre cada um desses fatores, assim como discussão crítica sobre os fatores de virulência mais promissores como alvos na terapia antivirulência, destacando suas vantagens e eventuais desvantagens, são também apresentados. Por fim, na discussão deste capítulo 1, nós disctutimos a possibilidade desta nova estratégia como

intervenção real em doenças infecciosas. Todos os fatores de virulência experimentalmente testados nesta dissertação, estão revisados neste capitulo 1, e portanto, não serão discutidos nesta introdução, para não tornar a leitura excessivamente repetitiva.

1.3 *GALLERIA MELLONELLA* COMO MODELO PARA ESTUDOS DE VIRULÊNCIA MICROBIANA

O estudo da virulência microbiana exige o uso de um modelo animal adequado. Como uma abordagem alternativa, diferentes modelos de infecção de invertebrados têm sido desenvolvidos, incluindo *Caenorhabditis elegans*, *Drosophila melanogaster* e *Galleria mellonella* como hospedeiros. Como resultado de semelhanças estruturais e funcionais entre o sistema imune inato de mamíferos e a resposta imune dos insetos, os novos modelos (*D. melanogaster* e *G. mellonella*) têm sido usados para avaliar a virulência de agentes patogênicos humanos e também a eficácia de agentes antimicrobianos.

As larvas do inseto *G. mellonella*, popularmente conhecida como traça da cera, estão sendo cada vez mais utilizadas para padronizar ensaios de virulência de uma série de importantes patógenos humanos, incluindo bactérias Gram-positivas e Gram-negativas (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Proteus vulgaris*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Acinetobacter baumanii*, *Escherichia coli*, *Helicobacter pylori and Burkholderia pseudomallei*, entre outros) e vários fungos (*Fusarium oxysporum*, *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, entre outros) (JUNQUEIRA, 2012; COOK e MCARTHUR, 2013). Independentemente da espécie, os resultados obtidos com as larvas de *G. mellonella* infectadas por injeção direta, consistentemente

correlacionam de forma positiva com os resultados de estudos em mamífero, ou seja, cepas que são atenuadas em modelos de mamíferos demonstram baixa virulência em *G. melonella* e cepas causadoras de infecções graves são também altamente virulentas no modelo (JANDER *et al.*, 2000; BRENNAN *et al.*, 2002; SLATER *et al.*, 2011).

O uso de insetos como modelo experimental para triagem tem atraído a atenção devido às inúmeras vantagens de invertebrados em relação aos modelos de mamíferos, incluindo: (i) aspectos éticos, sendo um invertebrado *G. mellonella* não está sujeita à limitação ética, (ii) aspectos logísticos, onde as larvas podem ser facilmente criadas e mantidas em laboratório e (iii) aspectos fisiológicos, visto que as larvas são suficientemente grandes, medindo em torno de 2 a 3 cm, o que permite a injeção, sem anestesia, de quantidade precisa de compostos ou de micro-organismos, assim como a obtenção de material histológico para estudos de dano tecidual, (iv) aspectos financeiros, com custo de manutenção relativamente baixo (GLAVIS-BLOOM *et al.*, 2012). Ao ser comparado com outros modelos invertebrados, os estudos de infecção com *G. mellonella* podem ser conduzidos em temperatura entre 15 a 37°C, o que permite mimetizar a temperatura corporal humana, diferentemente de *Drosophila* que tolera até 25°C (RAMARAO *et al.*, 2012). Na figura 1, encontram-se algumas imagens do ensaio de virulência por injeção.

Uma maior compreensão do sistema imunológico de *G. mellonella* também ajudou na caracterização deste organismo modelo (WOJDA, 2016). Embora insetos não possuam sistema imune adaptativo como os mamíferos, eles possuem uma sofisticada defesa celular e humoral, incluindo a produção de peptídeos antimicrobianos. Os hemócitos são o principais mediadores da defesa celular e são o tipo de células mais numerosas encontradas na hemolinfa de *G. mellonella* (MAK *et al.*, 2010). Estas células

são fagócitos profissionais e desempenham funções semelhantes a dos macrófagos humanos e neutrófilos (LAVINE e STRAND, 2002; BERGIN *et al.*, 2005).

Quando a literatura é revisada, se torna evidente que o número de estudos que utilizam *G. mellonella* como um modelo de hospedeiro tem aumentado significativamente nos últimos anos. Além disso, tem havido uma melhoria das técnicas utilizadas com este modelo, o que permite novas possibilidades para o desenvolvimento de outros estudos.

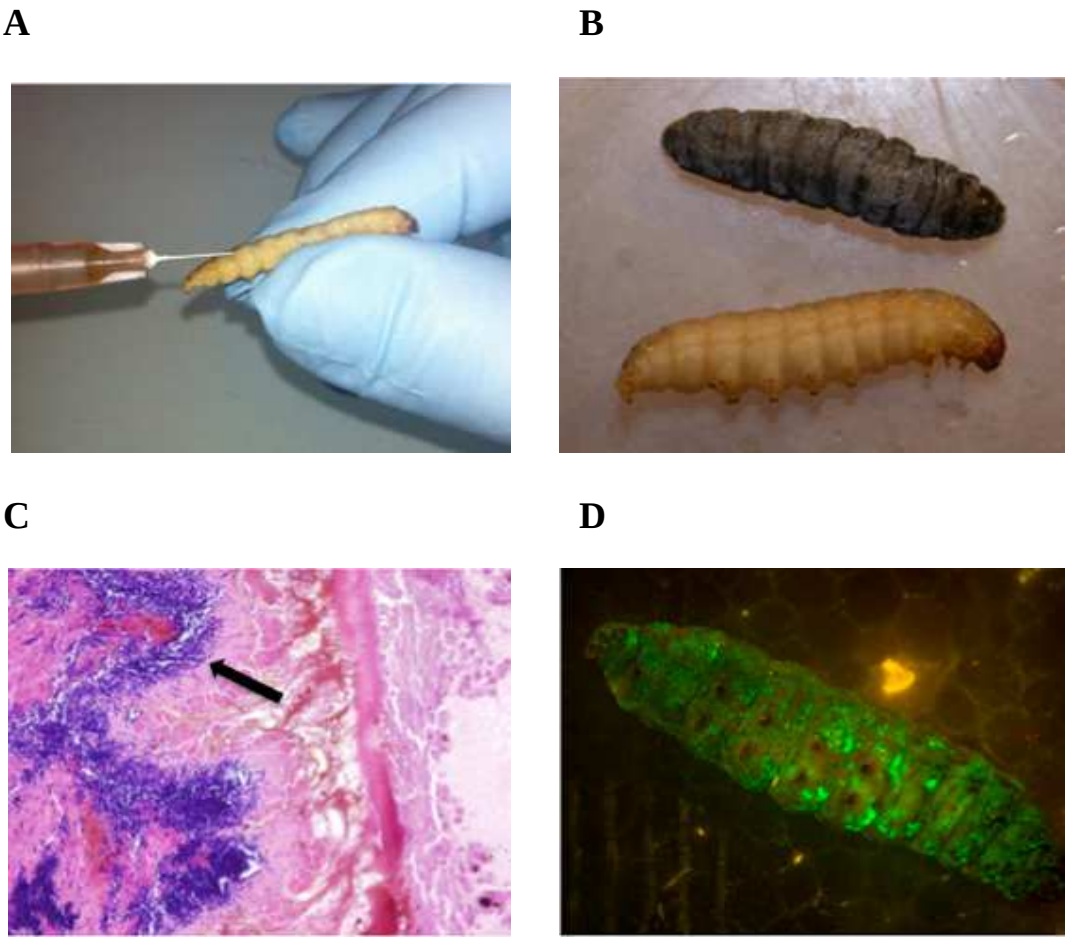

Figura 1. Imagens relacionadas à infecção no modelo de *G. melonella*. (A) Bactérias são inoculadas na hemolinfa de larvas de *G. mellonella*. (B) Comparação de uma larva morta (parte superior, preto) e viva (em baixo, branco). (C) Cortes longitudinais histológicos do inseto corados com hematoxilina, eosina e Gram. A fotografia de microscópica de luz mostra o intestino (rosa) e bacilos corados por Gram (violeta escuro) localizada na superfície intestinal apontada pela seta. (D) *Bacillus*

cereus expressando 407-GFP fluorescente foi introduzido em larvas de *G. mellonella* por alimentação forçada. Após 24 horas, as bactérias atingiram a hemolinfa e o cadáver do inseto é completamente preenchido pela proteína fluorescente verde (Adaptado de Ramarao et al., 2012).

1.4 INFECÇÕES ASSOCIADAS À *STAPHYLOCOCUS* SPP.

Cocos Gram-positivos são alguns dos agentes etiológicos mais frequentemente associados a infecções bacterianas encontradas na comunidade e adquiridas em ambientes hospitalares (ZIEBUHR *et al.*, 2006; STRYJEWSKI e CHAMBERS, 2008; DAVID e DAUM, 2010). O nome do gênero *Staphylococcus* refere-se ao fato de estes cocos Gram-positivos crescerem num padrão semelhante a um cacho de uvas. A maioria dos estafilococos possuem entre 0,5 e 1,5 μm de diâmetro sem apêndices especiais usados para motilidade, e são capazes de crescer em uma variedade de condições, aerobicamente e anaerobicamente, na presença de concentrações elevadas de sal e a temperaturas que variam entre 18 °C a 40 °C (MURRAY *et al.*, 2009).

O gênero *Staphylococcus* compreende cerca de 39 espécies, as quais podem ser divididas em dois grupos: os coagulase positivos, isto é cepas capazes de produzir coagulação da fibrina no sangue, no qual *S. aureus* é a principal espécie, e os coagulase negativos, no qual se enquadram as demais espécies (BECKER e EIFF, 2011). Infecções por estafilococos são geralmente causadas por *S. aureus*, que pode se comportar tanto como uma bactéria comensal como um patógeno oportunista. Os tipos mais comuns de infecções causadas por *S. aureus* incluem as seguintes:

- **Lesões superficiais ou de tecidos moles** (por exemplo, foliculite, furúnculos, impetigo, infecções de feridas, piomiosite, bursite séptica, artrite séptica)

- **Doenças toxinogênicas** (toxinoses) (intoxicação alimentar, síndrome do choque tóxico e síndrome da pele escaldada)
- **Infecções graves e/ou sistêmicas** (endocardite, osteomielite, pneumonia, meningite e bacteremia)
- **Infecções relacionadas a dispositivos protéticos** (por exemplo, articulações protéticas e válvulas cardíacas; shunts vasculares, enxertos, cateteres)

Esta variedade de manifestações clínicas está relacionada com uma vasta produção de fatores de virulência que lhe permite aderir às superfícies, evadir o sistema imunológico, e causar efeitos tóxicos prejudiciais às células (TONG *et al.*, 2015). Para obter acesso ao hospedeiro, *S. aureus* expressa distintas classes de proteínas de superfície que promovem a fixação, sendo uma destas conhecidas coletivamente como MSCRAMMs (*Microbial Surface Components Recognizing Adhesive Matrix Molecules* - componentes da superfície microbiana que reconhecem moléculas adesivas de matriz), que são ancoradas por uma enzima chamada sortase específica de bactérias Gram-positivas (FOSTER *et al.*, 2013). Estas proteínas MSCRAMMs são responsáveis por reconhecer os componentes mais importantes da matriz extracelular ou plasma do sangue humano, incluindo fibrinogênio, fibronectina e colágeno (VENGADESAN e NARAYANA, 2011). Na superfície da bactéria encontram-se, portanto, diversas proteínas que são covalentemente ancoradas na parede celular de peptidoglicano. Na última década, essas proteínas estão recebendo especial atenção por serem consideradas alvos terapêuticos interessantes no *design* de vacinas a fim de conferir proteção contra infecções por *S. aureus* (KUKLIN *et al.*, 2006; STRANGER-JONES *et al.*, 2006; GLOWALLA *et al.*, 2009).

Não menos importantes, as exoproteínas produzidas por *S. aureus* também contribuem para a sua capacidade de invadir e causar doença. Quase todas as cepas secretam um grupo de enzimas e citotoxinas que inclui hemolisinas (alfa, beta, gama e delta), nucleases, proteases, lipases, hialuronidases e colagenases. A função principal destas proteínas pode ser facilitar a destruição tecidual, muitas destruindo membranas biológicas e lisando células do sistema imune, com o objetivo de converter os tecidos do hospedeiro em nutrientes necessários para o seu crescimento e invasão. Algumas cepas produzem ainda uma ou mais toxinas, que incluem a toxina da síndrome do choque tóxico (TSST-1), as enterotoxinas estafilocócicas (SEA, SEB, SEC, SED, SEE, SEG, SEH, e SEI), as toxinas esfoliativas tóxicas (ETA e ETB) e leucocidinas (PVL, LukAB e LukDE) (DINGES *et al*., 2000; OTTO, 2014).

A expressão coordenada de diversos fatores de virulência em resposta a condições ambientais durante as infecções (por exemplo, a expressão prévia de adesinas durante a colonização *versus* a produção de toxinas tardiamente para facilitar a propagação no tecido) aponta para a existência de reguladores globais que controlam a expressão de muitos genes não relacionados. Em *S. aureus* a produção destes fatores é controlada por vários *loci* de reguladores globais, tais como *agr*, *sar*A, *sae*, *Sig*B, *alr* e vários homólogos de *Sar*A. Desta forma, um gene de virulência alvo pode estar sob a influência de vários reguladores que "cruzam informações" para garantir que o gene específico seja expresso apenas quando necessário (BRONNER *et al.*, 2004; CHEUNG *et al.*, 2004).

Em contraste, *Staphylococcus epidermidis* não é reconhecido por produzir determinantes de virulência como *S. aureus*, mas sim, fatores que promovem sua persistência, particularmente a formação de biofilmes. Esta espécie também normalmente reside na pele e membranas mucosas humanas saudáveis sendo que

raramente causa doença. Contudo, *S. epidermidis* tem sido frequentemente identificado como agente etiológico de infecções relacionadas a dispositivos biomédicos (ZIEBUHR *et al.*, 2006; OTTO, 2009). *S. epidermidis* possui uma habilidade destacada para a formação de biofilmes, estilo de vida na forma de grupamentos bacterianos envoltos por matriz exopolissacarídica, como a PIA, que inibe os mecanismos principais de defesa do hospedeiro (OTTO, 2009).

O rápido aumento de infecções sistêmicas graves e o aparecimento de muitas cepas resistentes, como *S. aureus* resistente à meticilina (MRSA) e *S. aureus* resistente à vancomicina (VRSA), tem tornado o tratamento destas infecções um grande desafio em ambientes hospitalares e na comunidade, podendo ser por vezes fatais (LOWY, 2003; LOOMBA *et al.*, 2010). Importante salientar que a diminuição da sensibilidade aos antibibacterianos contribui também para a persistência de infecções associadas a biofilme, tais como as associados com os dispositivos implantados (HØIBY *et al.*, 2010). A classe mais comumente prescrita de antibacterianos para estas infecções foram até então os β-lactâmicos, os quais incluem penicilinas e cefalosporinas, contudo o agente de escolha para cada tratamento é dependente da extensão, do sítio da infecção e do perfil de resistência (TONG *et al.*, 2015). Sem dúvida, o desenvolvimento de novos agentes antimicrobianos e de novas abordagens terapêuticas são urgentemente necessários.

1.5 INFECÇÕES ASSOCIADAS A BIOMATERIAIS

Os avanços na medicina moderna, bem como o aumento da sua disponibilidade global, tiveram como principal consequência melhorias na qualidade de vida humana, resultando em um aumento do número de pessoas que vivem mais de 80 anos. Simultaneamente, o uso de marca-passo, sensores de glicose contínua, equipamentos

cardiovasculares e oculares, implantes ortopédicos, e muitos outros biomateriais aumentaram o bem-estar e qualidade de vida de muitas pessoas, particularmente em UTIs. O uso de dispositivos médicos, em geral, tem assistido a um crescimento exponencial em aplicações clínicas nas últimas cinco décadas (AVULA e GRAINGER, 2015). Estima-se que o mercado global de dispositivos médicos continue a crescer, atingindo cerca de US$ 434 bilhões até 2017, com uma taxa de crescimento anual de cerca de 7,1% entre 2012 e 2017 (RAO *et al.*, 2015).

Entretanto, o aumento do uso destes implantes, resultou em concomitante aumento de infecções bacterianas relacionadas a biomateriais. A formação de biofilmes na superfície destes dispositivos é geralmente reconhecida como a agente destas infecções (DONLAN, 2001). Os patógenos são normalmente introduzidos durante a implantação do dispositivo ou são transportados para a superfície do biomaterial por uma bacteremia temporária. A presença de grandes estruturas tridimensionais na forma de biofilmes já foi demonstrada por microscopia eletrônica de varredura em diferentes dispositivos como cateteres e próteses vocais (Figura 2).

A **B**

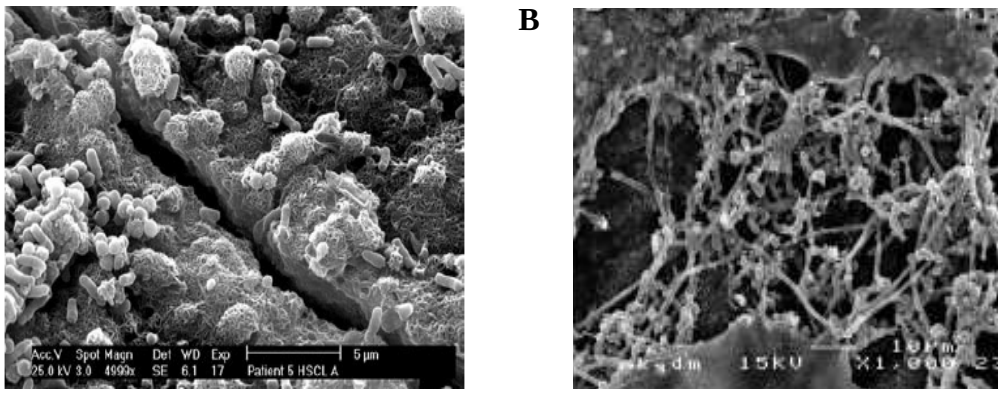

Figura 2. Biofilmes em dispositivos médicos. A) Desenvolvimento de biofilme cristalino em cateter de látex apenas 5 dias após ter sido inserido, onde bacilos e cocos pode ser vistos. B) Formação de biofilme em prótese vocal constituída de poliuretano proveniente de pacientes laringectomizados (adaptado de Stickler, 2008; Leonhard et al., 2010).

A colonização e o desenvolvimento de infecção em dispositivos médicos é dependente de muitos fatores incluindo a química do biomaterial, as propriedades físicas da superfície, o desenho do dispositivo médico, o local anatômico implantado, a extensão da invasão cirúrgica, o tempo de aplicação e a resposta do hospedeiro (RIMONDINI *et al.*, 2005). Devido à alta incidência (Tabela 2), os cateteres urinários e vasculares são os dispositivos médicos que mais trazem preocupação, pois estima-se que 5 a 25% dos pacientes cateterizados apresentam complicações relacionadas a estas infecções (DIONI *et al.*, 2014; NICOLLE, 2014).

Tabela 2. Incidência de infecções associadas aos biomateriais em diferentes dispositivos. Dados de incidência referem-se ao longo de todo o implante ou vida do dispositivo, salvo indicação contrária (adaptado de Busscher et al., 2012).

Local/Tecido próximo ao implante	**Implante ou dispositivo**	**Incidência de infeção durante o período de uso (%)**
Trato urinário	Cateter	33 (por semana)
Percutâneo	Cateter venoso central	2 - 10
	Marca-passo temporário	4
	Cateter interno curto	0 - 3
	Cateter de diálise peritoneal	3 - 5
	Pino de fixação ou parafuso	5 - 10
	Suturas	1 - 5
	Prótese vocal	25 (por mês)
	Implante dentário	5 - 10
Subcutâneo	Marca-passo cardíaco	1 - 7
	Prótese peniana	2 - 5
Tecidos moles	Prótese mamaria	1 - 7
	Parede abdominal	1 - 16
	Lentes intraoculares	0.1
Olho	Lentes de conato	0.1 – 0.5
Sistema circulatório	Prótese valvular cardíaca	1 - 3
	Enxerto vascular	1.5
Ossos	Prótese de quadril	2 - 4
	Prótese de joelho	3 - 4
	Prego tibial	1 - 7

Uma vez que a quimioterapia antibacteriana não é frequentemente capaz de curar estas infecções, apesar do uso de agentes antibacterianos com comprovada atividade *in vitro*, a remoção do dispositivo implantável é muitas vezes inevitável e tem sido uma prática clínica normal. Entretanto, em alguns casos a remoção do dispositivo médico infectado é difícil de ser realizada ou apresenta grande risco, deixando como única opção a administração de terapias antimicrobianas. Como os biofilmes possuem níveis maiores de resistência a agentes antimicrobianos, podendo ser 10 a 1000 vezes mais resistentes, esta abordagem pode falhar na erradicação destas infecções e ainda contribuir para aumento de cepas resistentes (VON EIFF *et al.*, 2005).

1.6 ESTRATÉGIAS PARA PREVENIR INFECÇÕES ASSOCIADAS A BIOMATERIAIS

Os impactos sociais, clínicos e econômicos importantes destas infecções relacionadas com dispositivos vem promovendo esforços para evitar essas doenças graves. Atualmente, não há uma única estratégia que possa eliminar totalmente a incidência de infecções associadas aos biomateriais. Infecções relacionadas a dispositivos podem ser evitadas com sucesso através da realização de uma série de ações complementares, cada uma contribuindo para diminuir a taxa de incidência. As normas aplicadas nos hospitais para lidar com as infecções pós-cirúrgicas incluem controle de assepsia, procedimentos de esterilização, protocolos adequados de profilaxia antibacteriana perioperatória e gestão adequada de antibacterianos. Juntamente com todas estas medidas preventivas, uma importante estratégia que tem progressivamente se destacado ao longo dos anos é o uso de biomateriais que são menos suscetíveis a infecções bacterianas. Ou seja, apesar de os fatores microbianos serem provavelmente

os mais importantes na patogênese da infecção, as propriedades dos dispositivos são as mais passíveis de modificação com o objetivo de prevenir a infecção (CAMPOCCIA, D. *et al.*, 2013).

A adesão bacteriana em superfícies de biomateriais é conhecida por ser regulada através de múltiplos mecanismos, alguns dos quais são transversais a todas as espécies microbianas, enquanto outros são específicos de cada espécie. O mecanismo inicial da adesão ocorre via adsorção passiva das células bacterianas sobre o material sólido através de interações físico-químicas de superfície do material e da célula. Posteriormente, se sobrepõem os mecanismos ativos de adesão mediada por estruturas bacterianas denominadas adesinas. Por conseguinte, há acumulação de múltiplas camadas bacterianas, formando estruturas conhecidas como biofilmes. Os avanços tecnológicos atuais têm como objetivo eliminar ou reduzir substancialmente estes mecanismos, ampliando o *design* de novos biomateriais e superfícies dotados de propriedades anti-infecciosas (ARCIOLA *et al.*, 2012; CAMPOCCIA, DAVIDE *et al.*, 2013).

Materiais resistentes à infecção podem ser construídos por vários métodos. A aplicação de revestimentos de superfície e a modificação da composição química da superfície dos substratos são geralmente considerados como sendo uma abordagem química para modificação superficial (como polimerização de superfície e funcionalização), ao passo que, a modificação da arquitetura da superfície do substrato pode ser considerada uma abordagem física (HASAN *et al.*, 2013). Os revestimentos antiaderentes e/ou bactericidas têm sido desenvolvidos com o intuito de assegurar novas propriedades anti-infecciosas desejáveis na interface do tecido com o biomaterial sem comprometer a qualidade das características do próprio material. Assim, é desejado um equilíbrio correto entre os efeitos bactericidas e propriedades de biocompatibilidade,

especialmente a citotoxicidade, citocompatibilidade e imunocompatibilidade. Resumidamente, diferentes estratégias são propostas a fim de reduzir a vulnerabilidade dos dispositivos médicos ao desenvolvimento de infecções (Figura 3).

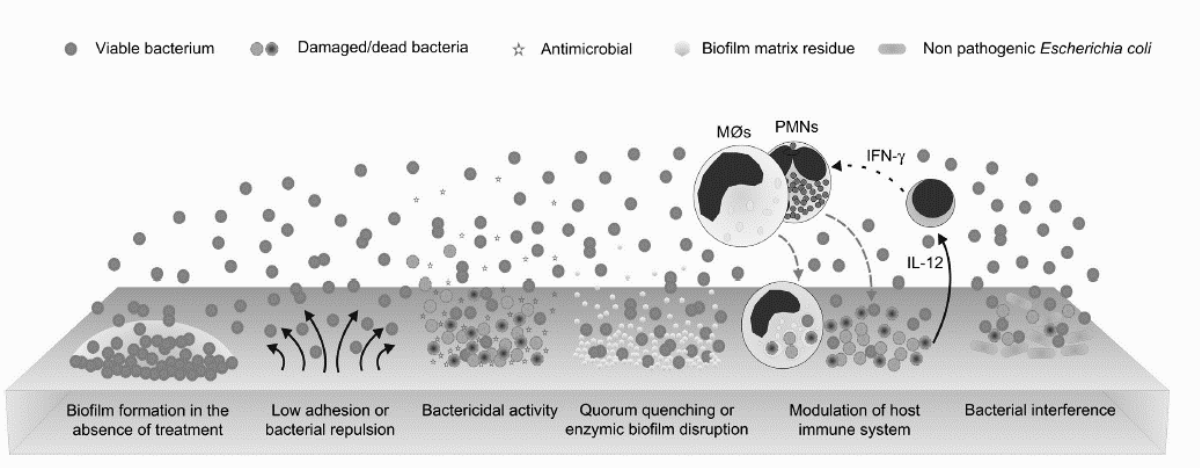

Figura 3. Diferentes mecanismos de ação propostos para o desenvolvimento de superfícies anti-infecciosas (adaptado de Campoccia *et al*., 2013a).

1.7 PLANTAS DA CAATINGA COMO FONTE DE COMPOSTOS ANTIADERENTES

A Caatinga brasileira, localizada na região nordeste do Brasil, é uma floresta tropical sazonalmente seca, composta por um mosaico de vegetação arbustiva e manchas de floresta seca. Este bioma é submetido a um clima quente e seco característico de regiões semiáridas. Ele cobre a maior parte dos estados do Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia e parte do nordeste de Minas Gerais, ocupando uma área de aproximadamente 735.000 km^2. A Caatinga é a única região totalmente inserida no território nacional brasileiro, o que significa que grande parte do seu patrimônio biológico não pode ser encontrado em nenhum outro lugar do planeta. Apesar de suas condições climáticas extremas, a

Caatinga abriga uma biodiversidade faunística e florística heterogênea e diversificada (LEAL *et al.*, 2005; SANTOS *et al.*, 2011).

Entretanto, este bioma tem sua diversidade de recursos vegetais ainda pouco estudada. As comunidades locais fazem uso de práticas medicinais tradicionais para tratar uma variedade de doenças, incluindo distúrbios gastrointestinais, tuberculose, infecções do trato urinário, entre outras (AGRA, M. F. *et al.*, 2007; AGRA, M F *et al.*, 2007). Devido às condições ambientais extremas encontradas no semiárido, é esperado que estas plantas possuam grande diversidade metabólica, sugerindo seu potencial como fontes de biomoléculas com atividades biológicas importantes, incluindo potencial antimicrobiano e antivirulência.

TRENTIN *et al.* (2011) e TRENTIN *et al.* (2014) conduziu as primeiras triagens de plantas da Caatinga com potencial atividade antibiofilme contra dois importantes patógenos como *S. epidermidis* e *Pseudomonas aeruginosa*, onde foram investigadas 24 espécies de plantas, destacando os extratos de *Commiphora leptophloeos*, *Myracrodruoun urundeuva, Anadenanthera colubrina* e *Pityrocarpa moniliformis*. Os taninos condensados identificados nos extratos de *C. leptophloeos, A. colubrina* e *M. urundeuva* foram responsáveis pela inibição do biofilme de *P. aeruginosa* por meio de propriedades bacteriostáticas, danificando a membrana bacteriana e impedindo a produção de matriz (TRENTIN *et al.*, 2013). Diferentemente, os taninos encontrados como ativos no extrato de *P. moniliformis* não apresentaram atividade antimicrobiana, sendo capazes de inibir a adesão de *S. epidermidis* através de diferentes mecanismos como repulsão electrostática, elevada hidrofilicidade e impedimento espacial (TRENTIN *et al.*, 2015).

Posteriormente, no estudo de SILVA *et al.* (2015) outras 14 espécies também tiveram suas atividades testadas contra os mesmos patógenos, onde os extratos de *Harpochilus neesianus*, *Apuleia leiocarpa* and *Poincianella microphylla* apresentaram citotoxicidade limitada contra células mamíferas e alto potencial antibiofilme.

2.1 OBJETIVOS GERIAS

Investigar a ação de fitocompostos como agentes atenuadores da virulência bacteriana, contribuindo para o desenvolvimento de estratégias alternativas no combate de infecções.

2.2 OBJETIVOS ESPECÍFICOS

- Rastrear fitocompotos da biblioteca do laboratório em diferentes ensaios antivurulência *in vitro*.
- Realizar o fracionamento bioguidado de um dos extratos de plantas da Caatinga com atividade antibiofilme promissora visando a purificação dos compostos bioativos.
- Estudar as potenciais vias de ação dos compostos puros na adesão e em outros fatores de virulência
- Determinar a toxicidade dos compostos no modelo de larvas de *G. mellonella.*
- Investigar a ação dos compostos antivirulência *in vivo*, utilizando como hospedeiro as larvas de *G. mellonella.*

Os dados desta dissertação são apresentados sob a forma de capítulos referentes aos manuscritos em construção e/ou submetidos. Decidiu-se por apresentar as figuras e tabelas no decorrer do texto devido à praticidade de leitura e correção.

3.0 CAPÍTULOS

CAPÍTULO 1

PLANT NATURAL PRODUCTS TARGETING BACTERIAL VIRULENCE FACTORS. SILVA LN, ZIMMER KR, MACEDO AJ, TRENTIN DS.

Manuscrito aceito no periódico **CHEMICAL REVIEWS** (*in press*)

CHEMICAL REVIEWS

Review

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Plant Natural Products Targeting Bacterial Virulence Factors

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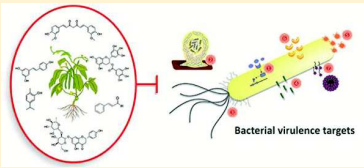
ABSTRACT: Decreased antimicrobial efficiency has become a global public health issue. The paucity of new antibacterial drugs is evident, and the arsenal against infectious diseases needs to be improved urgently. The selection of plants as a source of prototype compounds is appropriate, since plant species naturally produce a wide range of secondary metabolites that act as a chemical line of defense against microorganisms in the environment. Although traditional approaches to combat microbial infections remain effective, targeting microbial virulence rather than survival seems to be an exciting strategy, since the modulation of virulence factors might lead to a milder evolutionary pressure for the development of resistance. Additionally, anti-infective chemotherapies may be successfully achieved by combining antivirulence and conventional antimicrobials, extending the lifespan of these drugs. This review presents an updated discussion of natural compounds isolated from plants with chemically characterized structures and activity against the major bacterial virulence factors: quorum sensing, bacterial biofilms, bacterial motility, bacterial toxins, bacterial pigments, bacterial enzymes, and bacterial surfactants. Moreover, a critical analysis of the most promising virulence factors is presented, highlighting their potential as targets to attenuate bacterial virulence. The ongoing progress in the field of antivirulence therapy may therefore help to translate this promising concept into real intervention strategies in clinical areas.



CONTENTS

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1. INTRODUCTION

Bacterial pathogenicity is a complex process involving a wide range of extracellular and cell wall components that are coordinately expressed during different stages of infection, disrupting the balance between bacteria and host. Bacterial pathogenesis is generally considered to be multifactorial due to the combined action of virulence factors through the following

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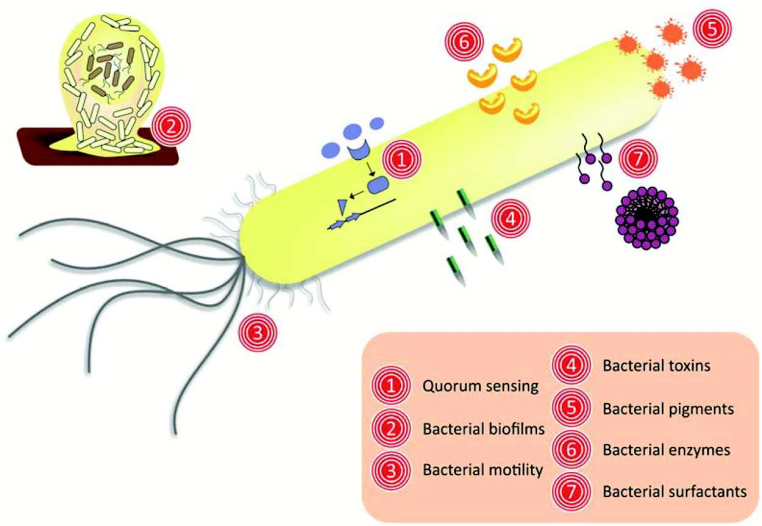

Figure 1. Bacterial virulence factors addressed in this review as targets for antivirulence agents.

steps: (i) host invasion, (ii) tissue colonization, (iii) tissue damage, and (iv) host defenses evasion.[1] Traditional approaches to combat microbial infections are based on interruption of cell growth, more specifically by inhibiting cell wall synthesis, destabilizing components of membrane, inhibiting DNA replication, or restricting protein synthesis. Although these strategies remain effective and have been of great importance to treat infectious diseases, microorganisms presenting multidrug resistance to conventional antimicrobials become more common and today are considered a major public health problem.[2] We are nowadays facing a *post-antibiotic* era, in which some antibiotics are no longer effective against various microbial threats in healthcare institutions.[3] Data published by the American Centers for Diseases Control and Prevention point to over 2 million illnesses caused by bacteria and fungi that are resistant to at least a few classes of antibiotics.

Antivirulence therapy is an alternative approach to controlling bacterial infections, which target the virulence rather than the viability of pathogens, leading to less severe infections of a level that can be cleared by the host's immune system. Antivirulence therapy has a number of advantages when compared to classical antibiotic therapy, since virulence factors are not essential for microbial survival and therefore have the potential (i) to produce a mild evolutionary pressure for development of resistance, (ii) to provide an increased repertoire of pharmacological targets, and (iii) to generate antimicrobial agents presenting novel action mechanisms.[4] Antitoxin agents have been the object of clinical trials, such as monoclonal antibodies against toxin A from *Clostridium difficile*, shiga-like toxin produced by *Escherichia coli*, and an organo-selenium compound that blocks the toxic effects of *C. difficile* toxin B.[5−7] In this regard, the path to improve therapies may lie in combining antivirulence drugs with already established antibacterials in order to increase efficacy and potentially reduce bacterial resistance development to these existing agents. Considering the diversity of bacterial virulence targets that could be blocked, the combination of agents with synergistic effect may shed light on several possibilities for future treatments.

Despite the wide variety of molecules derived from combinatorial chemistry designed by the pharmaceutical industry, natural products still play a key role in the development of pharmaceuticals.[8] Following the so-called "Golden Age of Antibiotics (1940−1960)", when several antibiotics were revealed to be produced especially by microorganisms, new classes of antibiotics have not emerged in the past 50 years. However, microorganisms remain a valuable source of bioactive natural metabolites, particularly marine microorganisms, which represent an underexplored niche in the drug discovery scenario.[8,9] Notably, natural compounds presenting anti-infective properties, not only for the direct treatment of human diseases but also as lead compounds in drug design, account for about 80% of the pharmaceutical entities recognized by the Food and Drug Administration over 30 years.[10] The 2015 Nobel Prize in Physiology or Medicine brought special attention to medically natural products from plants. Searching for antimalarial drugs, Tu and collaborators screened about 2000 Chinese herbal remedies. The isolation of the artemisinin, which has saved millions of lives, highlights the importance of the immense diversity of products in nature.[11] From the ecological standpoint, the selection of plants as source material of antimicrobial compounds is an appropriate strategy, since plants naturally produce a wide range of secondary metabolites that act as a chemical line of defense against microorganisms in the environment.[12] Additionally, many natural products are chemical entities endowed with uniquely diverse features, including complex structures that could not be easily obtained by the synthetic pathway, which may grant a totally different array of mechanisms of action as compared with antibacterials.

Here we present an updated and complete review of natural products isolated from plants with chemically characterized structures and significant activity against the major bacterial virulence factors, addressing all original papers published up to June 2015 (Table 1). Whenever possible, we brought up the compounds' pathways of action, as discussed by the authors of each paper. The report has been divided first on the basis of bacterial target and then chemical classes, which allows comparative evaluations of structures for each virulence target. We systematically covered the action of plant natural products, including 371 chemical structures, against seven well-recognized bacterial virulence factors, such as (1) quorum sensing,

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Table 1. Summary of Plant-Derived Compounds Displaying Antivirulence Activity[a]

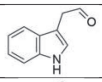
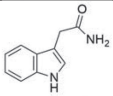
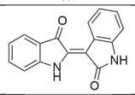
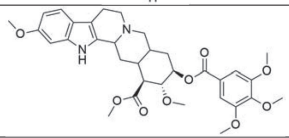
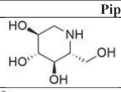
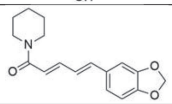
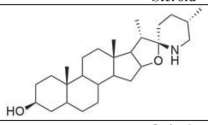
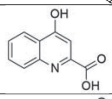
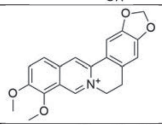
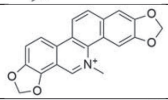
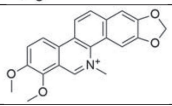
N°	Compound	Structure	Bacteria	Target	Activity	Ref
		Alkaloids and derivatives				
		Indole and dihydroindole				
(**1**)	Indole		*P. aeruginosa*	TOX	1 mM	135
(**2**)	3-Indolylacetonitrile		*P. aeruginosa*	BF	100 μg/mL	134
			E. coli	BF	25 - 150 μg/mL	
			P. aeruginosa	PIG	100 μg/mL	
			P. aeruginosa	BF TOX	1 mM	135
(**3**)	Indole-3-carbinol		*S. aureus*	BE	400 - 2000 μg/mL	29
				MOT	400 μg/mL	
			E. coli	BE	800 - 4000 μg/mL	
				MOT	800 μg/mL	
			C. violaceum	QS	250 - 5000 μg/mL	
			P. aeruginosa	TOX	1 mM	135
(**4**)	Indole-3-acetaldehyde		*P. aeruginosa*	TOX	1 mM	135
(**5**)	Indole-3-carboxyaldehyde		*P. aeruginosa*	BF	1 mM	135
(**6**)	Indole-3-acetamide		*P. aeruginosa*	BF TOX	1 mM	135
(**7**)	Indirubin		*L. monocytogenes*	BF	50 μM	136
(**8**)	Isatin		*P. aeruginosa*	BF TOX	1 mM	135
(**9**)	Reserpine		*E. coli*	MOT	0.5 - 10 μg/mL	259
			K. pneumoniae	BF	0.0078 - 0.0156 mg/mL	141
		Piperidine				
(**10**)	Deoxynojirimycin		*S. mutans*	BF	1 - 8 mg/L	137,138
(**11**)	Piperine		*E. coli*	MOT	0.5 - 10 μg/mL	259
			H. pylori	MOT	50 - 100 μM	260
		Steroid				
(**12**)	Tomatidine		*S. aureus*	TOX	8 - 12.8 mg/L	32
				QS	12.8 mg/L	
		Quinole				
(**13**)	Kinurenic acid		*C. violaceum*	QS	50 - 200 μg/mL	47
(**14**)	Berberine		*S. aureus*	ENZ	5 - 40 ug/mL	348
			S. epidermidis	BF	15 - 75 μg/mL	139,140
			K. pneumoniae	BF	0.0078 - 0.0625 mg/mL	141
			C. perfringens	ENZ	1 - 100 μM	167
(**15**)	Sanguinarine		*S. aureus* *S. epidermidis*	BF	0.1 - 1000 μM	142
(**16**)	Chelerythrine		*S. aureus* *S. epidermidis*	BF	0.1 - 1000 μM	142
				BE	1 - 1000 μM	

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Table 1. continued

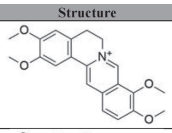
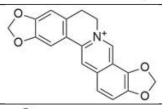
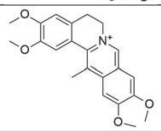
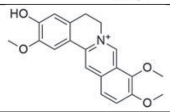
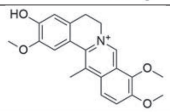
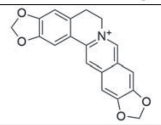
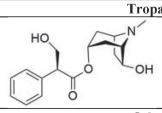
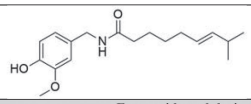
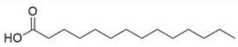
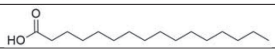
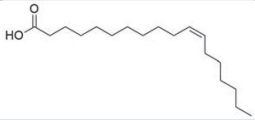
N°	Compound	Structure	Bacteria	Target	Activity	Ref
			S. aureus	ENZ	5 - 40 μg/mL	348
(**17**)	Palmatine		*C. perfringens*	ENZ	1 - 100 μM	167
(**18**)	Coptisine		*C. perfringens*	ENZ	1 - 100 μM	167
(**19**)	Pseudodehydro-corydaline		*C. perfringens*	ENZ	1 - 100 μM	167
(**20**)	Jatrorrhizine		*C. perfringens*	ENZ	1 - 100 μM	167
(**21**)	Dehydrocorybulbine		*C. perfringens*	ENZ	1 - 100 μM	167
(**22**)	Pseudocoptisine		*C. perfringens*	ENZ	1 - 100 μM	167
	Tropane					
(**23**)	Anisodamine		*S. aureus*	TOX	50 mg/kg in mice	293
	Others					
(**24**)	Caffeine		*P. aeruginosa*	QS	0.1 - 1.0 mg/mL	30
				MOT	0.1 - 0.3 mg/mL	
			C. violaceum	QS	0.1 - 0.3 mg/mL	
			P. aeruginosa	QS	25 - 200 μg/mL	31
				BF	25 - 200 μg/mL	
				MOT	25 - 200 μg/mL	
				ENZ	25 - 200 μg/mL	
				PIG	25 - 200 μg/mL	
			C. violaceum	QS	25 - 200 μg/mL	
(**25**)	Capsaicin		*S. aureus*	TOX	2 - 16 μg/mL	34
	Fatty acids and derivatives					
(**26**)	11-Methyldodecanoic acid (iso-C13:0)		*P. aeruginosa*	MOT	5 μg/mL	261
(**27**)	Lauric acid		*P. mirabilis*	MOT	499 μM	262
				TOX		
(**28**)	Myristic acid		*P. mirabilis*	MOT	55 – 437 μM	262
				TOX	437 μM	
(**29**)	Palmitic acid		*P. mirabilis*	MOT	389 μM	262
				TOX		
(**30**)	Stearic acid		*P. mirabilis*	MOT	175 μM	262
				TOX		
(**31**)	Vaccenic acid (cis-11-octadecenoic acid)		*P. aeruginosa*	MOT	5 μg/mL	261

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Table 1. continued

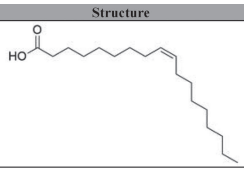
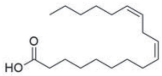
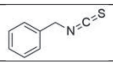
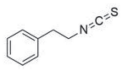
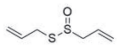
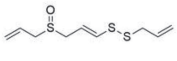
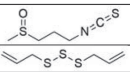
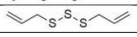
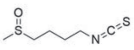
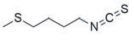
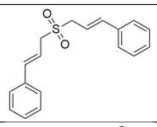
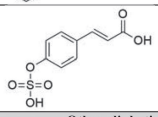
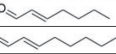
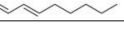
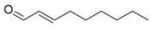
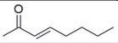
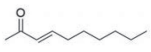
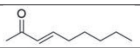
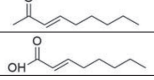
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(32)	Oleic acid (cis -9-octadecenoic acid)		*S. aureus*	BF	0.001% - 0.1%	143
			S. mutans	BE	100 μg/mL	144
			P. aeruginosa	MOT	5 μg/mL	261
(33)	Linoleic acid (cis-9, cis-12-octadecadienoic acid);		*S. mutans*	BF	100 - 200 μg/mL	145
				BE		
				BE	100 μg/mL	144
			K. pneumoniae	BF	0.0078 - 0.0312 mg/mL	141
Organosulfurs and derivatives						
(34)	Allylisothiocyanate		*C. violaceum*	QS	350 - 1000 μg/mL	35
			S. aureus *L. monocytogenes* *P. aeruginosa* *E. coli*	BF	1000 μg/mL	146
				BE		
				MOT		
(35)	Benzylisothiocyanate		*C. violaceum*	QS	15 - 250 μg/mL	35
(36)	2-Phenylethyl-isothiocyanate		*C. violaceum*	QS	250 - 1000 μg/mL	35
			S. aureus *L. monocytogenes* *P. aeruginosa* *E. coli*	BF	1000 μg/mL	146
				BE		
				MOT		
(37)	Allicin		*S. aureus*	TOX	2 - 16 μg/mL	36
			S. epidermidis	BF	0.098 - 25 μg/mL	147
				BF	0.25 - 4 mg/L	148
			S. pneumoniae	TOX	0.06 - 1.84 μmol/mL	294
			P. mirabilis	BF	2 - 32 μg/mL	149
				BE	64 - 512 μg/mL	
				ENZ	1 - 50 μg/mL	
(38)	Ajoene		*P. aeruginosa*	QS	10 - 80 μg/mL	38
				BF	100 μg/mL	
				SUR	10 – 80 μg/mL	
(39)	Iberin		*P. aeruginosa*	QS	4 - 250 μM	37
				SUR	50 - 400 μM	
(40)	Diallyl trisulphide		*H. pylori*	ENZ	4 - 14 mg/L	349
(41)	Sulforaphane		*P. aeruginosa*	QS	0.003 - 200 μM	39
				BF	0.2 - 333 μM	
				PIG	50 - 100 μM	
(42)	Erucin		*P. aeruginosa*	QS	0.003 - 200 μM	39
				BF	0.2 - 333 μM	
				PIG	50 - 100 μM	
(43)	Methyl-styryl sulfone		*V. harveyi*	QS	50 - 100 μM	40
(44)	Zosteric acid		*E. coli*	BF	500 mg/L	150
Other aliphatic compounds						
(45)	*trans*-2-Hexen-1-al		*V. harveyi*	QS	50 - 100 μM	40
(46)	*trans*-2-Heptenal		*V. harveyi*	QS	50 - 100 μM	40
(47)	*trans*-2-Octenal		*V. harveyi*	QS	50 - 100 μM	40
(48)	*trans*-2-Nonenal		*Vibrio* spp.	QS	50 - 100 μM	40
				PIG	100 μM	
				BF		
				ENZ		
(49)	*trans*-2-Decenal		*V. harveyi*	QS	50 - 100 μM	40
(50)	*trans*-2-Undecenal		*V. harveyi*	QS	50 - 100 μM	40
(51)	*trans*-2-Dodecenal		*V. harveyi*	QS	50 - 100 μM	40
(52)	*trans*-Tridecenal		*V. harveyi*	QS	50 - 100 μM	40
(53)	*trans*-3-Octen-2-one		*V. harveyi*	QS	50 - 100 μM	40
(54)	*trans*-3-Decen-2-one		*Vibrio* spp.	QS	50 - 100 μM	40
				PIG	100 μM	
				BF		
				ENZ		
(55)	*trans*-3-Nonen-2-one		*V. harveyi*	QS	50 - 100 μM	40
(56)	2-Octenoic acid		*V. harveyi*	QS	50 - 100 μM	40

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Table 1. continued

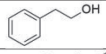
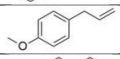
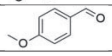
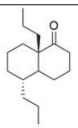
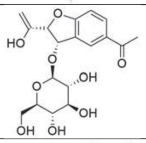
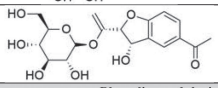
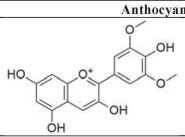
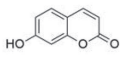
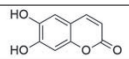
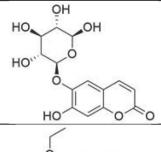
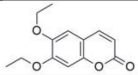
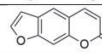
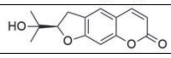
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(57)	*cis*-3-Nonen-1-ol		*C. violaceum* *P. aeruginosa*	QS PIG	0.062 – 0.125 mg/mL	41
Other cyclic compounds						
(58)	Phenylethyl alcohol		*S. aureus*	TOX	0.01 - 0.25 %	295
(59)	Estragole		*C. violaceum*	QS	0.032 - 0.062 mg/mL	41
(60)	p-Anisaldehyde		*C. violaceum*	QS	0.125 - 0.25 mg/mL	41
(61)	4-Hydroxy-2,5-dimethyl-3(2H)-furanone		*P. aeruginosa*	QS BF MOT PIG SUR ENZ	0.1 - 1.0 µM	42
(62)	5, 8a-Di-1-propyl-octahydronaphthalen-1-(2H)-one		*S. mutans*	BF	39.06 – 78.12 µg/mL	151
(63)	Gnaphaliol 3-*O*-β-D-glucopyranoside		*P. aeruginosa*	BF BE	128 µg/mL	152
(64)	Gnaphaliol 9-*O*-β -D-glucopyranoside		*P. aeruginosa*	BF BE	128 µg/mL	152
Phenolics and derivatives						
Anthocyanins						
(65)	Malvidin		*K. pneumoniae* *C. violaceum*	BF QS	5 - 20 µg/mL	43
Coumarins						
(66)	Coumarin		*E. coli*	BF MOT	5 - 100 µg/mL 50 µg/mL	153
			S. aureus *E. coli* *V.anguillarum* *E. tarda*	BF	1.36 - 1.71 mM	44
			P. aeruginosa	BF MOT PIG	1.0 - 10 mM 1.36 - 1.71 mM 1.0 - 2.0 mM	
			S. maltophilia *B. cepacia*	ENZ	1.36 - 1.71 mM	
			A. fischeri	QS	1.0 - 2.0 mM	
			S. marcescens *C. violaceum* *A. tumefaciens*	QS QS QS	25 -150 µg/TLC plate	
(67)	Umbelliferone (7-hydroxycoumarin)		*S. aureus*	BE MOT	200 -1000 µg/mL 200 µg/mL	29
			E. coli	BE MOT	800 - 4000 µg/mL 800 µg/mL	
			C. violaceum	QS	250 to 5000 µg/mL	
			E. coli	BF MOT	5 - 100 µg/mL 50 µg/mL	153
(68)	Esculetin		*P. aeruginosa*	BF	200 µM	154
(69)	Esculin		*P. aeruginosa*	BF	200 µM	154
(70)	Dimethyl-esculetin		*C. violaceum*	QS	50 - 200 µg/mL	47
(71)	Psoralen		*P. aeruginosa*	BF	200 µM	154
(72)	Nodakenetin		*P. aeruginosa* *S. malthophilia*	BF	200 µM	86

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Table 1. continued

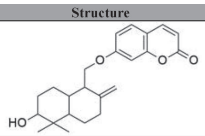
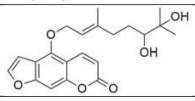
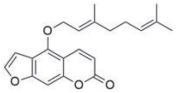
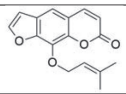
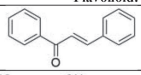
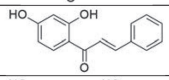
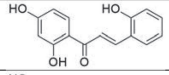
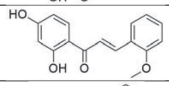
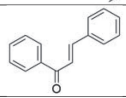
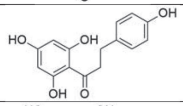
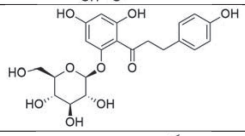
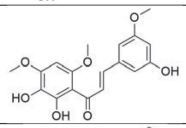
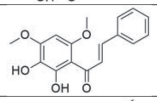
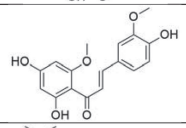
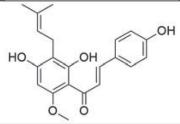
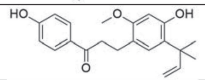
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**73**)	Coladonin		*E. coli*	BF	0.5 - 100 µg/mL	153
(**74**)	Dihydroxy-bergamottin		*P. aeruginosa* *E. coli* *S. typhimurium*	BF	100 µg/mL	48
			V. harvey	QS		
(**75**)	Bergamottin		*P. aeruginosa* *E. coli* *S. typhimurium*	BF	100 µg/mL	48
			V. harvey	QS		
			E. coli	BF	3.125 - 100 µM	155
				MOT	100 µM	
(**76**)	Imperatorin		*E. coli*	BF	3.125 - 100 µM	155
				MOT	100 µM	
		Flavonoid: Chalcones				
(**77**)	Chalcone		*S. mutans*	BF	5 - 500 µM	156
				ENZ	20 - 100 µM	
(**78**)	2',4'-Dihydroxychalcone		*S. aureus*	BF	400 µM	157
				BE		
(**79**)	2,2',4'-Trihydroxychalcone		*S. aureus*	BF	400 µM	157
				BE		
(**80**)	2',4'-Dihydroxy-2-methoxychalcone		*S. aureus*	BF	400 µM	157
				BE		
(**81**)	*trans*-Benzylidene acetophenone		*P. aeruginosa*	QS	4 mM	49
				ENZ		
(**82**)	Phloretin		*S. aureus*	TOX	50 µg/mL	177
			E. coli	BF	5 - 100 µg/ml	159
(**83**)	Phloridzin		*C. violaceum*	QS	1000 µg/mL	35
(**84**)	2',3',5-Trihydroxy-4',6',3-trimethoxychalcone		*V. harveyi*	BF	15.6 – 500 µM	50
				QS		
(**85**)	2',3'-Dihydroxy-4',6'-dimethoxychalchone		*V. harveyi*	BF	15.6 – 500 µM	50
				QS		
(**86**)	2',4',4-Trihydroxy-3,6'-dimethoxychalchone		*V. harveyi*	BF	15.6 – 500 µM	50
				QS		
(**87**)	Xanthohumol		*S. aureus*	BF	3.9 - 7.8 µg/mL	158
				BE	15 - 30 µg/mL	
(**88**)	Licochalcone A		*S. aureus*	TOX	0.25 - 2 µg/mL	51,296
				QS		

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Table 1. continued

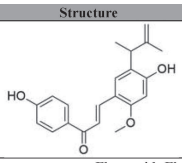
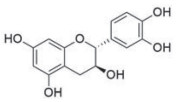
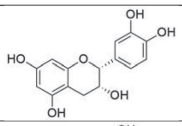
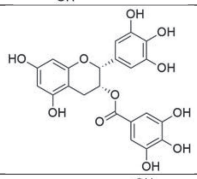
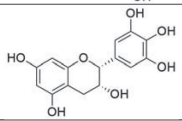
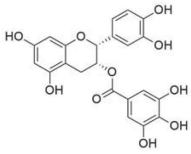
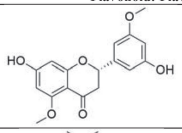
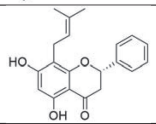
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**89**)	Licochalcone E		*S. aureus*	TOX		
				QS	0.06 - 0.5 µg/mL	52
		Flavonoid: Flavanols				
(**90**)	Catechin		*P. aeruginosa*	BF	4 µg/mL	160
				PIG	0.1125 mg/mL	180
				BF	4 mM	53
				PIG	0.125 - 16 mM	
				ENZ	4 mM	
			C. violaceum	QS	0.125 - 16 mM	
(**91**)	Epicatechin		*P. aeruginosa*	ENZ	4 mM	53
			C. violaceum	QS	1000 µg/mL	35
(**92**)	Epicatechin gallate		*S. aureus*	TOX	3.12 - 25 µg/mL	297
				ENZ	25 µg/mL	
(**93**)	Epigallocatechin		*P. aeruginosa*	BF	4 µg/mL	160
(**94**)	Epigallocatechin gallate		*S. aureus* *S. epidermidis*	BF	62.5 - 250 µg/mL	161
			S. mutans	BF	7.8 - 31.25 µg/mL	162
				BE	156.25 - 625 µg/mL	
				BF	1.95 - 1000 µg/mL	163
				BE	9.77 - 1.250 µg/mL	
			E. faecalis	BE	125 - 500 µg/mL	166
			P. aeruginosa	MOT	1.5 - 200 mg/L	263
			S. maltophilia	BF	2 - 4 mg/L	164
				BE	2 - 8 mg/L	
			V. harveyi	QS	23.25 - 93.75 µg/mL	54
			C. jejuni	BF	23.25 - 93.75 µg/mL	
				MOT	7.75 – 93.75 µg/mL	
			E. coli	QS	1.25 - 40 µg/mL	55
			P. putida	QS	2.5 - 40 µg/mL	
			B. cepacia	BF	5 - 40 µg /mL	
				MOT	40 µg/mL	
			P. gingivalis	BE	0.5 - 5 mg/mL	165
				BF	1 - 100 mg/L	
		Flavonoid: Flavanones				
(**95**)	(-)-(2S)-7,5'-Dihydroxy-5,3'-dimethoxyflavanone		*V. harveyi*	BF		
				QS	15.6 - 500 µM	50
(**96**)	Glabranine		*S. aureus*	BF		
				BE	100 µM	157

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Table 1. continued

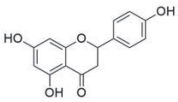
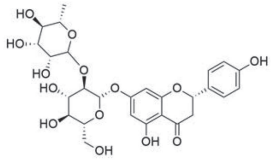
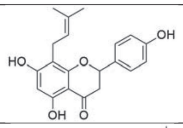
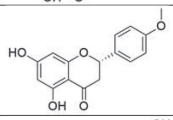
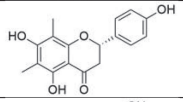
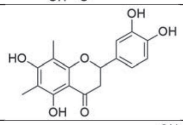
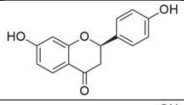
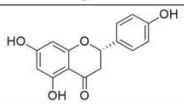
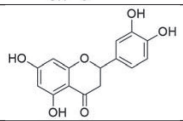
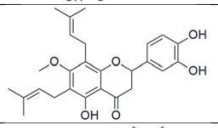
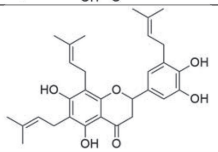
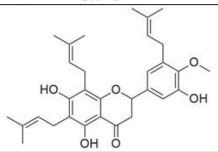
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**97**)	Naringenin		*S. aureus*	TOX	1 - 16 μg/mL	61
			L. monocytogenes	TOX	186.57 μg/mL	304
			S. typhimurium	MOT	100 μg/L	264
			P. aeruginosa *C. violaceum*	QS	4 mM	49
			P. aeruginosa	PIG		
				ENZ		
			E. coli	BF	6.25- 100 μg/mL	56
			V. harveyi	QS		
				BF		
(**98**)	Naringin		*P. aeruginosa*	BF	1.000 μg/mL	183
				MOT		
				TOX		
				ENZ		
			E. coli	BF	6.25 - 100 μg/mL	56
			V. harveyi	QS		
				BF		
			Y. enterocolitica	QS	200 μg/mL	57
				BF	95 μg/mL	
				MOT	400 μg/mL	
			C. violaceum	QS	100 - 400 μg/mL	
(**99**)	8-Prenylnaringenin		*S. aureus*	BF	100 μM	157
				BE		
(**100**)	Isosakuranetin		*S. aureus*	BF	400 μM	157
(**101**)	Farrerol		*S. aureus*	TOX	0.5 - 8 μg/mL	66
				QS		
(**102**)	Cyrtominetin		*S. aureus*	TOX	2 - 256 μg/mL	298
(**103**)	Liquiritigenin		*S. aureus*	TOX	4 - 32 μg/mL	64
				QS		
(**104**)	Pinocembrin		*S. aureus*	TOX	1 - 16 μg/mL	63
				QS		
(**105**)	Eriodictyol		*P. aeruginosa* *C. violaceum*	QS	4 mM	49
			P. aeruginosa	PIG		
				ENZ		
(**106**)	Amoradicin		*C. perfringens*	ENZ	1 - 100 μM	372
(**107**)	Amorisin		*C. perfringens*	ENZ	1 - 100 μM	372
(**108**)	Isoamoritin		*P. aeruginosa*	BF	6.25 - 25 μM	372
			C. perfringens	ENZ	1 - 100 μM	

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Table 1. continued

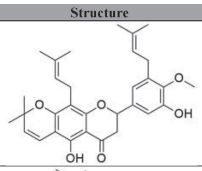
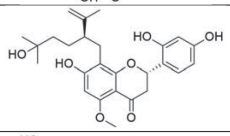
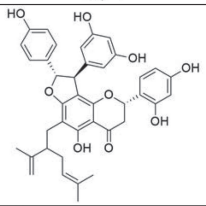
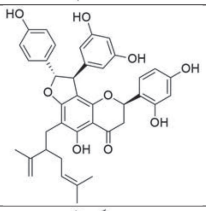
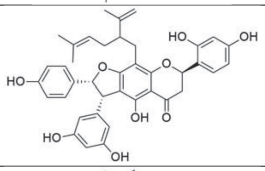
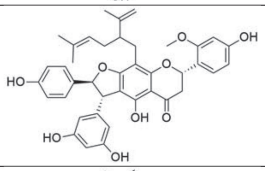
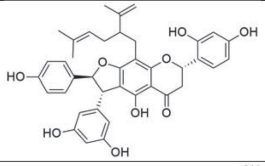
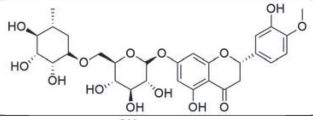
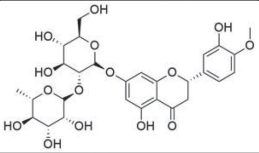
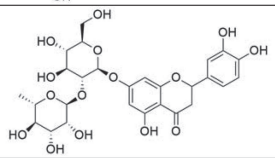
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**109**)	Amoricin		*C. perfringens*	ENZ	1 - 100 µM	372
(**110**)	Kurarinol		*S. aureus*	ENZ	25 - 100 µg/mL	350
(**111**)	Alopecurone H		*S. epidermidis*	BF	1.56 - 100 µg/mL	168
(**112**)	Alopecurone I		*S. epidermidis*	BF	1.56 - 100 µg/mL	168
(**113**)	Alopecurone J		*S. epidermidis*	BF	1.56 - 100 µg/mL	168
(**114**)	Alopecurone D		*S. epidermidis*	BF	1.56 - 100 µg/mL	168
(**115**)	Alopecurone A		*S. epidermidis*	BF	1.56 - 100 µg/mL	168
(**116**)	Hesperidin		*E. coli* *Y. enterocolitica* *C. violaceum*	BF BF / QS QS	6.25 - 100 µg/mL 200 µg/mL 100 - 400 µg/mL	56 57
(**117**)	Neohesperidin		*E. coli* *V. harveyi* *Y. enterocolitica* *C. violaceum*	BF QS / BF QS / BF QS	6.25 - 100 µg/mL 200 µg/mL 100 - 400 µg/mL	56 57
(**118**)	Neoeriocitrin		*E. coli* *V. harveyi*	BF / QS BF	6.25 - 100 µg/mL	56

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Table 1. continued

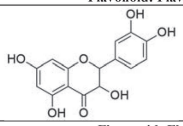
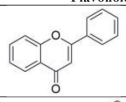
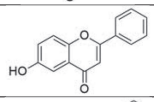
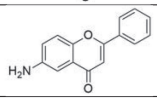
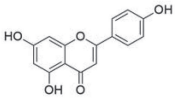
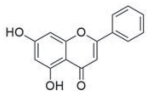
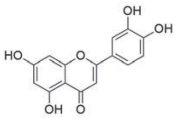
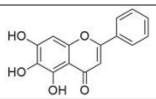
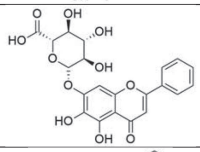
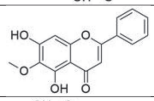
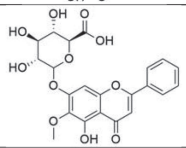
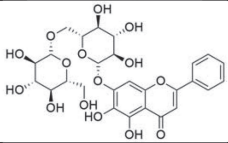
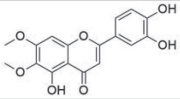
N°	Compound	Structure	Bacteria	Target	Activity	Ref
		Flavonoid: Flavanonols				
(**119**)	Taxifolin		*P. aeruginosa*	QS	4 mM	49
				PIG		
				ENZ		
			C. violaceum	QS		
		Flavonoid: Flavones				
(**120**)	Flavone		*S. aureus*	PIG	50 μg/mL	177
				TOX	20 - 50 μg/mL	170
					25 - 50 μg/mL	177
(**121**)	6-Hydroxyflavone		*S. aureus*	TOX	50 μg/mL	177
(**122**)	6-Aminoflavone		*S. aureus*	TOX	50 μg/mL	177
(**123**)	Apigenin		*S. aureus*	BF	20 - 50 μg/mL	170
				TOX	20 μg/mL	
				TOX	50 μg/mL	177
				TOX	1 - 8 μg/mL	62
				QS		
			S. mutans	BE	1.33 mM	169
			E. coli	BF	50 μg/mL	159
			E. coli	BF	6.25 - 100 μg/mL	56
			V. harveyi	QS		
				BF		
(**124**)	Chrysin		*S. aureus*	BF	20 - 50 μg/mL	170
				TOX	2 - 16 μg/mL	58
					20 μg/mL	170
				QS	2 - 16 μg/mL	58
			L. monocytogenes	TOX	8 – 128 μg/mL	304
			E. coli	BF	50 μg/mL	159
(**125**)	Luteolin		*S. aureus*	BF	20 - 50 μg/mL	170
				TOX	20 μg/mL	
				TOX	25 μg/mL	177
				TOX	2 - 16 μg/mL	65
				QS	2 - 16 μg/mL	65
				BF	20 - 50 μg/mL	176
			E. coli	BF	12.5 - 50 μg/mL	172
				MOT		
(**126**)	Baicalein		*P. aeruginosa*	BF	0.2 - 200 μM	154
(**127**)	Baicalin		*S. aureus*	TOX	2 - 128 μg/mL	300
			L. monocytogenes	TOX	0.5 - 128 μg/mL	304
			P. aeruginosa	BF	200 μM	154
			H. pylori	ENZ	0.42 - 1 mM	357
(**128**)	Oroxylin A		*S. aureus*	TOX	1 - 8 μg/mL	299
					2 - 32 μg/mL	302
(**129**)	Oroxylin A 7–*O*-glucoronide		*S. aureus*	TOX	0.25 - 4 μg/mL	302
(**130**)	Oroxylin B		*S. aureus*	TOX	4 - 64 μg/mL	302
(**131**)	3′,4′,5-Trihydroxy-6,7-dimethoxy-flavone		*S. aureus*	BF	40 - 80 μg	173
				BE	80 μg	

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Table 1. continued

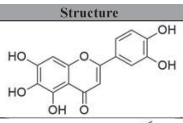
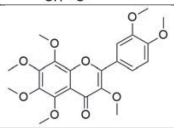
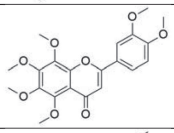
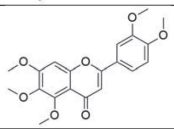
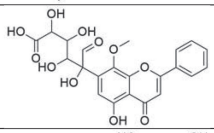
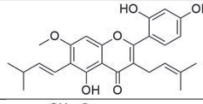
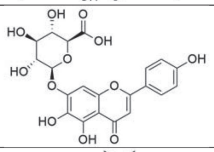
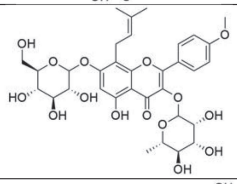
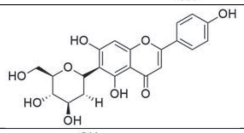
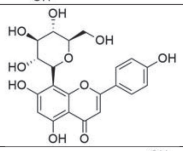
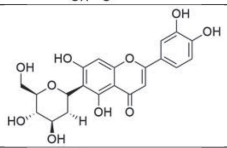
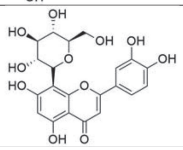
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**132**)	5,6,7,3,4′-Pentahydroxy-flavone		*S. aureus*	BF	40 - 80 μg	173
				BE	80 μg	
(**133**)	Heptamethoxy-flavone		*E. coli*	BF	3.125 - 100 μM	155
				MOT	100 μM	
(**134**)	Nobiletin		*E. coli*	BF	3.125 - 100 μM	155
				MOT	100 μM	
(**135**)	Sinensitin		*E. coli*	BF	6.25- 100 μg/mL	56
			V. harveyi	QS		
				BF		
			E. coli	BF	3.125 - 100 μM	155
				MOT	100 μM	
(**136**)	Wogonoside		*S. aureus*	TOX	2 - 16 μg/mL	300
(**137**)	Artocarpin		*S. pneumoniae*	BF	0.78 - 50 μM	174
				ENZ	2.6 - 7.7μM	
(**138**)	Scutellarin		*H. pylori*	ENZ	0.42 - 1 mM	357
(**139**)	Icariin		*P. acnes*	BE	0.01 – 0.08 % w/v	175
(**140**)	Isovitexin		*E. coli* *C. violaceum*	QS	7.5 - 100 μg/disc	67
(**141**)	Vitexin		*E. coli* *C. violaceum*	QS	7.5 – 100 μg/disc	67
(**142**)	Isoorientin		*E. coli* *C. violaceum*	QS	7.5 – 100 μg/disc	67
(**143**)	Orientin		*E. coli* *C. violaceum*	QS	7.5 – 100 μg/disc	67

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Table 1. continued

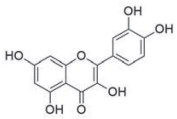
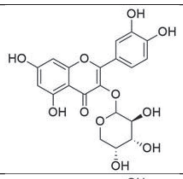
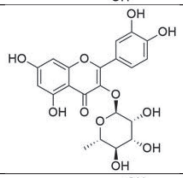
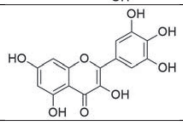
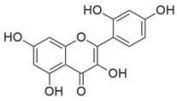
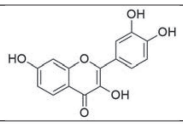
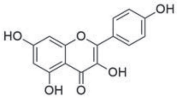
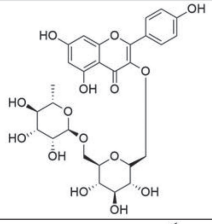
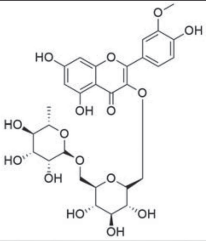
N°	Compound	Structure	Bacteria	Target	Activity	Ref
		Flavonoid: Flavonols				
(**144**)	Quercetin		*S. aureus*	BF	20 - 50 µg/mL	170
				TOX	20 µg/mL	
				TOX	50 µg/mL	177
				ENZ	33.3 - 52.7 µM	351
				BF	1 - 20 µg/mL	176
				TOX	5 - 10 µg/mL	
			S. mutans	BF	1.5 mg/ml	179
			P. aeruginosa	BF	0.085 mg/mL	180
				MOT		
			E. coli	BF	6.25 - 100 µg/mL	245
			V. harveyi	QS		
				BF		
			C. violaceum	QS	25 - 100 µg/mL	68
(**145**)	Quercetin-3-*O*-arabinoside		*S. mutans*	BF	0.0078 - 2 mg/mL	178
			C. violaceum	QS	25 - 100 µg/mL	68
(**146**)	Quercitrin		*S. aureus*	ENZ	16 - 256 µg/mL	352
			S. mutans	BF	32 µg/mL	137
(**147**)	Myricetin		*S. aureus*	ENZ	36.9 - 44 µM	351
			S. aureus *E.coli*	BF	1 - 400 µM	181
(**148**)	Morin		*S. aureus*	ENZ	8.5 - 37.4 µM	351
				TOX	2 - 16 µg/mL	301
			S. mutans	BF	30 µM	182
			L. monocytogenes	TOX	0.5 - 128 µg/mL	304
			P. aeruginosa	BF	30 µg/mL	183
				MOT		
				ENZ		
				TOX	750 µg/mL	
(**149**)	Fisetin		*S. aureus*	BF	20 - 50 µg/mL	170
				TOX	50 µg/mL	177
			L. monocytogenes	TOX	0.88 – 28 µM	303
(**150**)	Kaempferol		*S. aureus*	BF	20 - 50 µg/mL	170
				TOX	20 µg/mL	
				BF	20-50 µg/mL	176
			E. coli	BF	6.25 - 100 µg/mL	56
			V. harveyi	QS		
				BF		
(**151**)	Kaempferol-3-rutinoside		*S. mutans*	ENZ	60.7 µg/mL	353
(**152**)	Isorhamnetin 3-*O*-β-D-rutinoside		*S. mutans*	ENZ	60.2 - 64 µg/mL	353

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Table 1. continued

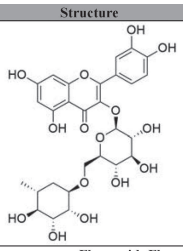
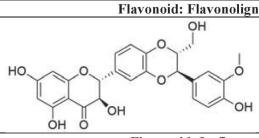
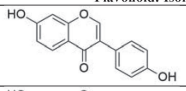
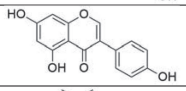
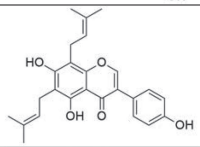
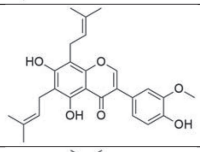
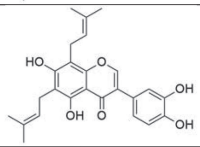
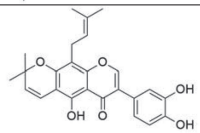
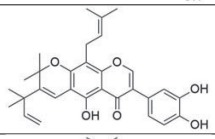
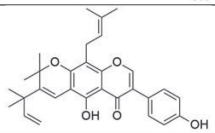
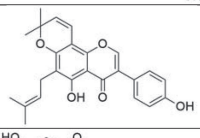
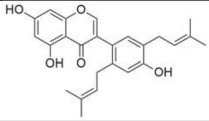
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(153)	Rutin		*E. coli* *C. violaceum*	QS	7.5 - 100 µg/disc	67
			E. coli	BF	6.25 - 100 µg/mL	56
			V. harveyi	QS BF		
			Y. enterocolitica *E. carotovora* *C. violaceum*	QS	50 - 200 µg/mL	47
Flavonoid: Flavonolignans						
(154)	Silibinin		*S. aureus*	TOX QS	4 - 32 µg/mL	59
Flavonoid: Isoflavones						
(155)	Daidzein		*E. coli*	BF	50 µg/mL	159
			C. violaceum	QS	50 - 200 µg/mL	47
(156)	Genistein		*S.aureus*	BF	500 µg/mL	171
				TOX	50 µg/mL	177
			E. coli	BF	50 µg/mL	159
(157)	8-γ,γ-Dimethyl-allylwighteone		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354
(158)	Flemingsin		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354
(159)	6,8-Diprenylorobol		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354
(160)	Auriculasin		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354
(161)	Flemiphilippinin A		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354
(162)	Flemiphilippinin E		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354
(163)	Osajin		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354
(164)	5,7,30,40-Tetrahydroxy-20,50-di(3-methylbut-2-enyl)isoflavone		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354

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Table 1. continued

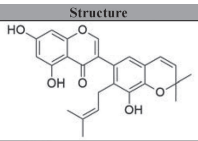
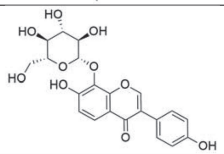
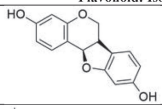
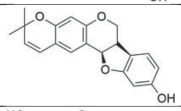
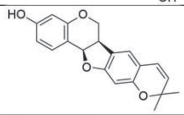
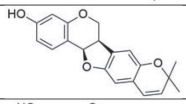
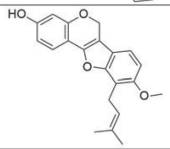
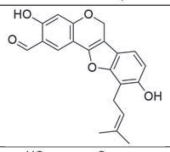
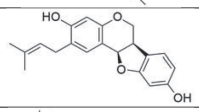
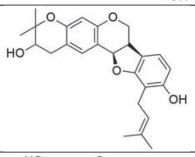
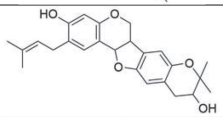
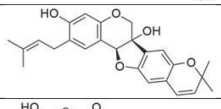
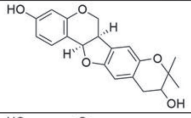
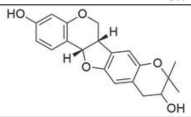
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**165**)	5,7,30-Trihydroxy-20-(3-methylbut-2-enyl)-40,50-(3,3-dimethylpyrano) isoflavone		*C. perfringens*	ENZ	0.05 - 80 µg/mL	354
(**166**)	Puerarin		*S. aureus*	TOX QS	2 - 16 µg/mL	60
	Flavonoid: Isoflavonoids					
(**167**)	Demethylmedicarpin		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**168**)	Neorautenol		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**169**)	Isoneorautenol		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**170**)	Phaseollin		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**171**)	Eryvarin D		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**172**)	Erythribyssin O		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**173**)	Calopocarpin		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**174**)	Erythribyssin L		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**175**)	Erysubin D		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**176**)	Erysubin E		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**177**)	Erythribyssin D		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(**178**)	Erythribyssin M		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355

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Table 1. continued

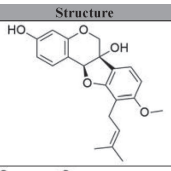
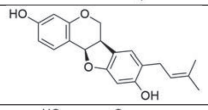
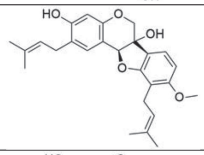
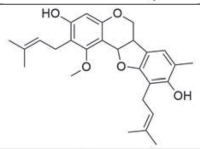
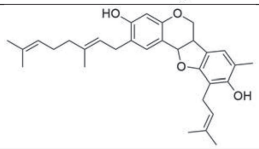
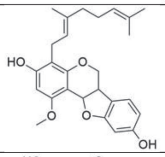
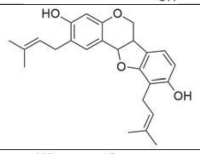
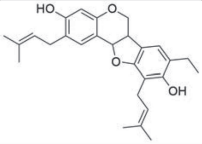
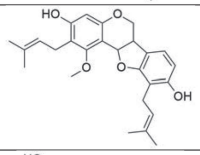
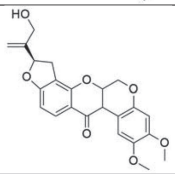
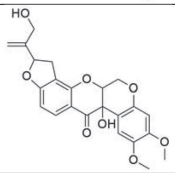
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(179)	Cristacarpin		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(180)	Sophorapterocarpan A		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(181)	Erystagallin A		*V. cholerae* *C. perfringens*	ENZ	0.1 - 1000 µM	355
(182)	Bicolosin A		*C. perfringens*	ENZ	1 - 10 µM	356
(183)	Bicolosin B		*C. perfringens*	ENZ	1 - 10 µM	356
(184)	Bicolosin C		*C. perfringens*	ENZ	1 - 10 µM	356
(185)	Erythrabyssin II		*C. perfringens*	ENZ	1 - 10 µM	356
(186)	Lespebuergine G4		*C. perfringens*	ENZ	1 - 10 µM	356
(187)	1-Methoxy-erythrabyssin II		*C. perfringens*	ENZ	1 - 10 µM	356
(188)	Amorphigeni		*C. perfringens*	ENZ	0 - 100 µM	372
(189)	Dalbinol		*P. aeruginosa* *C. perfringens*	BF ENZ	6.25 - 25 µM 0 - 100 µM	372

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Table 1. continued

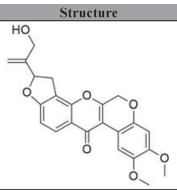
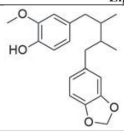
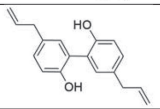
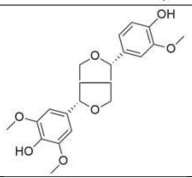
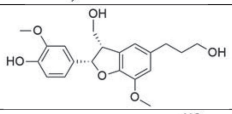
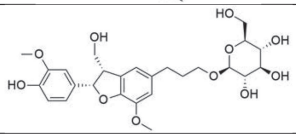
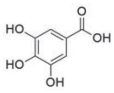
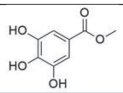
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**190**)	6-Ketodehydro-amorphigenin		*C. perfringens*	ENZ	0 - 100 µM	372
		Lignans				
(**191**)	Macelignan		*S. mutans* *S. sanguis* *A. viscosus*	BF BE	0.2 - 10 µg/mL	184
(**192**)	Magnolol		*S. aureus*	BF	64 - 2048 µg/mL	185
				TOX	1 - 8 µg/mL	305
(**193**)	Medioresinol		*S. aureus* *P. acnes*	BE	5 - 50 µg/mL	186
			E. faecalis *E. coli*	BE	2.5 - 50 µg/mL	
			P. aeruginosa	BE	20 - 200 µg/mL	
(**194**)	(7S,8S)-Dihydro-dehydrodiconiferyl alcohol		*S. mutans*	ENZ	35.9 µM	358
(**195**)	(7S,8S)-Dihydro-dehydrodiconiferyl alcohol 9'-*O*-β-D-glucopyranoside		*S. mutans*	ENZ	37.3 µM	358
		Phenolic acids				
(**196**)	Gallic acid		*S. aureus*	BF BE	1 - 4 mg/mL	187
				TOX	0.5 - 2 µg/mL	
			S. mutans	BF BE	0.0625 - 4 mg/mL	179,191
			S. mutans *E. coli*	BF	8 mg/mL	189
			S. aureus *L.monocytogenes* *E. coli* *P. aeruginosa*	BF BE	1000 µg/mL	188
			L. monocytogenes *P. aeruginosa* *E.coli*	MOT	100 - 1000 µg/mL	
			P. aeruginosa	MOT	50 - 500 mg/L	263
			C. violaceum	QS	50 - 200 µg/mL	47
			C. violaceum	QS	1000 µg/mL	35
			C. violaceum	BF	30 - 1000 µg/mL	190
				MOT	15 - 500 µg/mL	
(**197**)	Methyl gallate		*S. mutans*	BF BE	0.0625 - 4 mg/mL	191
(**198**)	Salicylic acid		*S. mutans*	BF	3.8 mg/mL	179
			S. aureus	BE	1600 - 8000 µg/mL	29
				MOT	1600 µg/mL	
			E coli	BE	3200 - 16000 µg/mL	
				MOT	3200 µg/mL	
			C. violaceum	QS	250 - 5000 µg/mL	
			P. aeruginosa	BF	4 µg/mL	160
				BF	0.05 - 1.0 mM	192
				PIG	0.1 - 1mM	
				ENZ	1 mM	
			P. aeruginosa	QS MOT ENZ	30 mM	70
			E. coli *C. violaceum*	QS	0.3 mg/mL	71

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Table 1. continued

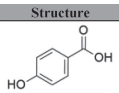
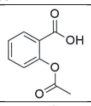
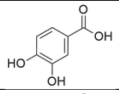
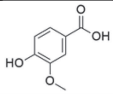
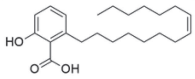
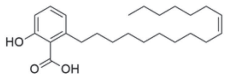
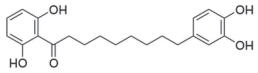
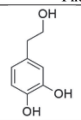
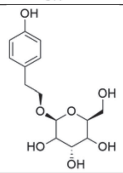
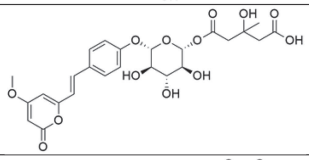
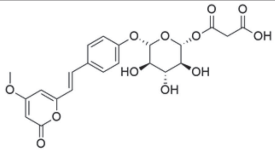
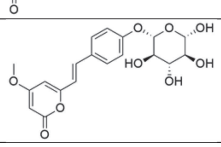
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**199**)	*p*-Hydroxybenzoic acid		*S. aureus*	BF	1000 μg/mL	171
(**200**)	Acetyl salicylic acid		*P. aeruginosa*	PIG ENZ	1.0 mM	192
(**201**)	Methyl salicylate		*P. aeruginosa*	PIG ENZ	1.0 mM	192
(**202**)	Salicylamide		*P. aeruginosa*	PIG ENZ	1.0 mM	192
(**203**)	Benzoic acid		*P. aeruginosa*	PIG ENZ	1.0 mM	192
(**204**)	Protocatechuic acid		*S. aureus* *H. pylori*	BF ENZ	1500 μg/mL 8 - 48 mg/L	171 349
(**205**)	Vanillic acid		*C. violaceum* *C. violaceum* *A.tumifaciens* *A. hydrophila*	QS QS BF	50 - 200 μg/mL 0.063 - 0.25 mg/mL	47 69
(**206**)	Ginkgolic acid C15:1		*S. aureus* *E. coli*	BF BF MOT	0.5 - 10 μg/mL 1 - 10 μg/mL 10 μg/ml	193
(**207**)	Ginkgolic acid C17:1		*S. aureus* *E. coli*	BF BF	0.5 - 10 μg/mL 1 - 10 μg/mL	193
(**208**)	Malabaricone C		*P. aeruginosa* *C. violaceum*	BF PIG QS	1 - 3 mg/mL	72
		Phenylethanoids				
(**209**)	4-Hydroxytyrosol		*S. aureus*	TOX	5 - 200 ng/mL	306
(**210**)	Salidroside		*P. acnes*	BE	0.02 - 2.5 %	175
(**211**)	Desmethylyangonine-4'-*O*-[6"-*O*-(3-hydroxy-3-methylglutaryl)]-β-D-glucopyranoside		*P. aeruginosa*	BF	128 μg/mL	152
(**212**)	Desmethylyangonine-4'-*O*-(6"-*O*-malonyl)-β-D-glucopyranoside		*P. aeruginosa*	BF	128 μg/mL	152
(**213**)	Desmethylyangonine-4'-*O*-β-D-glucopyranoside		*P. aeruginosa*	BF	128 μg/mL	152

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Table 1. continued

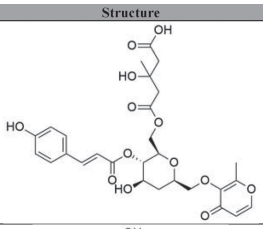
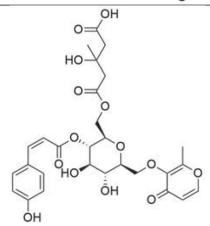
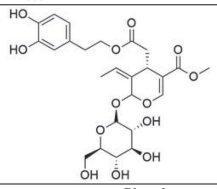
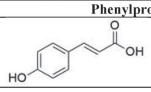
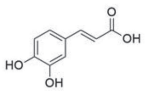
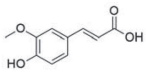
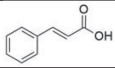
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**214**)	Maltol 3-*O*-(4'-*O*-*p*-coumaroyl-6'-*O*-(3-hydroxy-3-methylglutaroyl))-β-glucopyranoside		*S. mutans*	ENZ	92.4 – 96.2 µg/mL	353
(**215**)	Maltol-3-*O*-(4'-*O*-*cis*-*p*-coumaroyl-6'-*O*-(3-hydroxy-3-methylglutaroyl))-β-glucopyranoside		*S. mutans*	ENZ	57 – 58.6 µg/mL	353
(**216**)	Oleuropein glucoside		*C. violaceum*	QS	1000 µg/mL	35
		Phenylpropanoids				
(**217**)	*p*-Coumaric acid		*C. violaceum* *A. tumefaciens* *P. chlororaphis*	QS	0.6 – 9.1 mM	85
(**218**)	Caffeic acid		*S. aureus*	BF	1 - 4 mg/mL	187
				BE		
				TOX	7.8125 - 125 µg/mL	
			S. epidermidis	BF	0.4 - 4 mg/mL	194
			S. mutans	ENZ	20.2 µM	358
			C. violaceum	QS	1000 µg/mL	35
(**219**)	Ferulic acid		*S. aureus* *L. monocytogenes* *P. aeruginosa* *E.coli*	MOT	100 - 1000 µg/mL	188
			S. aureus *P. aeruginosa* *E. coli*	BF	1000 µg/mL	
				BE	1000 µg/mL	
			C. violaceum	QS	1000 µg/mL	35
(**220**)	Cinnamic acid		*V. harveyi*	QS	50 - 100µM	40
(**221**)	Cinnamaldehyde		*S. aureus*	BE	0.0625 - 6.25 %	198
			S. pyogenes	BF	0.2 - 1mM	197
			L. monocytogenes	BF	0.50 - 0.75 mM	199
				BE	5.0 - 10.0 mM	
				MOT TOX ENZ	0.5 - 0.75 mM	266
			P. aeruginosa	QS	0.005 - 0.02%	74
				BF	0.01 - 0.0.05%	
				MOT	0.005%	
				TOX PIG	0.005 - 0.02%	
			C. violaceum	QS	0.005 - 0.01%	
			E. coli	BF	0.01 - 0.05 %	
			P. aeruginosa	PIG	0.1- 0.3 mg/mL	71
			E. coli *C. violaceum*	QS	0.3 - 3.0 mg/mL	
			E. coli *P. mirabilis*	BF	0.1 - 0.5%	200
				BE	1 - 1.5%	
			C. violaceum *Y. enterocolitica* *E. carotovora*	QS	50 - 200 µg/mL	47
			Vibrio spp.	QS	1 - 100 µM	73
				BF PIG ENZ	100 µM	
				QS	50 - 100µM	40
				BF PIG ENZ	100 µM	

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Table 1. continued

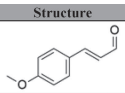
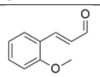
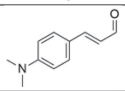
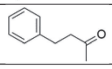
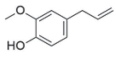
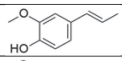
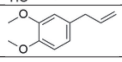
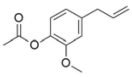
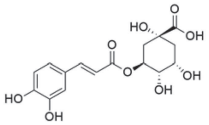
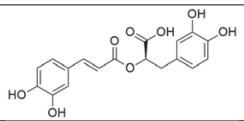
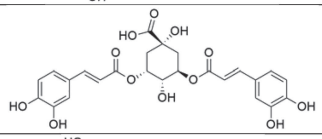
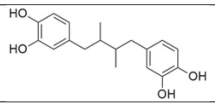
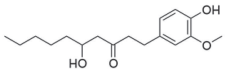
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**222**)	4-Methoxy-cinnamaldehyde		*V. harveyi*	QS	50 - 100μM	40
			Vibrio spp.	QS	1 - 100 μM	73
				BF ENZ	100 μM	
(**223**)	2-Methoxi-cinnamaldehyde		*Vibrio* spp.	QS	1 - 100 μM	73
(**224**)	4-Dimethylamino-cinnamaldehyde		*Vibrio* spp.	QS	50 - 100 μM	40
					1 - 100 μM	73
(**225**)	4-Phenyl-2-butanone		*V. harveyi*	QS	50 - 100 μM	40
(**226**)	Eugenol		*S. aureus*	BF BE	0.02 - 0.08%	201
				TOX	16 - 128 μg/mL	76
				QS	128 μg/mL	
			L. monocytogenes	BF	1.8 - 2.5 mM	199
				BE	18.5 - 25 mM	
			P. aeruginosa	QS	0.005 - 0.05%	74
				BF	0.01- 0.1%	
				MOT	0.005%	
				TOX PIG	0.005 -0.05%	
			E. coli	BF	0.001 -0.01%	
			C. violaceum	QS	0.005 -0.01%	
			P. aeruginosa	BF	50 - 400 μM	75
				MOT	200 μM	
				PIG	50 - 200 μM	
				ENZ	50 - 400 μM	
			C. violaceum	QS	60 - 360 nM	
			K. pneumoniae	BF	7.8 - 62.5 μg/mL	141
(**227**)	Isoeugenol		*C. violaceum*	QS	0.032 - 0.062 mg/mL	41
			P. aeruginosa	PIG		
(**228**)	Methyl eugenol		*P. aeruginosa*	BF	2.5 - 10 μg/mL	78
			V. harveyi *C. violaceum*	QS		
(**229**)	Eugenyl acetate		*S. aureus*	TOX PIG	37.5 - 150 μg/mL	77
			P. aeruginosa	PIG ENZ		
			C. violaceum	QS		
(**230**)	Chlorogenic acid		*S. aureus*	BF BE	1- 4 mg/mL	187
				TOX	62.5 - 250 μg/mL	
				TOX	0.16 - 1.28 mg/mL	307
			S. epidermidis	BF	0.4 - 4 mg/mL	194
			P. aeruginosa	BF BE	128 μg/mL	152
				BF MOT TOX ENZ	750 μg/mL	183
			S. maltophilia	BE	8 - 32 μg/mL	195
			E. coli *C. violaceum*	QS	7.5 - 100 μg/disc	67
			C. violaceum	QS	50 - 200 μg/mL	47
			S. mutans	ENZ	20.1 μM	358
(**231**)	Rosmarinic acid		*P. aeruginosa*	BF MOT TOX ENZ	750 μg/mL	183
(**232**)	3,5-Dicaffeoylquinic acid		*P. aeruginosa*	BF BE	128 μg/mL	152
(**233**)	Nordihydroguaiaretic acid		*S. epidermidis* *S. aureus*	BE	256 mg/L	196
(**234**)	6-Gingerol		*P. aeruginosa*	QS BF PIG ENZ SUR	0 - 1.000 μM 0 - 100 μM	81
			C. violaceum	QS		

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Table 1. continued

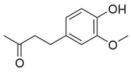
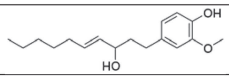
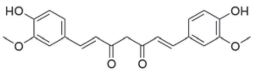
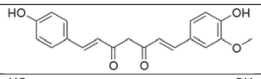
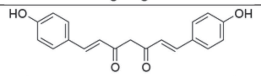
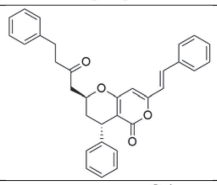
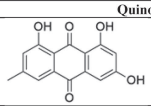
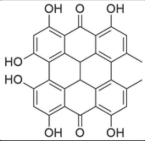
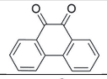
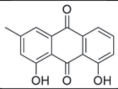
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(235)	Zingerone		*P. aeruginosa*	BF BE MOT	10 mg/mL	202
			P. aeruginosa	QS	100 - 1000 µg/ml	80
				BF MOT PIG TOX ENZ SUR	10 mg/mL	
			C. violaceum	QS	100-500 ppm	79
(236)	6-Shogaol		*C. violaceum*	QS	100 - 500 ppm	79
(237)	Curcumin		*S. aureus*	ENZ	13.8 µg/mL	360
			S. mutans	ENZ	10.2 µM	203,359
				ENZ	10 - 15 µM	203
				BF	15 µM	
			P. aeruginosa	QS BF PIG ENZ	1-3 µg/mL	82
			K. pneumoniae	BF	0.0078 - 0.25 mg/mL	141
			E. coli	BF	50 µg/mL	159
			E. coli *P. mirabilis*	BF	25 - 100 µg/mL	84
				MOT	50 - 100 µg/mL	
			S. marcescens	MOT	50 - 100 µg/mL	
				BF	25 - 100 µg/mL	
				PIG		
			P. aeruginosa	MOT	50 - 100 µg/mL	
				BF	25 - 100 µg/mL	
				SUR		
			C. violaceum	QS	25 - 100 µg/mL	
			V. harveyi *V. parahaemolyticus* *V. vulnificus*	BF	25 - 100 µg/mL	83
				BE MOT	100 µg/mL	
			V. harveyi	QS	25 - 100 µg/mL	
			V. harveyi *V. vulnificus*	ENZ	25 - 100 µg/mL	
(238)	Demethoxy-curcumin		*S. aureus*	ENZ	23.8 µg/mL	360
(239)	Bisdemethoxy-curcumin		*S. aureus*	ENZ	31.9 µg/mL	360
(240)	Katsumadain A		*S. pneumoniae*	ENZ	0.7 – 3.2 µM	174
		Quinones				
(241)	Emodin		*S. mutans*	BF	0.1 - 5 µg/mL	204
			P. aeruginosa *S. malthophilia*	BF	5 - 200 µM	86
			A. tumefaciens	QS	0.3 - 30 mM	
(242)	Hypericin		*S. mutans*	BF	5 µg/mL	204
(243)	Quinone		*P. aeruginosa* *E. coli* *S. typhi*	BF	2 – 10 µg/mL	205
(244)	Chrysophanol		*P. aeruginosa* *S. malthophilia*	BF	200 µM	86
(245)	Thymoquinone		*S. aureus*	BF	20.5 – 75 µg/mL	206
			S. epidermidis	BF	40 – 109 µg/mL	
			E. faecalis	BF	44 – 349 µg/mL	

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Table 1. continued

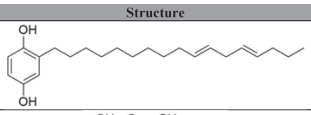
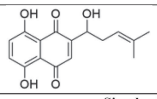
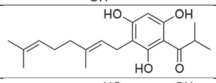
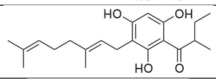
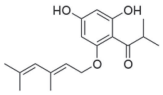
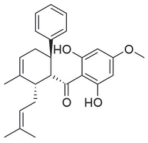
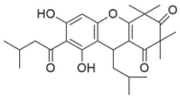
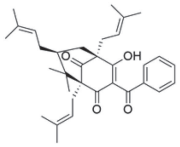
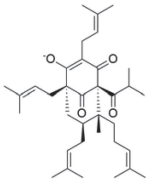
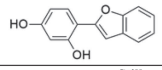
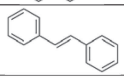
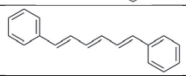
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**246**)	10'(Z),13'(E)-Heptadecadienylhydroquinone		*P. mirabilis*	MOT TOX QS	36 - 145 μM 72.6 μM 36 - 145 μM	87
(**247**)	Shikonin		*P. aeruginosa* *S. malthophilia*	BF	200 μM	86
	Simple phenols					
(**248**)	Resorcinol		*S. aureus*	BF TOX	100 μg/mL 10 μg/mL	207
(**249**)	3-Geranyl-1-(2-methylpropanoyl) phloroglucinol		*S. aureus* *S. epidermidis*	BF	1.95 - 7.81 μg/mL	208
(**250**)	3-Geranyl-1-(2-methylbutanoyl) phloroglucinol		*S. aureus* *S. epidermidis*	BF	1.95 - 7.81 μg/mL	208
(**251**)	2-Geranyloxy-1-(2-methylpropanoyl) phloroglucinol		*S. aureus* *S. epidermidis*	BF	1.95 - 7.81 μg/mL	208
(**252**)	Panduratin A		*S. mutans* *S. sanguis*	BF BE	0.5 - 40 μg/mL 1 - 60 μg/mL	209
(**253**)	Rhodomyrtone		*S. aureus*	PIG	0.0625 - 0.25 μg/mL	324
(**254**)	7-Epiclusianone		*S. mutans*	BF	12.5 - 250 μg/mL	210-212
(**255**)	Hyperforin		*S. aureus,* *E. faecalis*	BF	25 μg/mL	213
(**256**)	Dihydroxybenzofurane		*S. aureus,* *S. epidermidis*	BF BE	10 - 1000 μM 100 - 1000 μM	142
	Stilbenoids					
(**257**)	*Cis*-stilbene		*S. aureus*	TOX	10 μg/mL	207
(**258**)	*Trans*-stilbene		*S. aureus*	BF TOX	50 - 200 μg/mL 1 - 100 μg/mL	207
(**259**)	Dicinnamyl		*S. aureus*	BF	100 μg/mL	207

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Table 1. continued

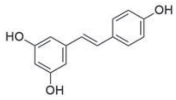
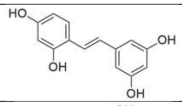
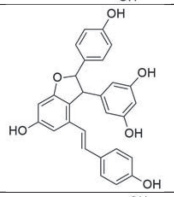
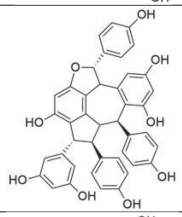
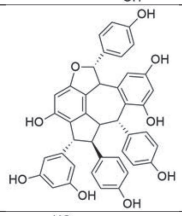
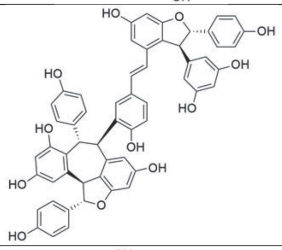
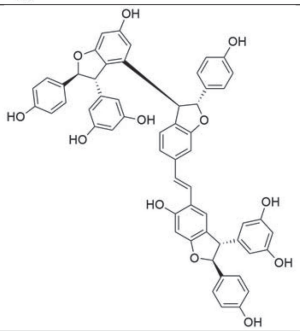
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**260**)	Resveratrol		*S. aureus*	TOX	20 μg/mL	170
				TOX	1 - 100 μg/mL	207
			P. acnes	BE	0.02 – 0.32 % w/v	175
			P. aeruginosa	BF	2 - 100 μg/mL	214
			P. aeruginosa *E. coli*	BF	5 - 50 μg/mL	217
			E. coli	BF	0.01 - 0.05 mg/mL	216
				MOT	20 μg/mL	
			V. cholerae	BF	10 - 30 μg/mL	215
			P. mirabilis	MOT	15 - 60 μg/mL	89
				QS		
				TOX	30 - 60 μg/mL	
				ENZ		
			C. violaceum	QS	20 - 80 μg/mL	88
			C. violaceum *Y. enterocolitica* *E. carotovora*	QS	50 - 200 μg/mL	47
(**261**)	Oxyresveratrol		*S. aureus*	BF	100 μg/mL	207
(**262**)	ε-Viniferin		*S. aureus*	TOX	1- 10 μg/mL	207
			P. aeruginosa	BF	2 - 50 μg/mL	214
			E. coli	BF	2 - 25 μg/mL	
			P. aeruginosa *E. coli*	BF	5 - 50 μg/mL	217
			C. perfringens	ENZ	83 – 86.4 μM	361
(**263**)	Suffruticosol A		*P. aeruginosa* *E. coli*	BF	5 - 50 μg/mL	361
			C. perfringens	ENZ	5.7 -8.1 μM	217
(**264**)	Suffruticosol B		*P. aeruginosa* *E. coli*	BF	5 - 50 μg/mL	361
			C. perfringens	ENZ	6.4 - 8 μM	217
(**265**)	Vitisin A		*S. aureus*	TOX	1- 10 μg/mL	217
			P. aeruginosa *E. coli*	BF	5 - 50 μg/mL	207
(**266**)	Vitisin B		*S. aureus*	TOX	1 - 10 μg/mL	217
			P. aeruginosa *E. coli*	BF	5 - 50 μg/mL	207

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Table 1. continued

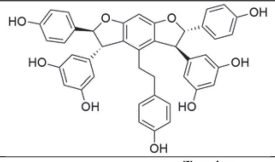
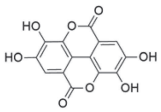
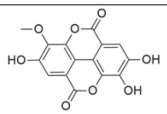
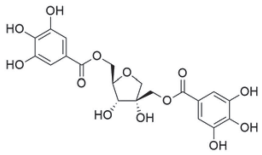
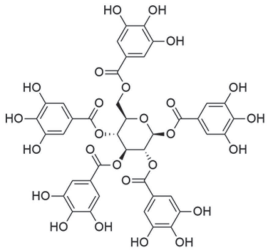
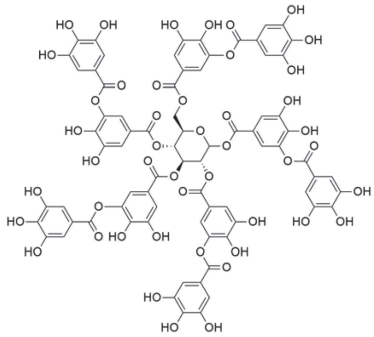
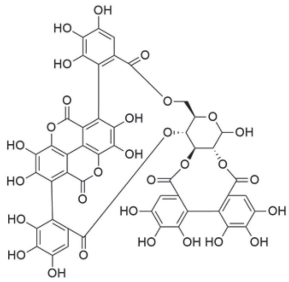
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**267**)	*trans*-Gnetin H		*C. perfringens*	ENZ	2.8 – 4.6μM	361
		Tannins				
(**268**)	Ellagic acid		*S. aureus*	BF	15 - 40 μg/mL	219
			E. coli	BF	15 - 40 μg/mL	218,219
			E. coli	QS	2.5 - 40 μg/mL	55
			P. putida	QS	1- 30 μg/mL	
			B. cepacia	BF	5 - 40 μg/mL	
				MOT	20 μg/mL	
			C. violaceum *Y. enterocolitica* *E. carotovora*	QS	4 - 17 μg/mL	47
(**269**)	3-*O*-Methyl ellagic acid		*S. marcescens*	QS BF	20 - 160 μg/mL	91
			C. violaceum	QS		
(**270**)	2,5-Di-*O*-galloyl-D-hamamelose (Hamamelitannin)		*S. aureus*	QS	25 - 50 μg	92
				TOX	300 μg/mL	
				BF	1.5 - 50 μg	
			S. epidermidis	QS	25 - 50 μg	
				BF	30 - 50 μg	
(**271**)	1,2,3,4,6-Penta-*O*-galloy-β-D-glucopyranose		*S. aureus*	BF	1.56 - 50 μM	220
(**272**)	Tannic acid		*S. aureus*	BF	0.8 - 20 μg/mL	221
					20 - 50 μg/mL	170
					1- 50 μg/mL	176
			S. mutans	BF	0.4 mg/mL	179
			P. aeruginosa	BF	4 μg/mL	160
				MOT	1 - 100 mg/L	263
			E. coli	BF	30 μg/mL	218
					0.05 - 0.1 mg/mL	216
			E. coli *C. violaceum*	QS	0.3 mg/mL	71
			E. coli	QS	5 - 60 μg/mL	55
			P. putida	QS	5 - 30 μg/mL	
			P. mirabilis	BF	100 - 200 μg/mL	93
			C. violaceum	QS		
			C. violaceum	BF	15 - 125 μg/mL	190
				MOT		
(**273**)	Punicalagin		*S. typhimurium*	MOT	16 - 32 μg/mL	94
			C. violaceum *S. typhimurium*	QS		
			P. aeruginosa	MOT	5 - 100 μg/mL	265

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Table 1. continued

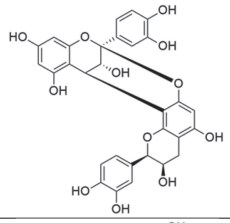
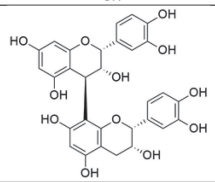
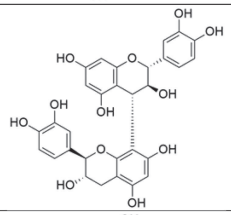
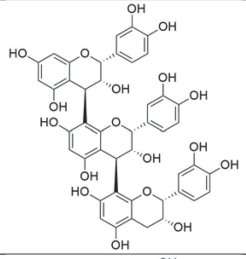
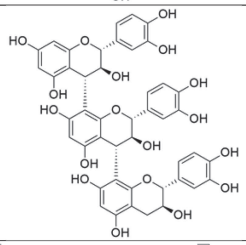
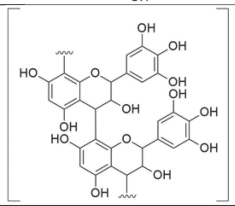
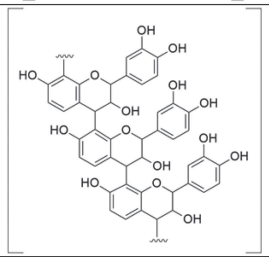
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**274**)	Proanthocyanidin A2		*S. aureus* *S. epidermidis*	BF	0.1 – 1000 µM	142
(**275**)	Procyanidin B2		*H. pylori*	ENZ	165 µg gallic acid equivalents/ mL	362
(**276**)	Procyanidin B3		*H. pylori*	ENZ	117 µg gallic acid equivalents/ mL	362
(**277**)	Procyanidin C1		*H. pylori*	ENZ	112 µg gallic acid equivalents/ mL	362
(**278**)	Procyanidin C2		*H. pylori*	ENZ	66 µg gallic acid equivalents/ mL	362
(**279**)	B-type linked proanthocyanidins	 up to 5	*S. epidermidis*	BF	0.125 - 4 mg/mL	222
(**280**)	B-type linked proanthocyanidins	 up to 3	*P. aeruginosa*	BF	0.125 - 4 mg/mL	223

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Table 1. continued

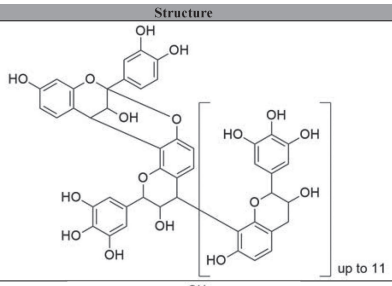
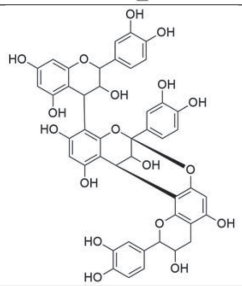
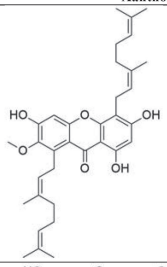
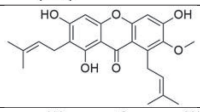
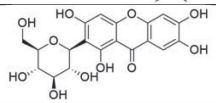
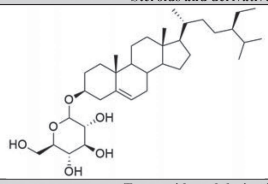
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**281**)	A and B-type linked proanthocyanidins	 up to 11	*P. aeruginosa*	BF	0.125 - 4 mg/mL	223
(**282**)	A-type proanthocyanidins (from trimers to oligomers with high polymerization degree)		*S. mutans*	BE	0.5 - 1.5 mg/mL	224-226
				BF	0.001 - 100 µg/mL	227
				BE		
				MOT	100 µg/mL	
			P. aeruginosa	MOT	5 - 100 µg/mL	265
Xanthones						
(**283**)	Mangostana-xanthone I		*C. violaceum*	QS	2 mg/mL	95
(**284**)	α-Mangostin		*S. mutans*	BF	150 µM	95
			C. violaceum	QS	2 mg/mL	228
(**285**)	Mangiferin		*P. aeruginosa*	BF	1000 µg/mL	183
				MOT		
				TOX		
Steroids and derivatives						
(**286**)	β -Sitosterol – 3-*O*-glucopyranoside		*S. aureus*	ENZ	18.3 µg/mL	363
			E. coli	BF	3.125 - 100 µM	155
				MOT	100 µM	
Terpenoids and derivatives						
Monoterpenes						
(**287**)	α-Phellandrene		*C. violaceum*	QS	0.125 - 0.25 mg/L	41
			P. aeruginosa	PIG		
(**288**)	*p*-Cymene		*C. violaceum*	QS	0.25 - 0.5 mg/L	41
			P. aeruginosa	PIG		

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Table 1. continued

N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**289**)	Thymol		*S. aureus*	TOX	8 - 64 µg/mL	99
				QS		
				TOX	0.15 - 0.3 µL/mL	308
				ENZ		
				TOX	0.67 mM	295
			S. aureus *S. epidermidis*	BF	0.125 - 0.5 %	229
				BE		
			L. monocytogenes	BF	0.33 - 0.5 mM	199
				BE	3.3 – 5 mM	
				MOT	0.33 - 0.5 mM	266
				TOX		
				ENZ		
			P. aeruginosa *P. fluorescens*	BF	0.0625 – 0.25 µl/mL	230,231
			C. violaceum	QS	0.016 - 0.032 mg/mL	41
			P. aeruginosa	PIG		
(**290**)	Carvacrol		*S. aureus* *S. epidermidis*	BF	0.125 - 0.5 %	229
				BE		
			S. aureus	TOX	0.15 - 0.3 µL/mL	308
				ENZ		
			S. aureus *S. typhimurium*	BF	0.5 - 2 mM	96
			C. violaceum	QS	0.2 - 0.8 mM	
				BF		
			L. monocytogenes	MOT	0.5 - 0.65 mM	266
				TOX		
				ENZ		
			L. monocytogenes	BF	0.5 - 0.65 mM	199
				BE	5 - 10 mM	
			P. aeruginosa *P. fluorescens*	BF	0.0625 - 0.25 µl/mL	230,231
			C. violaceum	QS	0.016 - 0.032 mg/mL	41
			P. aeruginosa	PIG		
			E. coli	MOT	0.3 – 1mM	267
			C. jejuni	MOT	0.1 - 0.4 mM	268
(**291**)	α-Terpineol		*S. aureus*	TOX	0.65 – 6.5 mM	295
			C. violaceum	QS	0.062 - 0.125 mg/mL	41
			P. aeruginosa	PIG		
			P. mirabilis	MOT	0.06 - 0.75 mg/L	269
				TOX	0.5 mg/L	
(**292**)	Thujone		*P. aeruginosa*	QS	0.125 - 0.25 mg/mL	41
				PIG		
			C. violaceum	QS		
			C. violaceum	QS	0.25 - 0.5 mg/mL	41
			P. aeruginosa	PIG		
(**293**)	Citral		*P. aeruginosa*	QS	0.008 - 0.016 mg/mL	41
				PIG		
			C. violaceum	QS		
			C. violaceum	QS	0.25 - 0.5 mg/mL	41
			P. aeruginosa	PIG		
			P. mirabilis	MOT	0.06 - 0.75 mg/L	269
				TOX	0.5 mg/L	
(**294**)	Citronellol		*P. mirabilis*	MOT	0.06 - 0.75 mg/L	269
				TOX	0.3 mg/L	
(**295**)	Citronellal		*P. mirabilis*	MOT	0.3 mg/L	269
(**296**)	Geraniol		*P. mirabilis*	MOT	0.06 - 0.75 mg/L	269
				TOX	0.3 mg/L	
			C. violaceum	QS	0.062 - 0.125 mg/mL	41
			P. aeruginosa	PIG		
(**297**)	(-)-Carvone		*C. violaceum*	QS	0.125 - 0.25 mg/mL	41
			P. aeruginosa	PIG		
(**298**)	Pulegone		*P. mirabilis*	MOT	0.06 - 0.75 mg/L	269
(**299**)	Menthone		*C. violaceum*	QS	0.25 - 0.5 mg/mL	41
			P. aeruginosa	PIG		
(**300**)	Menthol		*S. aureus*	TOX	8 – 64 µg/mL	98
				QS	64 µg/mL	
				TOX	3.2 mM	295
			P. aeruginosa	QS	100 - 800 µg/mL	100
				BF		
				ENZ		
				PIG		
				MOT		
			C. violaceum	QS	50 - 400 µg/mL	

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Table 1. continued

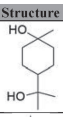
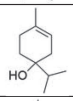
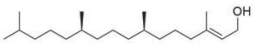
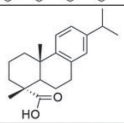
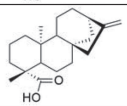
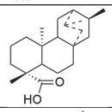
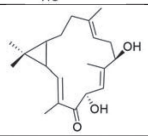
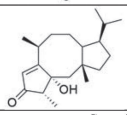
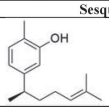
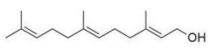
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**301**)	*p*-Menthane-1,8-diol		*S. aureus*	TOX	7.5 – 37.5 mM	295
(**302**)	Limonene		*E. coli*	BF	3 µL/mL	97
			B.cereus	BF	2 µL/mL	
			C. violaceum	QS	0.25 - 0.5 mg/mL	41
			P. aeruginosa	PIG		
(**303**)	Terpinene-4-ol		*E. coli*	BF	1.0 µL/mL	97
			P. putida	BF	2 µL/mL	
			B. cereus	BF	3 µL/mL	
			C. violaceum	QS	1 - 3 µL/disc	
			P. mirabilis	MOT	0.5 mg/L	269
(**304**)	1,8-Cineol		*P. mirabilis*	MOT	0.75 mg/L	269
(**305**)	Linalool		*S. aureus*	TOX	0.75 mM	295
			E. coli	BF	1,5 µL/mL	97
			B. cereus	BF	3 µL/mL	
			C. violaceum	QS	1 - 3 µL/disc	
			C. violaceum	QS	0.125 - 0.25 mg/mL	41
			P. aeruginosa	PIG		
(**306**)	α-Pinene		*E. coli*	BF	0.5 µL/mL	97
			B.cereus	BF	3 µL/mL	
			C. violaceum	QS	1 - 3 µL/disc	
(**307**)	Camphene		*C. violaceum*	QS	0.25 - 0.5 mg/mL	41
			P. aeruginosa	PIG		
(**308**)	Camphor		*C. violaceum*	QS	0.125 - 0.25 mg/mL	41
			P. aeruginosa	PIG		
(**309**)	(-) Borneol		*C. violaceum*	QS	0.125 - 0.25 mg/mL	41
			P. aeruginosa	PIG		
Diterpenes						
(**310**)	Phytol		*P. aeruginosa*	BF	2.375 - 9.5 µg/mL	232
				MOT	4.75 µg/mL	
				PIG		
(**311**)	Geranyllinalool		*P. aeruginosa*	QS	250 µM	101
				PIG		
(**312**)	Dehydroabietic acid		*S. aureus*	BF	30 - 60 µM	233
				BE	80 - 120 µM	
(**313**)	Kaurenoic acid		*S. mutans*	BF	3 - 4 µg/mL	234
(**314**)	*Ent*-trachyloban-19-oic acid		*S. mutans*	BF	14.1 - 125 µg/mL	236
(**315**)	Casbane diterpene (1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane)		*P. aeruginosa* *P. fluorescens* *E. coli* *K. pneumoniae*	BF	125 - 500 µg/mL	237
(**316**)	Anadensin		*P. aeruginosa*	BF	5 - 50 µg/mL	238
Sesquiterpenes						
(**317**)	Xanthorrhizol		*S. mutans*	BF	14.1 - 125 µg/mL	235,236
(**318**)	Farnesol		*S. aureus*	BF	50 - 300 µM	239
				BF	0.01 % (v/v)	241
				TOX	0.0005 % (v/v)	
			S. mutans	BE	0.25 - 1.33 mM	169,240
			P. aeruginosa	QS	25 - 250 µM	101
				PIG	250 µM	
			P. aeruginosa	QS	0.25 - 0.5 mg/L	41

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Table 1. continued

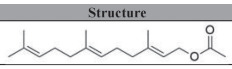
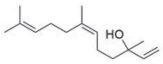
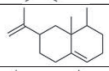
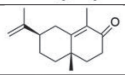
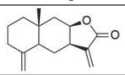
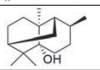
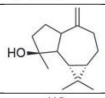
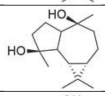
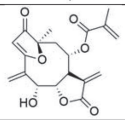
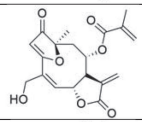
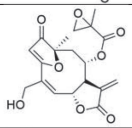
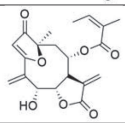
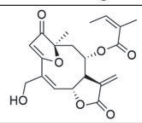
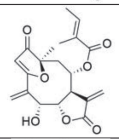
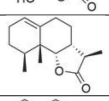
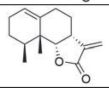
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**319**)	Farnesyl acetate		*P. aeruginosa*	QS PIG	250 µM	101
(**320**)	Nerolidol		*S. aureus*	BF TOX	0.001 - 0.05% (v/v) 0.0001 - 0.001% (v/v)	241
			C. violaceum *P. aeruginosa*	QS PIG	0. 25 - 0.5 mg/mL	41
(**321**)	Nerol		*C. violaceum* *P. aeruginosa*	QS PIG	0.062 - 0.125 mg/mL	41
(**322**)	Valencene		*S. aureus*	BF	0.01 % (v/v)	241
(**323**)	α-Cyperone		*S. aureus*	TOX QS	2 - 16 µg/mL	104
(**324**)	Isoalantolactone		*S. aureus*	TOX QS	1 – 8 µg/mL	103
(**325**)	Patchouli alcohol		*H. pylori*	ENZ	2.67 mM	364
(**326**)	*Ent*-spathulenol		*P. aeruginosa*	BF	5 - 50 µg/mL	238
(**327**)	*Ent*-4β,10α-dihydroxy-aromadendrane		*P. aeruginosa*	BF	5 - 50 µg/mL	238
(**328**)	Viridiflorol		*S. aureus* *P. aeruginosa*	BF ENZ BF	50 µg/mL	242
(**329**)	1-Oxo-3, 10-epoxy-5-hydroxy-8-metacryloyloxy-germacra-2,4(15),11(13)-trien-6,12-olide		*P. aeruginosa*	QS BF ENZ	0.5 - 200 µg/mL	102
(**330**)	1-Oxo-3, 10-epoxy-8-methacryloyloxy-15-hydroxygermacra-2,4,11(13)-trien-6,12-olide		*P. aeruginosa*	QS BF ENZ	0.5 - 200 µg/mL	102
(**331**)	1-Oxo-3,10-epoxy-8-epoxymethacryloyloxy-15-hydroxygermacra-2,4,11(13)-trien-6,12-olide		*P. aeruginosa*	QS BF ENZ	0.5 - 200 µg/mL	102
(**332**)	1-Oxo-3,10-epoxy-5-hydroxy-8-angeloyloxy-germacra-2,4(15),11(13)-trien-6,12-olide		*P. aeruginosa*	QS BF ENZ	0.5 - 200 µg/mL	102
(**333**)	1-Oxo-3,10-epoxy-8-angeloyloxy-15-hydroxygermacra-2,4,11(13)-trien-6,12-olide		*P. aeruginosa*	QS BF ENZ	0.5 - 200 µg/mL	102
(**334**)	1-Oxo-3,10-epoxy-5-hydroxy-8-tigloyloxy-germacra-2,4(15),11(13)-trien-6,12-olide		*P. aeruginosa*	QS BF ENZ	0.5 - 200 µg/mL	102
(**335**)	5-Epidilatanolide A		*P. aeruginosa*	BF	0.25 - 2.5 µg/mL	243
(**336**)	5-Epidilatanolide B		*P. aeruginosa*	BF	0.25 - 2.5 µg/mL	243

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Table 1. continued

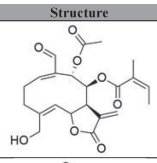
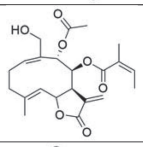
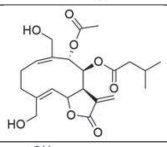
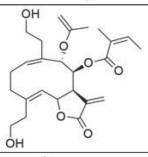
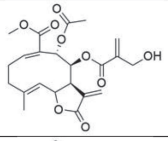
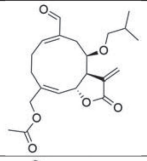
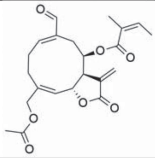
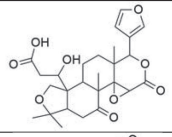
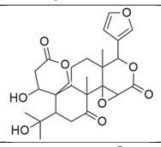
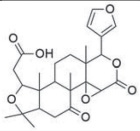
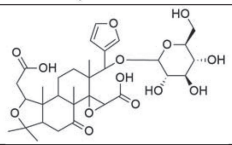
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**337**)	Lecocarpinolide B		*P. aeruginosa*	BF	0.25 -2.5 µg/mL	243
(**338**)	9r-Acetyloxy-8β-angeloyloxy-14-hydroxy-acanthospermolide		*P. aeruginosa*	BF	0.25 - 2.5 µg/mL	243
(**339**)	9r-Acetyloxy-14,15-dihydroxy-8β-(2-methylbutanoyloxy)-acanthospermolide		*P. aeruginosa*	BF	0.25 - 2.5 µg/mL	243
(**340**)	9r-Acetyloxy-14,15-dihydroxy-8β-angeloyloxy-Acanthospermolide		*P. aeruginosa*	BF	0.25 - 2.5 µg/mL	243
(**341**)	19-Hydroxy-15-desoxy-orientalide		*P. aeruginosa*	BF	0.25 - 2.5 µg/mL	243
(**342**)	15-Acetyloxy-8β-isobutanoyloxy-14-oxo-(4Z)-acanthospermolide		*P. aeruginosa*	BF	0.25 - 2.5 µg/mL	243
(**343**)	15-Acetyloxy-8β-angeloyloxy-14-oxo-(4Z)-acanthospermolide		*P. aeruginosa*	BF	0.25 - 2.5 µg/mL	243
	Nor-triterpenes					
(**344**)	Isolimonic acid		*E. coli*	BF	6.25 - 100 µg/mL	244
(**345**)	Ichangin		*E. coli*	BF	6.25 - 100 µg/mL	244
(**346**)	Isoobacunoic acid		*E. coli*	BF	6.25 - 100 µg/mL	244
(**347**)	Isoobacunoic acid glucoside		*E. coli*	BF	6.25 - 100 µg/mL	244

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Table 1. continued

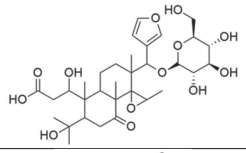
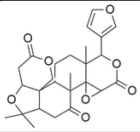
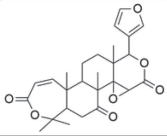
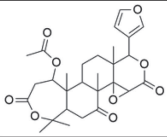
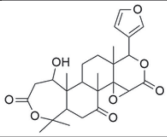
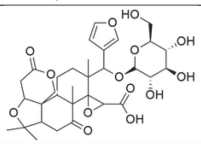
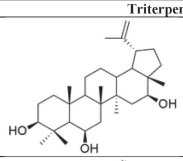
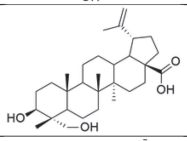
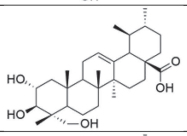
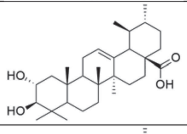
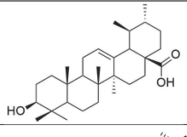
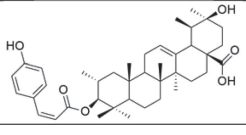
N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**348**)	Deacetyl nomilinic acid glucoside		*E. coli*	BF	6.25 - 100 µg/mL	244
(**349**)	Limonin		*E. coli* *V. harveyi*	BF	6.2 - 100 µg/mL	245
(**350**)	Obacunone		*E. coli* *V. harveyi*	BF	6.2 - 100 µg/mL	245
(**351**)	Nomilin		*E. coli* *V. harveyi*	BF	6.2 - 100 µg/mL	245
(**352**)	Deacetyl nomilin		*E. coli* *V. harveyi*	BF	6.2 - 100 µg/mL	245
(**353**)	Limonin 17-β-D-glucopyranoside		*E. coli* *V. harveyi*	BF	6.2 - 100 µg/mL	245
	Triterpenes					
(**354**)	3β,6 β,16 β-Trihydroxylup-20(29)-ene		*S. mutans* *S. mitis*	BF	0.9 - 7.8 µg /mL	246
			S. aureus	TOX	20 µg/mL	170
(**355**)	Betulinic acid		*P. aeruginosa*	ENZ	50 µg/mL	242
(**356**)	Asiatic acid		*P. aeruginosa*	BF	50 - 100 µg/mL	247
(**357**)	Corosolic acid		*P. aeruginosa*	BF	50 - 100 µg/mL	247
(**358**)	Ursolic acid		*P. aeruginosa*	BF	50 - 100 µg/mL	247
				ENZ	50 µg/mL	242
			P. aeruginosa *E. coli* *V. harveyi*	BF	5 - 30 µg/mL	249
(**359**)	3β-*O*-*Cis*-*p*-coumaroyl-20β-hydroxy-12-ursen-28-oic acid		*P. aeruginosa*	BF	10 µg/mL	248

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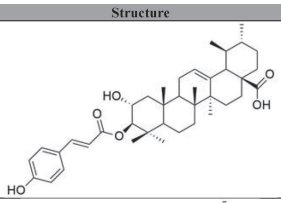
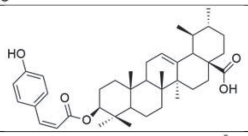
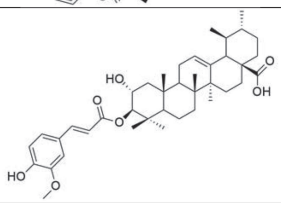
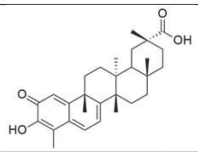
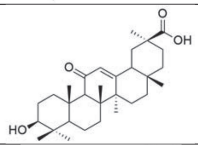
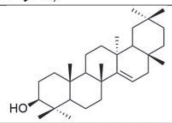
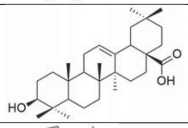
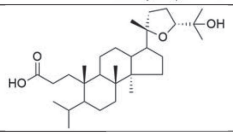
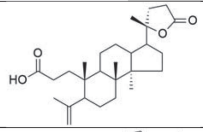
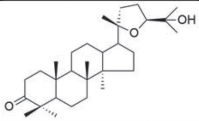
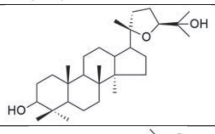
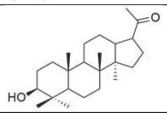
Table 1. continued

N°	Compound	Structure	Bacteria	Target	Activity	Ref
(**360**)	3β-*O*-*Trans*-*p*-coumaroyl-2α hydroxy-12-ursen-28-oic acid		*P. aeruginosa*	BF	10 μg/mL	248
(**361**)	3β-*O*-*Cis*-*p*-coumaroyl-2α hydroxy-12-ursen-28-oic acid		*P. aeruginosa*	BF	10 μg/mL	248
(**362**)	3β-*O*-*Trans*-feruloyl-2α-hydroxy-12-ursen-28-oic acid		*P. aeruginosa*	BF	10 μg/mL	248
(**363**)	Celastrol		*S. aureus* *S. epidermidis*	BE	256 mg/L	196
(**364**)	Glycyrrhetinic acid		*S. aureus*	TOX QS	1 – 8 μg/mL	105
(**365**)	Taraxerol		*S. aureus* *P. aeruginosa*	BF ENZ	50 μg/mL	242
(**366**)	Oleanolic acid		*P. aeruginosa*	ENZ	50 μg/mL	242
(**367**)	Shoreic acid		*S. aureus* *P. aeruginosa*	BF ENZ BF	50 μg/mL	242
(**368**)	Eichlerialactone		*P. aeruginosa*	ENZ BF	50 μg/mL	242
(**369**)	Cabraleone		*S. aureus* *P. aeruginosa*	BF ENZ BF	50 μg/mL	242
(**370**)	Cabraleadiol		*S. aureus* *P. aeruginosa*	BF ENZ BF	50 μg/mL	242
(**371**)	3-β-Hydroxy-nordammaran-20-one		*P. aeruginosa*	ENZ BF	50 μg/mL	242

[a]Abbreviations: QS, quorum-sensing; BF, biofilm formation; BE, biofilm eradication; MOT, motility; TOX, toxin; PIG, pigment; ENZ, enzyme; SUR, surfactant.

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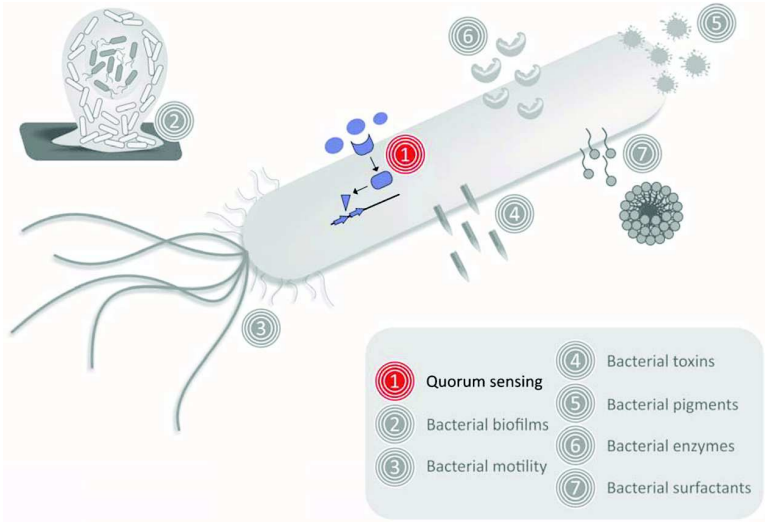

Figure 2. Quorum sensing as a bacterial target for antivirulence compounds.

(2) bacterial biofilms, (3) bacterial motility, (4) bacterial toxins, (5) bacterial pigments, (6) bacterial enzymes, and (7) bacterial surfactants (Figure 1). An important issue emerging from the analysis of the literature review is that the methods used to evaluate each virulence factor vary considerably. Additionally, we need to mention that a direct comparison between selected articles is difficult, mainly when such effort is based on several methodological variabilities: strain, inoculum concentration, aeration, culture media, temperature, incubation time, bioactive compound concentration (including the unit expressed), and substratum. In this sense, we have considered the conclusions and statistical analysis drawn by each author, since defining a general cutoff range might open a breach to data misinterpretation. We conclude this review with a discussion of compiled data, emphasizing the challenges and opportunities in developing antivirulence drugs for complementary treatment of infectious diseases.

2. PLANT-DERIVED NATURAL PRODUCTS AGAINST QUORUM SENSING

2.1. Overview

Bacterial pathogens express certain virulence genes at high cell density and communicate with each other using small diffusible signaling molecules called autoinducers. This chemical communication system, known as quorum sensing (QS), involves producing, releasing, detecting, and responding to autoinducers, the external concentrations of which increase as a function of increasing cell-population density.[13] Once a threshold concentration of the signal molecules is reached, the population is considered to be "quorate". The binding of the signal autoinducer molecules to the cognate receptors occurs within the bacterial cells, triggering a signal transduction cascade that results in population-wide changes in gene expression.[14] Thus, QS enables bacteria to monitor the environment for other bacteria and to act cooperatively, facilitating population-dependent adaptive behavior, including resistance to environmental stress.[15,16]

Although cell-to-cell communication is a process employed by numerous bacteria, the QS system is likely to differ among bacterial species, especially in signal types, receptors, mechanisms of signal transduction, and target outputs (for relevant details, see ref 17). In general, gram-negative bacteria recognize small molecules as autoinducers, like the (i) N-acylated L-homoserine lactones (AHLs), which are the most common class of autoinducers and the best understood bacterial systems at molecular level, and (ii) other molecules, the production of which depends on *S*-adenosylmethionine (SAM) as a substrate, including the autoinducers AI-2 and (*S*)-3-hyroxytridecan-4-one (CAI-1) (for relevant details, see refs 18 and 19). AHLs are membrane permeable and bind to a cytoplasmic receptor in order to exert a regulatory output. In contrast, gram-positive bacteria recognize peptides with diverse post-translational modifications as autoinducers, called autoinducer peptides (AIP), and use either a membrane-bound histidine kinase or cytoplasmic receptors (for relevant details, see ref 20). Additionally, it has been proposed that the autoinducer AI-2 is used by both gram-negative and gram-positive bacteria, representing a universal chemical language by which different bacterial species can communicate with each other, being considered an attractive broad spectrum target.

Many clinically relevant bacteria globally regulate the expression of several virulence genes through QS systems, which are not essential for bacterial survival. The disruption of QS (Figure 2) by interfering with signal molecules and their cognate receptors is termed "quorum quenching". Essentially, three different targets are found in QS systems: inhibition of the signal generator, degradation of the signal molecule, and blockade of the signal receptor. It is noteworthy that the broad influence on virulence factor expression and the species specificity of most QS systems make QS inhibitors (QSI) highly attractive.[19,21−23] However, caution is advised concerning the high mutation ability of pathogenic bacteria, in particular *Pseudomonas aeruginosa*, which has led to the emergence of strains resistant to QSI. Maeda and co-workers[24] reported that mutants of *P. aeruginosa* clinical isolates lacking the genes *mexR* or *nalC*, which encode repressors of the multidrug resistance efflux pump MexAB-OprM, eliminate the protective effects of the QSI furanone C-30. In addition, Garcia-Contreras and co-workers[25] recently showed that furanone C-30 may decrease the stress response of a bacterial population, paving the way to the selection of quorum-quenching-resistant mutants. The complexity of bacterial QS networks, with

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multiple global regulators and different influences on virulence factors expression, is still unclear and has therefore become the object of relevant investigation, imposing major challenges regarding strategies for QS inhibition. Consistent with this idea, recent researches have shown that *P. aeruginosa* can express alternate signaling pathways to bypass LasR mutations, independently of its hierarchical position in the QS circuitry, as opposed to what was previously thought.[26] The production of antibodies against bacterial signaling molecules is a recent approach in the development of novel antibacterial drugs resulting in decreased bacterial virulence. This therapy was initially tested in gram-positive bacteria, with very promising results.[27] Kaufmann and co-workers[28] were pioneers in the efficient production of AHL-sequestering antibodies, inhibiting the biosynthesis of pyocyanin through 3-oxo-C12-HSL sequestration.

Considering the plant-derived natural products active against the virulence factor QS, the following classes of active products were found:

2.2. Alkaloids and Derivatives

The indole alkaloid indole-3-carbinol (**3**), which can be found at relatively high levels in cruciferous vegetables, has been shown to inhibit pigment production by the sensor strain *Chromobacterium violaceum* in the study by Monte and co-workers.[29] By applying a similar screening of anti-QS properties using *C. violaceum* as biosensor, Norizan and co-workers[30] observed that caffeine (**24**) inhibited violacein production, without interfering with bacterial growth. Moreover, they showed that caffeine presented anti-QS properties against *P. aeruginosa* PA01, inhibiting the production of AHL signaling molecules.[30] The same phenomenon was observed for caffeine by Husain and co-workers,[31] who reported a reduction in the production of violacein and QS-regulated virulence factors in *C. violaceum* and *P. aeruginosa*, respectively.

Studying the biological activities of the steroidal alkaloid tomatidine (**12**) against *Staphylococcus aureus*, Mitchell and co-workers[32] showed that tomatidine blocked the expression of several genes normally influenced by the QS accessory gene regulation (*agr*) system (*hla*, *hld*, *geh*, *nuc*, *plc*, and *splC*), thereby altering the virulence of *S. aureus*, such as hemolysis production. The staphylococcal QS autoinducer molecule binds to a transmembrane protein, called AgrC, which acts as the sensor kinase of the bacterial two-component regulatory system. Upon this binding, AgrC activates the response regulator, AgrA, which in turn induces the transcription of RNAII and RNAIII. It is accepted that RNAIII is the effector molecule of the *agr* system and that the result of this QS regulatory cascade is the down-regulation of surface virulence factors, such as protein A, and up-regulation of secreted virulence factors, such as α-toxin hemolysin.[19,33] Although tomatidine has been shown to lack growth-inhibitory activity against *S. aureus*, it was demonstrated that this alkaloid specifically potentiated the inhibitory effect of aminoglycosides against clinical strains, including aminoglycoside-resistant bacteria, revealing strong bactericidal activity.[32] Capsaicin (**25**) substantially decreased the production of α-toxin by community-associated methicillin-resistant *S. aureus*. Qiu and co-workers[34] observed that capsaicin-treated *S. aureus* presented a decrease in expression of RNAIII. This finding indicated that the reduced α-toxin levels may be attributable to the inhibition of the *agr* two-component system, which regulates the transcription of target genes, such as *hla*.[34]

2.3. Fatty Acids and Derivatives

No compound found.

2.4. Organosulfurs and Derivatives

In a preliminary screening, products of the hydrolysis of glucosinolates, such as allylisothiocyanate (**34**), benzylisothiocyanate (**35**), and 2-phenylethylisothiocyanate (**36**), caused loss of purple pigment (violacein) by a specific *C. violaceum* strain, indicating QSI.[35] It was shown that these compounds reduced AHL production, although they were cytotoxic against mouse lung fibroblasts.[35]

Leng and co-workers[36] showed that allicin (**37**) inhibited the production of α-hemolysin by *S. aureus*. The transcriptional levels of *agrA*, which positively regulates the expression of *hla* (α-hemolysin gene), were inhibited, suggesting that the inhibition of the *agr* system is involved in the mode of action of this organosulfur compound.[36]

Jakobsen and co-workers[37] screened several plant extracts for QS inhibitors. They isolated the isothiocyanate iberin (**39**), a potent QSI compound produced by many members of the Brassicaceae family. Iberin, at concentrations that did not interfere with growth, inhibited the expression of the *lasB-gfp* fusion in *P. aeruginosa* and was shown to compete with AHL signal molecules of regulator proteins, although it does not exhibit strong structural similarity to AHL molecules. After iberin treatment, 49 genes controlled by QS were significantly down-regulated, including genes implicated in rhamnolipid production. Molecular changes in the structure of iberin, using four structural analogs, emphasized the importance of the sulfinyl or sulfonyl groups for QSI activity and highlighted the fact that the carbon chain should be three carbons long, rather than four or five.[37] Another study published by Jakobsen and co-workers[38] revealed that the inhibition of the *P. aeruginosa* QS system by crude garlic extract was due to the organusulfur compound ajoene (**38**). DNA microarray studies of ajoene-treated *P. aeruginosa* revealed a concentration-dependent attenuation of a few but central QS-controlled virulence factors, such as LasA protease, chitinase, and rhamnolipids, among others. Furthermore, in a mouse model of pulmonary infection, a significant clearing of infecting *P. aeruginosa* was detected in ajoene-treated mice, when compared to a nontreated control group.[38]

Using several reporter strains of *P. aeruginosa*, Ganin and co-workers[39] proved that sulforaphane (**41**) inhibited LasR activation, suggesting that sulphoraphane by itself may bind its presumed target (LasR). The deoxy precursor erucin (**42**) also presented attenuation in QS, but the inhibitory effects of this compound on QS were less severe.[39] Through *Vibrio harveyi* bioluminescence inhibition, Brackman and co-workers[40] screened for AI-2 inhibitors and found that methyl styryl sulfone (**43**) reduced bioluminescence when compared with a nontreated control.

2.5. Other Aliphatic Compounds

The potential of cinnamaldehyde analogs was investigated in order to evaluate their ability to interfere with AI-2 QS from *Vibrio* spp. Higher concentrations of *trans*-2-hexen-1-al (**45**) and 2-octenoic acid (**56**) poorly reduced AI-2-regulated bioluminescence. In turn, *trans*-2-heptenal (**46**) and *trans*-tridecenal (**52**) showed intermediary inhibitor activity, while *trans*-2-octenal (**47**), *trans*-2-decenal (**49**), *trans*-2-undecenal (**50**), *trans*-2-dodecenal (**51**), and *trans*-3-octen-2-one (**53**) led to a more pronounced inhibition of bioluminescence. *trans*-2-Nonenal (**48**), *trans*-3-decen-2-one (**54**), and *trans*-3-nonen-2-

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one (**55**) were the most active compounds.[40] Further analyses indicated that *trans*-2-nonenal and *trans*-3-decen-2-one inhibit QS by decreasing the DNA binding ability of LuxR, thereby affecting in vitro production of QS-regulated virulence factors by several strains of *Vibrio*, such as biofilm formation, matrix production, and protease activity. In addition, they were found to be nontoxic toward *Caenorhabditis elegans* and significantly increased *C. elegans* survival after infection.[40]

The activity of *cis*-3-nonen-1-ol (**57**) in reducing pigment production by *C. violaceum* was discussed by Ahmad and co-workers.[41] Among several volatile compounds from plants, this aliphatic alcohol was shown to affect the QS system of *C. violaceum*, although it was less active against *P. aeruginosa* QS.

2.6. Other Cyclic Compounds

A study that evaluated the anti-QS properties of several compounds commonly found in essential oils, using *C. violaceum* and *P. aeruginosa* as bioreporter strains, identified estragole (**59**) and *p*-anisaldehyde (**60**) as bioactive substances.[41] The authors emphasized that the mechanism of violacein inhibition by the essential oils is most likely associated with AHL mimicry in signal reception inhibition.[41] Employing a well diffusion assay and *C. violaceum* and *A. tumefaciens* as biosensors, Choi and co-workers[42] found that the production of BHL and OdDHL was decreased by 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (**61**) in a concentration-dependent manner. By the exogenous addition of AHLs, LasA protease activity, as well as the production of rhamnolipid and pyocyanin, was enhanced. These data suggested that 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone influences QS through reversible competition for the binding sites of BHL and OdDHL, in light of the rescue of virulence factors.[42]

2.7. Phenolics and Derivatives

2.7.1. Anthocyanins. Gopu and co-workers[43] used docking analysis to identify the anthocyanin malvidin (**65**) as the compound responsible for the QSI activity of *Syzygium cumini* extract. Results of computer-based studies indicated that the interaction between the LasR receptor protein and malvidin is of hydrophobic nature and takes place without hydrogen interactions. Malvidin activity was confirmed by inhibition of *C. violaceum* pigment production.

2.7.2. Coumarins. The effect of coumarin (**66**) on the quorum-quenching biosensor reporter strains (*Serratia marcescens* for the detection of short AHLs, *C. violaceum* for medium AHLs, and *Agrobacterium tumefaciens* for long AHLs) was analyzed by Gutierrez-Barranquero and co-workers.[44] It was demonstrated that this agent exhibits a strong QSI activity against all three biosensors, displaying different degrees of inhibition against several AHLs and decreases the expression of the QS-related genes *pqsA* and *rhlI*.[44] The *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone) system is one of the three QS circuits employed by *P. aeruginosa* to control virulence factor gene expression.[45] PQS is involved in iron scavenging, acting by trapping iron(III) at the cell surface, possibly facilitating siderophore-mediated iron transport.[46] Gutierrez-Barranquero and co-workers[44] also detected a reduction in *Aliivibrio fischeri* bioluminescence, related with the AI-2 QS system, after treatment with coumarin (**66**).

Truchado and co-workers[47] evaluated the capacity of several food phytochemicals to inhibit QS signals in the biosensor strain *C. violaceum*. To determine the efficacy of each compound, a preliminary screening of three different concentrations was carried out and dimethylesculetin (**70**), a coumarin derivative, was shown to inhibit QS via violacein reduction. 7-Hydroxycoumarin (**67**), a widespread natural product of the coumarin family, also inhibited *C. violaceum* pigment production.[29]

The ability of furocoumarins to disrupt AI-1 and AI-2 QS systems was tested using two strains of *V. harveyi*. Dihydroxybergamottin (**74**) and bergamottin (**75**) influenced cell−cell signaling, as demonstrated by the drop in bioluminescence.[48]

2.7.3. Flavonoids. Screening different flavonoids for QSI, Vandeputte and co-workers[49] showed that the chalcone *trans*-benzylideneacetophenone (**81**) reduced the expression of all *P. aeruginosa* QS-related genes (except *lasI*) at the beginning of incubation. Other three chalcones, 2′,3′,5-trihydroxy-4′,6′,3-trimethoxychalcone (**84**), 2′,3′-dihydroxy-4′,6′-dimethoxychalchone (**85**), 2′,4′,4-trihydroxy-3,6′-dimethoxychalchone (**86**), and flavanone (−)-(2*S*)-7,5′-dihydroxy-5,3′-dimethoxyflavanone (**95**), isolated from *Piper delineatum*, exhibited the remarkable ability to interfere with the QS signaling of *V. harveyi* in the micromolar range.[49] Also, 2′,4′,4-trihydroxy-3,6′-dimethoxychalchone and (−)-(2*S*)-7,5′-dihydroxy-5,3′-dimethoxyflavanone were particularly attractive, since they disrupted bacterial signaling by interaction with elements downstream of LuxO in the QS circuit of *V. harveyi*, without affecting bacterial growth.[50] The chalcone glucoside phloridzin (**83**) inhibited *C. violaceum* pigment and was not toxic against mouse lung fibroblasts.[35]

Licochalcone A (**88**) and E (**89**) were found to prevent toxin secretion by *S. aureus*.[51,52] The transcription of *agrA* was investigated, and it was demonstrated that *agrA* level was inhibited in a dose-dependent manner by both chalcones, which may explain the activities observed.

Studying extracts of leaves and barks of *Combretum albiflorum*, Vandeputte and co-workers[53] identified the flavanol catechin (**90**) as an inhibitor of pigment production by *C. violaceum*. Further assays confirmed that this molecule negatively interfered with the expression of *P. aeruginosa* QS-related genes such as *lasB*, *rhlA*, *lasI*, *lasR*, *rhlI*, and *rhlR*. Using RhlR- and LasR-based biosensors, the authors suggested that catechin might affect the detection of the QS signal *N*-butanoyl-L-homoserine lactone by RhlR, reducing QS-regulated factors.[53] The related compound epicatechin (**91**) also inhibited *C. violaceum* pigment production while being nontoxic to mouse lung fibroblasts.[35] Considering the epigallocatechin gallate (**94**), Castillo and co-workers[54] demonstrated a significant decrease in AI-2 activity using the *V. harveyi* reporter strain. Moreover, Huber and co-workers[55] investigated epigallocatechin gallate as QSI using two other different AHL biosensors, *Escherichia coli* and *Pseudomonas putida*, which also presented fluorescence signal reduction when exposed to the flavanol.

The flavonoids naringenin (**97**), naringin (**98**), neohesperedin (**117**), neoeriocitrin (**118**), apigenin (**123**), sinensetin (**135**), quercetin (**142**), kaempferol (**150**), and rutin (**153**) inhibited either HAI-1- (an acyl homoserine lactone intraspecies signal) or AI-2-mediated bioluminescence in *V. harveyi* reporter strains. Considering HAI-1, naringin and neohesperidin presented stronger inhibitory activity, while sinensetin was a more effective antagonist against AI-2-mediated bioluminescence.[56] Regarding flavanone glycosides, Truchado and co-workers[57] demonstrated that naringin, neohesperidin, and hesperidin (**116**) were able to inhibit modestly the production of violacein in biosensor strain *C. violaceum*. The same researchers detected a decrease in levels of lactones secreted

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by *Yersinia enterocolitica*. They reached the conclusion that the anti-QS property of the flavanone glycosides against *Y. enterocolitica* may be at least partly due to an inhibiting role in the production of the autoinducer signals by the bacterium, such as inhibition of the synthesis of AHLs at the protein level, inhibition of the transport and secretion of AHLs to media, or enzymatic degradation of AHLs.[57]

Naringenin (**97**), liquiritigenin (**103**), pinocembrin (**104**), apigenin, chrysin (**124**), silibinin (**154**), and puerarin (**166**) protected human cells against injury promoted by a hemolysin produced by *S. aureus*. This beneficial effect may partly be explained by considering the inhibition of the *agr* system, since RNAIII, the effector molecule of the *agr* response, was inhibited by these compounds in a dose-dependent manner.[58−64] Similarly, Qiu and co-workers[65,66] showed that luteolin (**125**) and farrerol (**101**) inhibited α-toxin production by *S. aureus*. The authors found that the expression of the *agr* locus was repressed by 6-fold, suggesting that the impact of luteolin and farrerol on α-toxin production by *S. aureus* may also depend on the inhibition of the *agr* regulatory system.[65,66]

Vandeputte and co-workers[49] performed a screening for compounds able to inhibit QS-dependent factors by *P. aeruginosa* and *C. violaceum*. Naringenin (**97**), eriodictyol (**105**), and taxifolin (**119**) reduced pigment production of both strains as well as elastase production by *P. aeruginosa*. Consistently, naringenin inhibited the production of 3-oxo-C12-HSL and C4-HSL autoinducers and influenced the perception of AHLs by *P. aeruginosa*. This flavanone significantly reduced the expression of both *P. aeruginosa* QS systems (*lasRI* and *rhlRI*), decreasing the expression of QS-related genes involved in the production of virulence factors such as *lasA*, *lasB*, *phzA1*, and *rhlA*. On the other hand, the flavanonol taxifolin significantly reduced the expression of the synthase genes *lasI* and *rhlI*, although none of the other QS-related genes (i.e., *lasB*, *lasR*, *rhlR*, and *rhlA*) were affected.

Evaluating the capacity of several food phytochemicals to interfere with the QS system using *C. violaceum*, Truchado and co-workers[47] highlighted the activity of the isoflavone daidzein (**155**) and of the flavonol rutin. Rutin also was able to reduce the concentration of *Y. enterocolitica* and *Ecarotovora carotovora* AHLs by degrading and inhibiting their synthesis, without affecting bacterial growth.[47] Additionally, Brango-Vanegas and co-workers[67] isolated the *C*-glycosyl flavones isovitexin (**140**), vitexin (**141**), isoorientin (**142**), and orientin (**143**) from *Cecropia pachystachya*, along with the *O*-glycosyl flavonol rutin. Using the biosensor strains *C. violaceum* and *E. coli*, the most active compound tested with both biosensors was rutin. Among the C-glycosylated flavonoids, vitexin was the most active substance against *C. violaceum*, while orientin was the most active one against bioluminescence produced by *E. coli*.[67] The flavonols quercetin (**144**) and quercetin-3-*O*-arabinoside (**145**) also fully inhibited pigment production by *C. violaceum*, as shown in the study by Vasavi and co-workers.[68]

2.7.4. Lignans. No compound found.

2.7.5. Phenolic Acids. Truchado and co-workers[47] evaluated the potential of several food phytochemicals to inhibit QS signals in the biosensor strain *C. violaceum*. A preliminary screening using three different concentrations showed that the phenolic acids gallic acid (**196**) and vanilic acid or vanillin (**205**) reduced violacein through QS inhibition. Borges and co-workers[35] also observed the QSI potential of gallic acid, which inhibited pigment production, although all concentrations tested were cytotoxic to mouse lung fibroblasts. Using both *C. violaceum* and *A. tumefaciens* as bioindicator strains, Ponnusamy and co-workers[69] proved that vanillin, a food flavoring agent, presented QSI activity against short-chain and long-chain AHL molecules. Considering short-chain AHLs, vannilic acid showed the highest inhibition against C4-HSL, oxo-C8-HSL, C6-HSL, and C8-HSL, while for long-chain AHLs, the highest inhibition observed was against C14-HSL followed by C10-HSL. According to the authors, vannilin could interfere with the binding of the short-chain AHLs to their cognate receptor, but it was not efficient enough to substantially reduce the binding of long-chain AHLs to the receptor.[69]

A disk diffusion assay was performed for QSI screening using the biosensor strain *C. violaceum*, and salicylic acid (**198**) was shown to inhibit pigment production by this sensor strain.[29] By using different techniques, such as the examination of thin layer chromatography (TLC) profiles as well as β-galactosidase activation in *A. tumefaciens* and *C. violaceum* reporter strains, Bandara and co-workers[70] also demonstrated that salicylic acid interferes with QS systems, reducing the expression of AHL signal molecules in *P. aeruginosa*. In another study, Chang and co-workers[71] screened for AHL inhibitors and pointed to salicylic acid as a potential QS-inhibiting agent from natural products. Using LC−MS and AHL-negative *E. coli* with functional AHL synthases of *P. aeruginosa* LasI and RhlI, salicylic acid was shown to inhibit efficiently AHL production via the RhlI circuit.[71]

According to Chong and co-workers,[72] malabaricone C (**208**), a resorcinol extracted from *Myristica cinnamomea* barks, presented anti-QS action by inhibiting *C. violaceum* pigment production. This finding suggests that malabaricone C may modulate the interaction between the *C. violaceum* CviR receptor and its cognate signaling molecules, although it did not inhibit the autoinducer production in *P. aeruginosa*.

2.7.6. Phenylethanoids. Evaluating the effects of selected phtytochemicals on QS inhibition, Borges and co-workers[35] showed that oleuropein glucoside (**216**) inhibited violacein production, although it was toxic at all concentrations tested against the mouse fibroblast cell line.

2.7.7. Phenylpropanoids. In order to investigate phenylpropanoids with QSI potential, the production of violacein by *C. violaceum* was quantified. Violacein was inhibited when bacteria were exposed to caffeic acid (**218**) and ferulic acid (**219**).[35] Regarding toxicity, lower concentrations of ferulic acid preserved mouse lung fibroblasts viability, while caffeic acid was cytotoxic in all concentrations tested.[35] Truchado and co-workers[47] performed a preliminary screening of food phytochemicals and observed that only the highest of three clorogenic acid (**230**) concentrations inhibited QS via violacein reduction. A qualitative study also evaluated chlorogenic acid as QSI, finding that the compound slightly inhibited violacein by *C. violaceum* and completely failed to inhibit *E. coli* bioluminescence.[67] It is important to highlight that, disregarding differences in methodology, the amount of compound tested in the last study was lower than that used in the work described above.

Cinnamaldehyde (**221**) has been the object of considerable research against bacterial QS systems. Truchado and co-workers[47] evaluated its potential to affect the QS using *C. violaceum* and two pathogenic bacteria, *Y. enterocolitica* and *E. carotovora*. Cinnamaldehyde inhibited violacein production and was able to reduce the concentration of AHLs due to both degradation and inhibition of synthesis of *Y. enterocolitica* and *E.*

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carotovora, without affecting bacterial growth.[47] On the basis of screening for synthetic molecules and natural products, Chang and co-workers[71] pointed to cinnamaldehyde as a strong QS-inhibiting agent from natural products. The authors used LC−MS and AHL-negative *E. coli* with functional AHL synthases of *P. aeruginosa* LasI and RhlI to demonstrate that cinnamaldehyde efficiently inhibits AHL production by RhlI. The authors also suggested that cinnamaldehyde is an inhibitor that specifically targets short-chain AHL synthase (RhlI), but not long-chain AHL synthase (LasI). In addition, they conducted molecular docking analysis and concluded that cinnamaldehyde binds to LasI and EsaI, occupying the substrate binding pocket for AHL production.[71] Brackman and co-workers[73] showed that cinnamaldehyde and its analogs 4-methoxycinnamaldehyde (**222**), 2-methoxicinnamaldehyde (**223**), and 4-dimethylamino-cinnamaldehyde (**224**) blocked the AI-2 QS system of *V. harveyi* in a concentration-dependent manner without inhibiting bacterial growth. In another study, the same authors showed that cinnamaldehyde, cinnamic acid (**220**), 4-methoxicinnamaldehyde, 4-dimethylamino-cinnamaldehyde, and 4-phenyl-2-butanone (**225**) affect the AI-2 QS of *Vibrio* spp.[40] Cinnamaldehyde was the most active phenylpropanoid tested against bioluminescence, followed by cinnamic acid, while other derivatives presented low activity. Cinnamaldehyde decreased the DNA binding ability of LuxR, suggesting that it acts as a LuxR ligand, and thus was proved to be an active blocker of virulence in vivo, using *C. elegans* as host model.[40]

Kim and co-workers[74] screened 83 essential oils and discovered that the main constituents of cinnamon bark oil, cinnamaldehyde and eugenol (**226**), lowered the production of 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) of *P. aeruginosa* and violacein in the biosensor strain *C. violaceum*. The anti-QS activity of eugenol also was evaluated by Zhou and co-workers.[75] In that report, a reduction in violacein production by *C. violaceum* cultures and a modulation of *lasB* and *pqsA* in *E. coli* were observed, providing evidence that eugenol directly inhibits Las- and PQS-controlled transcription.[75] Regarding the action on the QS system of gram-positive bacteria, Qiu and co-workers[76] described the antivirulence activity of eugenol, since it reduced the production of exotoxins by *S. aureus*. This compound induced the repression of *S. aureus agrA* transcription and decreased the transcription levels of QS-regulated exoproteins.[76] The structurally related compounds isoeugenol (**227**) and eugenyl acetate (**229**) significantly inhibited *C. violaceum* pigment production.[41,77] Interestingly, this action was associated with the decreasing production of virulence factors by *P. aeruginosa* for both compounds, while eugenyl acetate also modulated virulence by *S. aureus*.[41,77] Packiavathy and co-workers[78] screened vegetables, cereals, and spices for QSI action, using *C. violaceum* and *V. harveyi* as bionsensors, highlighting the activity displayed by *Cuminum cyminum* extract. Through molecular docking analysis, the QSI activity was attributed to a eugenol derivative, methyl eugenol (**228**).

Phenolic components of ginger (*Zingiber officinale*) rhizome, 6-gingerol (**234**), zingerone (**235**), and 6-shogaol (**236**), were proved to exhibit QSI, as detected in the *C. violaceum* bioassay by Kumar and co-workers.[79] 6-Shogaol showed the highest inhibition of violacein production, followed by 6-gingerol and zingerone. The authors attributed the low activity of zingerone to its reduced alkyl side chain, when compared to the other ginger compounds tested.[79] These data led the same authors to investigate the interference of zingerone in AHL production using other reporter strains (*A. tumefaciens*, *E. coli*, and two *P. aeruginosa*).[80] Zingerone treatment reduced OdDHL molecules and PQS production. This observation, followed by docking analyses, indicated that the compound may interact with the active site of QS receptors, such as TraR, LasR, RhlR, and PqsR, blocking the downstream signaling pathway. Therefore, the blockage of receptor−ligand interaction seems to be the way by which zingerone prevents the activation of virulence QS-dependent gene expression.[80] QS inhibition by 6-gingerol was also confirmed with two reporter bacterial strains, *C. violaceum* and *A. tumefaciens*. The treatment with this compound resulted in a reduction of violacein production by *C. violaceum* cultures and fading cyan color when *A. tumefaciens* was tested.[81]

Curcumin (**237**), a major constituent of turmeric *Curcuma longa* L. roots/rhizomes, had antivirulence action against *P. aeruginosa* through the reduction of QS signals, as shown by Rudrappa and Bais.[82] Treatment with curcumin reduced the production of 3-oxo-C12-HSL, in addition to changing the expression of several genes involved in transcriptional regulation and in type-III secretion factors. Furthermore, *P. aeruginosa* exposed to curcumin presented reduced pathogenicity in two infection models, *C. elegans* and *Arabidopsis thaliana*.[82] The effect of curcumin on QS was also demonstrated by Packiavathy and co-workers[83] via reduction of violacein in *C. violaceum* and by Packiavathy and co-workers[84] via inhibition of bioluminescence in *V. harveyi*.

Notably, Bodini and co-workers[85] showed that *p*-coumaric acid (**217**) strongly inhibited QS in reporter strains, after detecting the reduction of violacein in *C. violaceum*, of carboxylic acid in *Pseudomonas chlororaphis*, and of *β*-galactosidase in *A. tumefaciens*. The effect was observed in a concentration-dependent manner, without comparable drop in growth rate.

2.7.8. Quinones. On the basis of a computer-based analysis, Ding and co-workers[86] screened for putative novel QSI of *A. tumefaciens* from a database of known compounds used in traditional Chinese medicine. Six compounds were identified as putative QSI, based on molecular docking studies; however, in vitro assays showed that only the anthraquinone emodin (**241**) induced proteolysis of the QS signal receptor TraR in *E. coli*. This effect may be the likely explanation for the inhibitory activity of emodin against *P. aeruginosa* biofilm formation.

10′(*Z*),13′(*E*)-Heptadecadienylhydroquinone (**246**) was shown to inhibit *Proteus mirabilis* swarming in the wild-type and *rppA* mutant, but not in the *rcsB* mutant. These results indicated that this quinone inhibits motility through the RcsB-dependent pathway, a two-component signaling pathway negatively regulating swarming and virulence factor expression in *P. mirabilis*.[87]

2.7.9. Simple Phenols. No compound found.

2.7.10. Stilbenoids. Resveratrol (**260**) was shown to reduce violacein production by *C. violaceum*, using a simple phenotypic assay.[88] Truchado and co-workers[47] also evaluated the potential of revestratrol to inhibit QS signals in *C. violaceum* and two pathogenic bacteria, *Y. enterocolitica* and *E. carotovora*. Resveratrol inhibited QS via violacein reduction and was able to diminish the concentration of AHLs of both *Y. enterocolitica* and *E. carotovora* in such a way that the phenomenon could not be attributed to any bactericidal or bacteriostatic effect.[47] Another study conducted by Wang and co-workers[89] showed that resveratrol also disrupts virulence factors produced by *P. mirabilis*. The QS circuits of this bacterium have not been well-clarified; however, it seems that the RsbA (regulator of

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swarming behavior) sensor is homologous to membrane histidine kinases of the two-component family of regulatory proteins, acting as a sensor of environmental conditions.[90] Therefore, Wang and co-workers[89] suggested that the inhibition of virulence factors (swarming and urease and hemolysis production) by resveratrol was mediated through RsbA, since the complementation of the *rsbA*-defective mutant with *rsbA* gene restored its responsiveness to resveratrol.

2.7.11. Tannins. The hydrolyzable tannin ellagic acid (**268**) was described to inhibit QS via violacein reduction and to reduce the synthesis of AHLs by *Y. enterocolitica* and *E. carotovora*, without affecting bacterial growth.[47] The structurally related tannin 3-*O*-methyl ellagic acid (**269**), isolated from *Anethum graveolens*, also reduced pigment production by *C. violaceum* and significantly interfered with *S. marcescens* QS, as demonstrated by the modulation of prodigiosin pigment, biofilm, and protease production in a concentration-dependent manner.[91]

Kiran and co-workers[92] performed a virtual screening to search for RNAIII-inhibiting peptide (RIP) analogs. RIP is known to be a heptapeptide that interferes with the staphylococcal QS system. The authors identified 2,5-di-*O*-galloyl-D-hamamelose, or hamamelitannin (**270**), a nonpeptide RIP analog produced by barks of *Hamamelis virginiana*. In all *S. aureus* and *S. epidermidis* strains tested, hamamelitannin down-regulated RNAIII production, which is part of the *agr* QS system. Therefore, bacteria exposed to hamamelitannin had virulence factors reduced in vitro and the coating with hamamelitannin completely prevented device-associated infections in vivo.

On the basis of an evaluation of violacein production by *C. violaceum*, tannic acid (**272**), another hydrolyzable tannin, has also been shown to antagonize the QS system. Its potential as QS antagonist was observed in biofilm prevention and disruption in a dose-dependent manner, while the viability of planktonic cells was unaffected by the compound.[93] Through a rapid screening for AHL inhibitors, Chang and co-workers[71] suggested that tannic acid is a strong QSI. By LC−MS analysis and using recombinant *E. coli* carrying *P. aeruginosa* LasI and RhlI, tannic acid prevented AHL production by RhlI. The authors indicated that this compound specifically targets short-chain AHL synthases (RhlI), not long-chain AHL synthases (LasI).[71] Punicalagin (**273**), another hydrolyzable tannin, demonstrated anti-QS activity at subinhibitory concentrations, which was indicated by the drop in the production of violacein.[94] The anti-QS property was evidenced when two QS-related genes (*sdiA* and *srgE*) of *Salmonella typhimurium* were activated by AHL, and punicalagin was able to reduce their expression. Interestingly, no detectable cytotoxic effects were observed when human colonic cell line HT-29 was treated with punicalagin.[94]

2.7.12. Xanthones. Commonly known as mangosteen, *Garcinia mangostana* was shown to contain two xanthones with QSI activity, α-mangostin (**284**) and the newly discovered mangostanaxanthone I (**285**). Though weak, the bioactivity of both compounds was revealed using the biosensor bacterium *C. violaceum*.[95]

2.8. Steroids and Derivatives

No compound found.

2.9. Terpenoids and Derivatives

Burt and co-workers[96] found that carvacrol (**290**) inhibits *C. violaceum cviI* gene expression, a gene coding for the *N*-acyl-*L*-homoserine lactone synthase, indicating that this compound hinders the production of AHL at the level of its synthase gene. This monoterpene significantly reduced the production of violacein and chitinase by *C. violaceum*.[96] The evaluation of violacein production by *C. violaceum* also was conducted for terpinen-4-ol (**303**), linalool (**305**), and α-pinene (**306**), the last of which was shown to be the most active.[97]

The monoterpenes thymol (**289**) and menthol (**300**) also reduced the production of *S. aureus* exotoxins, such as α-hemolysin, enterotoxins A and B, and the toxic shock syndrome toxin.[98,99] It was found that transcript levels of *agrA* decreased when the highest concentration of thymol and menthol was tested, which is consistent with the results since *agr* locus positively regulates transcription of toxin-encoding genes.[98,99] Menthol action against QS of gram-negative strains was also investigated by Husain and co-workers.[100] These authors used molecular docking and observed that QSI activity exhibited by peppermint essential oil may be attributed to menthol. Following in vitro analysis, menthol exhibited a concentration-dependent decrease in QS-regulated violacein production by *C. violaceum* and directly inhibited Las- and PQS-controlled transcription, supporting its broad-spectrum anti-QS activity.[100]

Ahmad and co-workers[41] investigated anti-QS properties of 29 compounds commonly found in essential oils using two bioreporter strains (*C. violaceum* and *P. aeruginosa*). Among them, 22 compounds presented varying levels of QSI activity, including the monoterpenes α-phellandrene (**287**), *p*-cimene (**288**), thymol, carvacol, geraniol (**296**), menthone (**299**), linalool (**293**), camphene (**307**), and camphor (**308**) and the sesquiterpenes farnesol (**318**), nerolidol (**320**), and nerol (**321**). The performance of α-terpineol (**291**) was emphasized as violacein inhibitor, similarly to thujone (**292**) and citral (**293**), which were shown to be pyocyanin inhibitiors. Interestingly, it was observed that the (+)-enantiomers of some monoterpenes increased violacein and pyocyanin production, while their levorotary analogues (−) carvone (**297**), (−) limonene (**302**), and (−) borneol (**309**) inhibited their production. As stated by the authors, although the QS systems of both strains consist of the LuxI/LuxR homologues, *P. aeruginosa* has three other QS systems, namely Las, Rhl, and Pqs. Therefore, the differences observed in violacein and pyocyanin inhibition by essential oil constituents could be attributed to the effect on different QS systems.

Cugini and co-workers[101] showed that the sesquiterpene farnesol and the structurally related compounds geranyllinalool (**311**) and farnesyl acetate (**319**) inhibited the PQS and pyocyanin production, without affecting bacterial growth. The authors suggested that the reduction in PQS production promoted by that farnesol is due to the decreasing of *pqsA* transcript in *P. aeruginosa*, which interferes with the PqsR-mediated transcriptional activation.[101] Additionally, the sesquiterpene lactones **329**−**334** from *Centratherum punctatum* were able to inhibit the production of AHLs in *P. aeruginosa*.[102] It was suggested that the inhibition of *P. aeruginosa* biofilm formation by sesquiterpenes occurs due to an interference in the QS.[102]

The inhibition of *S. aureus* α-toxin production by the sesquiterpene lactone isoalantolactone (**324**), by the sesquiterpene α-cyperone (**323**), and by the triperpene glycyrrhetinic acid (**364**) was described.[103−105] Encoded by *hla* gene, α-toxin is secreted in the late exponential phase of bacterial growth under the control of the accessory gene regulator (*agr*). Luo and co-workers[104] found that the transcription of *agrA* gene

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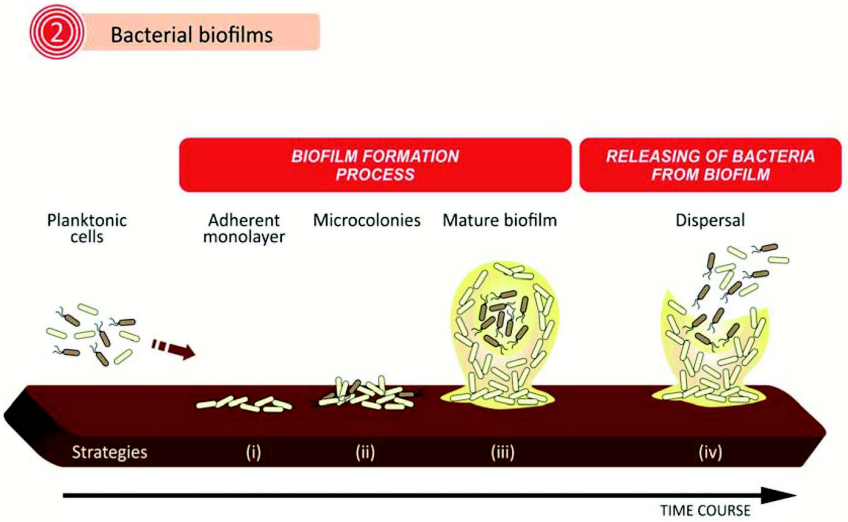

Figure 3. Schematic outlining the key stages in biofilm development, highlighting the strategies used to inhibit and/or to eradicate biofilm at specific stages: (a) prevention of bacterial adhesion, (b) inhibition of biofilm maturation, (c) disruption of the biofilm EPS matrix, and the (d) killing microorganism in mature biofilms.

was reduced by α-cyperone in a dose-dependent manner by up to 5.8-fold, while Qiu and co-workers[103] and Li and co-workers[105] observed that the transcription of RNAIII, the effector molecule of the Agr response, dropped 8.5 and 5.4 times, respectively, when treated with glycyrrhetinic acid and isoalantolactone. On the basis of this, the mode of action of these terpenoids may be partly explained due to the inhibition of the Agr regulatory system.

3. PLANT-DERIVED NATURAL PRODUCTS AGAINST BACTERIAL BIOFILMS

3.1. Overview

Currently available antibacterials have been conceived on the basis of the free-living-state microbial behavior. Millions of lives have been saved by these antibacterials, which represent one of the most important breakthroughs in modern medicine. However, new challenges have emerged since scientists realized that the vast majority of bacteria are living organized in structures called biofilms.[106] Biofilms can be defined as communities of microbial cells enclosed in a self-produced matrix of extracellular polymeric substances (EPS) required for cellular attachment to both biotic or abiotic surfaces. This lifestyle occurs in almost all natural environments, and constitutes the most common form of microbial organization.[107,108] Biofilm community organization exhibits significant morphological, physiological, and genetic differences from free-living (planktonic) bacteria,[109] posing a significant challenge in several areas, especially medical, odontological, and food industry settings.

In recent years, biofilms have attracted considerable attention, especially due to their enormous impact on medicine and public health. Bacteria in the biofilm form contribute to the chronicity of persistent infections, such as those associated with implanted medical devices. This lifestyle allows pathogens to evade the host immune defenses and resist antibacterial treatments, in an interesting trait that has been correlated with their resilience in most medical settings.[110,111] Devices that are prone to biofilm formation include central venous catheters, contact lenses, endotracheal tubes, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, replacement joints, and urinary catheters.[112,113] Although indwelling medical devices are often primarily colonized by single bacterial species, after a short time, a multispecies consortium quickly develops.[114] *Staphylococcus epidermidis* and *S. aureus* are recognized as the most frequent causal agents of nosocomial infections and infections on indwelling medical devices, which characteristically involve biofilms. This has been linked to the fact that staphylococci are a frequent commensal bacteria on the human skin and mucous surfaces.[115] Together with the gram-positive strains, *E. coli* is one of the most common bacteria detected in patients with indwelling or implanted foreign materials and a common microbial agent in recurrent urogenital infections.[116] Of equivalent importance, the opportunistic bacterial pathogen *P. aeruginosa* is a common agent that colonizes the lungs of cystic fibrosis patients, causing persistent biofilm-based infections, even when long-term antibacterial therapy is prescribed.[117]

Microbial attachment to food-processing surfaces is a rather fast process due to the nutritional richness of the substrates involved. Bacteria are commonly attached to the food product or industrial equipment surfaces, causing hygiene issues that result not only in human foodborne illness, but also in economic loss due to food spoilage. The insufficient or incorrect disinfection of food-processing surfaces prompted a large number of reports about the persistence of numerous foodborne pathogens on surfaces and product contamination.[118,119] These pathogens include *Salmonella* spp., *Listeria monocytogenes*, *Y. enterocolitica*, *Campylobacter jejuni*, and *E. coli* O157:H7, which are considered important causes of contamination of food products or transmission of diseases.[120]

Oral biofilms are primary etiologic agents in dental caries and periodontal disease. Although uncalcified biofilms can be removed by routine oral hygiene or by professional dental instruments, they have the potential to calcify into dental calculus or even forming subgingival plaque, which makes removal difficult.[121] Clinical studies have shown that caries are associated with increased counts of acidogenic and aciduric (acid-tolerant) bacteria, especially *Streptococcus mutans* and lactobacilli.[122]

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Essentially, four strategies are known to combat biofilms (Figure 3), namely, (a) prevention of bacterial adhesion (prevention of biofilm formation), (b) inhibition of biofilm maturation, (c) disruption of the biofilm EPS matrix, and the (d) killing of microorganism in mature biofilms.[106]

Consensus has been reached on the notion that inhibition of bacterial adhesion seems to be the most promising strategy, where bacteria in planktonic lifestyle are likely to be more sensitive (Figure 3, strategy a). One alternative regarding nonbiotic surfaces, such as medical devices, is to produce materials less prone to microbe adhesion. In this regard, several physical and chemical features of a given surface may be modified in order to achieve the best anti-infective performance while safe biocompatibility is maintained, following the so-called concept *race for the surface*.[123,124] Briefly, surface modifications may be carried out on the basis of (i) a chemical coating or grafting of the surface using antiadhesive or antimicrobials agents, and (ii) physical treatments of the surface, such as those induced by ionic plasma discharge.[125−128] Another scenario in this context is to inhibit adhesion by interfering with the production of microbial appendages, such as pili, curli, and flagella. The second messenger c-di-GMP has also been implicated in bacterial adhesion, including the stimulation of the synthesis of adhesins (for relevant details, see refs 129 and 130). Once microorganisms adhere, the formation of bacterial clusters is followed by EPS matrix production (Figure 3, strategy b). This step is highly influenced by gram-negative and -positive QS systems and may also be coordinated by c-di-GMP signaling. Therefore, compounds that are able to block these circuits are likely to affect biofilm maturation.

When a mature biofilm is established, a new treatment perspective is required. The strategies to disrupt or eradicate mature biofilms (Figure 3, strategy c) or to kill bacteria inside biofilms (Figure 3, strategy d) are likely to be the most significant in clinical practice. If the biofilm is accessible, as in the oral cavity, a combination of mechanical and physical methods such as UV and ultrasound might be applied. However, most biofilm infections are not easily accessible, and therefore, biochemical strategies to disrupt biofilms should be considered, including (i) the use of hydrolytic enzymes that degrade extracellular matrix components (for a relevant review see ref 131) and (ii) the use of compounds able to bind or block the c-di-GMP intracellular messenger, decreasing its level and promoting biofilm dispersion.[132,133] The release of bacterial cells from biofilms (Figure 3, strategy c) should render cells susceptible to being killed by classic antimicrobials. Finally, although no efficient and specific treatment has so far been identified aiming to eradicate bacteria in biofilms (Figure 3, strategy d), the combination of different antibacterials with antivirulence agents that promote biofilm disruption seems to be a promising approach.

Considering the plant-derived natural products active against the virulence factor biofilm, the following classes of active products were found:

3.2. Alkaloids and Derivatives

Indole derivatives from cruciferous vegetables were investigated as inhibitors of biofilm produced by *E. coli* and *P. aeruginosa*. Interestingly, 3-indolylacetonitrile (**2**) effectively reduced *E. coli* and *P. aeruginosa* biofilm formation, along with reduction in polymeric matrix production, showing that this derivative is more effective than its precursor.[134] DNA microarray analysis indicated that the compound repressed genes involved in curli formation (*csgA* and *csgB*) and glycerol metabolism (*glpD*, *glpF*, *glpK*, and *glpT*) at the same time that it induced indole-related genes (*tnaC*) and prophage genes (*z2978* and *z3345*) in *E. coli*. It has also been suggested that 3-indolylacetonitrile inhibited biofilm formation by *E. coli*, through the reduction of curli formation and induction of indole production. *P. aeruginosa* whole-transcriptomic data also showed that 3-indolylacetonitrile repressed virulence-related genes (*pqsE* and *pvcC*) and motility-related genes (*z2200*, *pilI*, *flhF*, and *motD*), though it also induced several small molecule transport genes.[134] Plant-derived indoles were also screened for their ability to inhibit biofilm formation by several bacteria. 3-Indolylacetonitrile, indole-3-carboxyaldehyde (**5**), indole-3-acetamide (**6**), and isatin (**8**) significantly decreased the ability of *P. aeruginosa* to form biofilms.[135] Indole-3-carbinol (**3**), another metabolite that can be found at relatively high levels in cruciferous vegetables, eradicated preformed biofilms, the percentages of biomass removal and inactivation always being higher for *E. coli* than for *S. aureus* for all concentrations tested.[29] The indole alkaloid indirubin (**7**) was able to inhibit biofilm formation by *L. monocytogenes* in a dose-dependent manner, without affecting planktonic cell density.[136]

The peperidine alkaloid deoxynojirimycin (**10**) from *Morus alba* showed anticariogenic potential against *S. mutans*, displaying different antivirulence effects. In addition to inhibition of biofilm formation, deoxynojirimycin also reduced the production of glycolytic acid by *S. mutans*, the synthesis of both water-soluble and -insoluble glucans as well as of the surface protein antigen SpaP, and the expression profile of several virulence genes.[137,138]

Berberine (**14**) is an isoquinoline-type alkaloid isolated from *Coptidis rhizoma* and other herbs that has been found to prevent the formation of *S. epidermidis* biofilm in both titanium alloy and polystyrene surfaces. The mechanism of action has been linked to the interaction of berberine with bacterial membrane and DNA.[139,140] Magesh and co-workers[141] showed the potential of both berberine and reserpine (**9**) on biofilm inhibition activity against high-biofilm-producing and multi-drug-resistant clinical isolates of *Klebsiella pneumoniae*. Reserpine is an indole alkaloid found in the dried roots of *Rauwolfia serpentine* and *Rauwolfia vomitoria* and has been reported to be an inhibitor of gram-positive bacterial efflux.[141] Furthermore, sanguinarine (**15**) and chelerythrine (**16**) were purified from *Macleya cordata* and presented a dose−response curve of biofilm formation inhibition and mature biofilm disruption against *S. aureus* and *S. epidermidis*.[142] It was inferred that both compounds were able to inhibit biofilm formation through a mechanism other than the mere killing of bacterial cells, preventing *S. aureus* transition to the sessile phenotype and acting on some elements of the bacterial cytoskeleton. Both compounds exhibited poor activity against mature biofilms.[142]

Caffeine (**24**), isolated from the leguminosae *Trigonella foenum-graecum*, caused noticeable disruption of biofilm formation by *P. aeruginosa*, without any inhibitory growth effect on the bacterial pathogen tested.[31]

3.3. Fatty Acids and Derivatives

It is known that fatty acids interact with biological membranes, increasing permeability and releasing different proteins or even sections of this layer. Stenz and co-workers[143] showed that oleic acid (**32**) drastically affected *S. aureus* biofilm production during primary adhesion, although once primary adhesion

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developed oleic acid stimulated biofilm formation. Oleic acid also significantly reduced the number of surviving biofilm cells, inhibiting biofilm accumulation and acid production rate by *S. mutans*.[144] Linoleic acid (**33**), a structurally related compound, is a major component of the *n*-hexane fraction from *Dryopteris crassirhizoma* able to reduce *S. mutans* biofilm accumulation during both initial and mature biofilm development in a dose-dependent manner. Moreover, when preformed biofilms were treated, linoleic acid did not affect the viability of *S. mutans* biofilm cells, although the dry weight of biofilms was significantly diminished.[145] Confirming these data, Pandit and co-workers[144] observed that linoleic acid strongly diminished dry weight, EPS biovolume, and thickness of *S. mutans* biofilms. Antibiofilm activity has also been demonstrated for linoleic acid against biofilms produced by *K. pneumoniae* clinical isolates.[141]

3.4. Organosulfurs and Derivatives

The effect of allylisothiocyanate (**34**) and 2-phenylethylisothiocyanate (**36**) against biofilms formed by *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* and the eradication thereof was investigated by Borges and co-workers.[146] Allylisothiocyanate prevented biofilm formation by *P. aeruginosa*, *E. coli*, and *L. monocytogenes*, while 2-phenylethylisothiocyanate significantly prevented biofilm formation by *P. aeruginosa*, *E. coli*, and *S. aureus*. However, total biofilm prevention was observed only for *E. coli* treated with allylisothiocyanate. Analyzing biomass and metabolic activity, these researchers also tested the capacity of these two molecules to control aged biofilms. Allylisothiocyanate and 2-phenylethylisothiocyanate caused the greatest reduction in biomass of *E. coli*. Similarly, 2-phenylethylisothiocyanate was more active than allylisothiocyanate to remove *E. coli*, *P. aeruginosa*, and *S. aureus* biofilms. Both compounds reduced the metabolic activity of all the biofilms tested, except that formed by *P. aeruginosa*.

Allicin (**37**) diminished biofilm formation by *S. epidermidis*, including clinical isolates, in a concentration-dependent manner.[147,148] Wu and co-workers[147] demonstrated that the architecture of biofilms produced by allicin-treated bacteria varied, especially in terms of biofilm thickness, which was accompanied by loss of viability of biofilm bacteria. Concomitantly, the authors observed down-regulation of *aap* and *icaA* gene expression in the biofilm, which has been associated with adhesion and bacterial accumulation in a biofilm, suggesting the involvement of a polysaccharide-intercellular-adhesion (PIA)-independent mechanism in the inhibition of *S. epidermidis* biofilm formation.[147] Ranjbar-Omid and co-workers[149] showed that allicin may be an excellent therapeutic agent for the treatment of *P. mirabilis*, because it inhibited biofilm development, disrupted established biofilms, and decreased urease activity in vitro.

Ajoene (**38**), a QS-interfering compound, presented a clear synergistic effect with tobramycin on *P. aeruginosa* biofilm killing.[38] Sulforaphane (**41**) and erucin (**42**), which are natural isothiocyanates from broccoli, also strongly reduced biofilm formation by *P. aeruginosa*. Surprisingly, the inhibitory activity of erucin against biofilm formation was markedly lower than that induced by sulforaphane, while in *P. aeruginosa* QS assays the compounds displayed similar activities. This contrast can be attributed to the small difference in overall hydrophobicity between the two compounds, resulting in differences in permeation and distribution in the biofilm.[39]

Zosteric acid (**44**), commonly found in the eelgrass *Zostera marina*, significantly decreased the biomass and thickness of *E. coli* biofilms, without affecting cell growth.[150]

3.5. Other Aliphatic Compounds

According to Brackman and co-workers,[40] the aliphatic compounds *trans*-2-nonenal (**48**) and *trans*-3-decen-2-one (**54**), which are able to inhibit AI-2-mediated QS, affected biofilm formation by *Vibrio anguillarum*, *Vibrio vulnificus*, and *Vibrio cholerae*. The authors suggested that such prevention of biofilm formation occurs through the inhibition of matrix production and/or bacterial accumulation.

3.6. Other Cyclic Compounds

Interestingly, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (**61**), also known as Furaneol or strawberry furanone, efficiently inhibited *P. aeruginosa* biofilm formation and facilitated cell detachment from the preformed biofilm. Moreover, the thickness and architecture of the treated biofilm varied, and most cells were sparsely scattered on the substratum, in a thin monolayer.[42]

A novel compound, a naphthalene derivative (**62**) isolated for the first time from *Trachyspermum ammi* seeds, reduced *S. mutans* adherence and biofilm formation, insoluble glucan synthesis, and bacterial hydrophobicity by destroying cell wall integrity.[151] Gnaphaliol-3-*O*-β-D-glucopyranoside (**63**) and gnaphaliol-9-*O*-β-D-glucopyranoside (**64**), the benzofuran glycosides isolated from *Helichrysum italicum*, inhibited biofilm formation and were able to disperse established *P. aeruginosa* biofilms.[152] It was speculated that the mechanisms of the compounds could be linked with *P. aeruginosa* QS response, despite the involvement of growth inhibition in gnaphaliol-9-*O*-β-D-glucopyranoside activity.[152]

3.7. Phenolics and Derivatives

3.7.1. Anthocyanins. The anthocyanin malvidin (**65**) was identified as the compound responsible for the QSI activity of *S. cumini*, which reduced EPS production and biofilm formation in *K. pneumoniae*.[43]

3.7.2. Coumarins. Of nine coumarins tested, Lee and co-workers[153] found that coumarin (**66**), umbelliferone (**67**), and coladonin (**73**) were able to reduce *E. coli* biofilm formation, without diminishing bacterial cell growth. Scanning electron microscopy (SEM) revealed a reduction in fimbriae production by coumarin and umbelliferone.[153] Umbelliferone eradicated preformed biofilms, with percent biomass removal and inactivation always higher for *E. coli* than for *S. aureus*, at all concentrations tested.[29] Also, Gutierrez-Barranquero and co-workers[44] noted that coumarin was effective against biofilm formation of *E. coli*, *V. anguillarum*, *Edwarsiella tarda*, and *S. aureus*.

A virtual screening performed using the 3D crystal structure of TraR (*A. tumefaciens*) combined with computer-aided drug design and biological assays allowed the identification of three coumarins as potential QSIs against *P. aeruginosa*, namely, esculetin (**68**), esculin (**69**), and psoralen (**71**). All compounds inhibited biofilm formation by *P. aeruginosa*, and psoralen was the most potent antibiofilm agent in this group.[154] Investigating traditional Chinese medicines, Ding and co-workers[86] showed that the furanocoumarin nodakenetin (**72**) prevented biofilm formation by *P. aeruginosa* and *Stenotrophomonas maltophilia*. The furanocoumarins bergamottin (**75**) and imperatorin (**76**), isolated from *Citrus* spp., inhibited *E. coli* biofilm formation and motility, but only at the highest concentrations tested, without

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affecting cell viability.[155] The ability of furocoumarins to inhibit biofilm formation was also tested using strains of *E. coli*, *S. typhimurium*, and *P. aeruginosa*, against which dihydroxybergamottin (**74**) and bergamottin significantly affected biofilm mass, without influencing bacterial growth.[48]

3.7.3. Flavonoids. Wallock-Richards and co-workers[156] demonstrated that *S. mutans* biofilm formation was reduced by *trans*-chalcone (**77**) in a concentration-dependent manner, with efficacy tailing off at higher concentrations. Other chalcones (**78**−**80**) were identified in a high-throughput screening platform with over 500 flavonoids and were shown to be highly active against formation and disruption of biofilm produced by *S. aureus*.[157] Xanthohumol (**87**), a prenylated chalconoid isolated from *Humulus lupulus*, was likewise effective against *S. aureus* biofilm formation and in already-formed biofilms, reducing mature biofilm viability.[158] It was hypothesized that xanthohumol is a potent inhibitor of lipid metabolism, affecting the composition and stability of microbial cell wall/membrane.[158] Regarding gram-negative strains, phloretin (**77**) inhibited *E. coli* biofilm formation, reducing fimbria formation without inhibiting the growth of planktonic cells.[159] A number of chalcones (**84**−**86**) isolated from *P. delineatum* suppressed biofilm formation by *V. harveyi* in a dose-dependent way.[50] Since these compounds disrupted QS signaling, biofilm formation suppression was likely to occur through disruption of cell-to-cell communication, without affecting bacterial growth.[50]

Catechin (**90**) and epigallocatechin (**93**) are flavan-3-ols that showed excellent antibiofilm activities against *P. aeruginosa*.[53,160] Epigallocatechin-3-gallate (**94**), the main polyphenol component of teas, interfered with the polysaccharides, decreasing slime production, therefore inhibiting biofilm formation by staphylococcal clinical isolates.[161] This metabolite was able to inhibit the sucrose-dependent initial attachment of *S. mutans*, thus inhibiting dental plaque accumulation, and also *S. mutans* pre-established biofilms.[162,163] At sub-MIC (minimal inhibitory concentration) levels, epigallocatechin-3-gallate inhibited the acidogenicity and acidurity of *S. mutans*, possibly as a result of the effect on bacterial glycolytic pathways.[162] Other studies demonstrated that epigallocatechin-3-gallate induced a significant decrease in adhesion, biofilm formation, and cell viability of clinical isolates of *S. maltophilia* and prevented biofilm development by *Burkholderia cepacia* and *C. jejuni*.[54,55,164] Epigallocatechin-3-gallate also destroys established *Porphyromonas gingivalis*[165] and *Enterococcus faecalis* biofilms.[166] It has been proposed that the antibiofilm activity of epigallocatechin-3-gallate does not depend on metabolic activity; instead, it is associated with its ability to bind to the peptidoglycan, damaging the integrity of the bacterial cell wall and thereby interfering with the initial docking phase of biofilm formation and subsequent mature phase.

In a dose-dependent manner, the flavanone (−)-(2*S*)-7,5′-dihydroxy-5,3′-dimethoxyflavanone (**95**) strongly inhibited biofilm formation by *V. harveyi* via disruption of QS signaling and without affecting bacterial growth.[50] Testing several flavonoids, Vikram and co-workers[56] demonstrated that *V. harveyi* and *E. coli* biofilms were reduced by the flavanones naringenin (**97**), naringin (**98**), hesperidin (**116**), neohesperedin (**117**), and neoeriocitrin (**118**) and by the flavones apigenin (**123**) and sinensetin (**135**), as well as by the flavonols quercetin (**144**), kaempferol (**150**), and rutin (**153**). According to the authors, the type and position of a sugar moiety may be harmful to biological activity, as demonstrated by the decrease in potency of aglycons by the addition of a sugar moiety at the seventh position (naringin) and at the third position (rutin). Moreover, the presence of a double bond between the second and the third positions does not favor biological acitivity, while the addition of hydroxyl groups plays an important role in this activity.[56] The flavanone glycosides naringin, hesperidin, and neohesperidin inhibited biofilm formation by *Y. enterocolitica*, and the antibiofilm effect was restored with the direct addition of 3-oxo-C6HSL and C6HSL.[57] Isoamoritin (**108**) was shown to inhibit the production of *P. aeruginosa* biofilms in a dose-dependent way, with no growth inhibition.[167] It was supposed that the action of the compound upon neuraminidase is linked to the reduction in biofilm formation.[167] With regard to gram-positive biofilms, a systematic screening of natural and synthetic flavonoids, covering 500 chemical structures, pointed to the flavanones glabranine (**96**), 8-prenylnaringenin (**99**), and isosakuranetin (**100**) as the most active natural antibiofilms against *S. aureus*.[157] Such findings lead to speculation about the possibility that the lipophilicity of molecules could improve bacterial transmembrane transport, in addition to facilitating the interaction of the compounds with the biofilm matrix.[157] Five alopecurones (**111**−**115**) were isolated from roots of *Sophora alopecuroides* and exhibited antibiofilm formation activity against *S. epidermidis* at concentrations lower than that for growth inhibitory activity.[168]

An interesting investigation revealed that apigenin (**123**) caused a significant impact in the biomass and polysaccharide content of *S. mutans* biofilms, inhibiting glucosyltransferases and fructosyltransferase without major impact on bacterial viability.[169] Red wine components such as the flavones apigenin, chrysin (**124**), and luteolin (**125**) as well the flavonols quercetin (**144**), fisetin (**149**), and kaempeferol inhibited *S. aureus* biofilm formation, when quercetin was remarkably the most active flavonoid.[170] The same study also showed that antibiofilm activity could be linked to the number and position of the hydroxyl group in flavonoid structures, in which a high number of hydroxyl groups enhanced antibiofilm activity.[170] Different flavonoids were screened for biofilm inhibitors against enterohemorrhagic *E. coli*, and the flavones apigenin and chrysin as well as the isoflavones daidzein (**155**) and genistein (**156**) presented good activity without inhibiting the growth of planktonic cells.[159] Furthermore, Moran and co-workers[171] demonstrated the antibiofilm activity of the isoflavone genistein against *S. aureus*, but not against *S. epidermidis*. Luteolin also significantly disrupts biofilm formation of *E. coli* through down-regulating the expression of the adhesin gene *fimH*, reducing bacterial surface hydrophobicity and suppressing motility.[172] Two flavones (**131**−**132**), isolated from *Teucrium polium*, were active against *S. aureus* biofilm formation and were efficient in eradicating biofilm through bacteriostatic effects.[173]

The isoprenylated flavone artocarpin (**137**) exhibited a strong dose-dependent effect on biofilm formation by *S. pneumoniae* strains and significantly reduced adherence of bacteria to epithelial cells.[174] Icariin (**139**), a flavonol glycoside, showed concentration-dependent antibiofilm activity, eradicating *Propionibacterium acnes* biofilms.[175] Computer-based analyses using the crystal structure of TraR (*A. tumefaciens*) and in vitro assays identified the flavones baicalein (**126**) and baicalin (**127**) as potential QSIs against *P. aeruginosa*.[154] Both compounds inhibited biofilm formation by *P. aeruginosa*, but baicalein was the most potent antibiofilm agent. Moreover, in the presence of baicalein, *P. aeruginosa* was significantly

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susceptible to ampicillin, demonstrating that this compound exhibited a marked synergistic activity.[154] Three structurally related flavones (**133**−**135**), isolated from *Citrus* spp., inhibited *E. coli* biofilm formation and motility, without affecting cell viability, among which heptamethoxyflavone (**133**) was one of the most potent inhibitors.[155]

Lee and co-workers[176] found that the flavonol quercetin (**144**), a compound present in the extract of *Alnus japonica*, inhibited the formation of biofilms of three *S. aureus* strains. The effects of quercetin on the expression of genes linked to biofilm formation and virulence in *S. aureus* were investigated by transcriptional analyses, which demonstrated that *icaA*, *icaD*, *agrA*, *sigB*, and *sarA* were repressed by this compound.[177] Quercetin and quercetin-3-*O*-arabinoside (**145**), the latter being isolated from *Psidium guajava*, were reported to prevent biofilm formation of *S. mutans* via inhibition of enzymes involved in polysaccharide synthesis and other proteins required for aggregation.[178,179] The derived glycoside quercitrin (**146**) inhibited *S. mutans* biofilm, reducing the synthesis of both water-soluble and insoluble glucans, and suppressed expression of several virulence genes.[137] In addition, quercetin was described to reduce significantly *P. aeruginosa* biofilm formation, being more effective than the antibacterials streptomycin and ampicillin.[180]

A recent study by Arita-Morioka and co-workers[181] revealed that myricetin (**147**) prevents curli production and curli-dependent biofilm formation by *E. coli* in a concentration-dependent manner through inhibition of the molecular chaperone DnaK. Myricetin also suppressed biofilm formation by *S. aureus* strains, including clinically isolated methicillin-resistant strains. Morin (**148**), a flavonol that has been detected in several Chinese herbs, reduced biofilm formation by *S. mutans*.[182] Morin activity was not linked with a decrease in viability; rather, the compound was shown to prevent cell wall anchoring of Pac adhesion.[182] Another investigation based on in silico docking analysis of QSI highlighted five top ranking compounds, including the flavanol morin and the flavanone naringin, which effectively disrupted *P. aeruginosa* biofilm architecture and reduced its biofilm formation.[183]

In a dose-dependent way, the isoflavonoid dalbinol (**189**) prevented the production of *P. aeruginosa* biofilms, without affecting bacterial growth. It was suggested that the inhibition of neuraminidase activity promoted by the compound led to the reduction in biofilm formation.[167]

3.7.4. Lignans. Macelignan (**191**) was isolated from *Myristica fragrans* and presented antiplaque action, reducing biomass of oral biofilms produced by *S. mutans*, *Streptococcus sanguis*, and *Actinomyces viscosus*.[184] Magnolol (**192**) is a major component isolated from *Magnolia* spp., which inhibited *S. aureus* biofilm development and simultaneously affected the transcription of virulence factors, such as the PIA.[185] Medioresinol (**193**), a furofuran-type lignan isolated from *Sambucus williamsii*, demonstrated a potent inhibitory effect on preformed biofilms and synergistic effects when combined with conventional antibacterials against the gram-positives *S. aureus*, *E. faecalis*, and *P. acnes* and the gram-negatives *P. aeruginosa* and *E. coli*.[186]

3.7.5. Phenolic Acids. The antibiofilm potential of gallic acid (**196**) against *S. aureus* and clinical isolates of methicillin-resistant *S. aureus* (MRSA) was investigated by Luis and co-workers.[187] The results showed that gallic acid reduced the formation of biofilm by all the *S. aureus* strains tested.[187] Investigating gallic acid, Borges and co-workers[188] demonstrated that important changes in motility caused by this compound can be correlated with a decreased ability of bacteria to form biofilms, at subinhibitory concentrations. Gallic acid prevented biofilm formation by *L. monocytogens*, *P. aeruginosa*, *S. aureus*, and *E. coli*. Additionally, the authors showed that the compound has great higher potential to reduce preformed biofilm biomass by the gram-negative microorganisms, when compared to the gram-positive bacteria. Although total biofilm removal was not achieved, the metabolic activity of all biofilms was strongly reduced by this phenolic acid, indicating that most adherent cells are unviable.[188] Shao and co-workers[189] evaluated gallic acid action on *E. coli* and *S. mutans* biofilm formation, observing that it was significantly affected by nutrient level, temperature, and treatment time. Gallic acid was also able to inhibit biofilm formation of *C. violaceum*, but when in synergism with carbenicillin, the treatment in fact enhanced biofilm formation.[190] Gallic acid and its derivative methyl gallate (**197**) inhibited in vitro biofilm formation and plaque accumulation of *S. mutans*.[179,191]

Salicylic acid (**198**), a monohydroxybenzoic acid, prevented the formation of *S. mutans* biofilm by interfering with the glucan synthesis mechanism through inhibition of glucosyl and fructosyl transferases, which synthesize extracellular polymeric substances.[179] Found at relatively high levels in barks of many plants, this natural product eradicates preformed biofilms, with percent biomass removal and inactivation rates higher for *E. coli* than for *S. aureus*, independently of concentration.[29] Jagani and co-workers[160] and Prithiviraj and co-workers[192] reported that salicylic acid also significantly reduces biofilm formation by *P. aeruginosa*. The related compounds *p*-hydroxybenzoic acid (**199**) and protocatechuic acid (**204**) had an antibiofilm effect against *S. aureus* in a concentration-dependent manner; however, both stimulated *S. epidermidis* biofilm formation.[171] Vanillin (**205**), a major constituent of vanilla beans, was able to prevent *Aeromonas hydrophila* biofilm formation.[69]

The most abundant ginkgolic acids (**206** and **207**) in *Ginko biloba* extract, markedly and in a dose-dependent way, inhibited *E. coli* and *S. aureus* biofilm formation, without affecting cell growth.[193] The reduction of *E. coli* fimbriae was observed by SEM, confirming a transcriptome analysis that revealed that ginkgolic acids C15:1 clearly down-regulated curli genes.[193] Considering *P. aeruginosa*, malabaricone C (**208**) was able to inhibit several virulence factors, including biofilm formation, to a similar extent as the positive control catechin.[72]

3.7.6. Phenylethanoids. In a study by Coenye and co-workers,[175] several plant extracts were investigated against *P. acnes* biofilms, and salidroside (**210**) was found to be a potent biofilm eradication compound when tested at subinhibitory concentrations. Glycosylated phenylethanoids with the desmethylyangonine group in their structures (**211**−**213**) isolated from *H. italicum* inhibited *P. aeruginosa* biofilm formation, although they were not active against preformed biofilms.[152]

3.7.7. Phenylpropanoids. The dihydroxycinnamic acids caffeic acid (**218**) and chlorogenic acid (**230**) showed great inhibitory activity against *S. epidermidis* biofilm formation.[194] Caffeic acid stimulated bacterial cell growth, possibly due to the stress caused by the compound, while the antibiofilm activity of chlorogenic acid seems to be associated, at least in part, to bacterial growth inhibition.[194] The antibiofilm potential of caffeic and chlorogenic acids against *S. aureus* and clinical isolates of MRSA was investigated by Luis and co-workers.[187] The results showed that both acids reduced biofilm formation and affected 24-h-established biofilms of all *S. aureus* strains

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tested. The authors also used SEM to analyze biofilm and observed that these acids damage the cell wall of the bacteria, indicating that both metabolites affect bacterial viability. Additionally, they observed that caffeic acid influences the stability of the cell membrane and the metabolic activity of the cells of *S. aureus*.[187] Chlorogenic acid also acts against gram-negative biofilms. This compound exhibited activity against preformed biofilms produced by *S. maltophilia* clinical isolates, greatly diminishing biofilm viability.[195] Chlorogenic acid and 3,5-dicaffeoylquinic acid (**232**), both of which were isolated from the medicinal plant *H. italicum*, displayed the best inhibitory effects in a preliminary screening against *P. aeruginosa* biofilms.[152] Confirming the results of in silico docking analysis of QS inhibitors, five top ranking compounds, including chlorogenic acid and rosmanaric acid (**231**), effectively disrupted biofilm architecture and reduced biofilm formation by *P. aeruginosa*.[183] Nordihydroguaiaretic acid (**233**) was able to eradicate staphylococcal biofilms on the basis of a mechanism involving disruption of the biofilm matrix.[196]

Borges and co-workers[188] demonstrated that ferulic acid (**219**) caused important changes in bacteria motility, which can be correlated with a decreased ability of bacteria to form biofilms, at subinhibitory concentrations. Ferulic acid prevented biofilm formation by *P. aeruginosa*, *S. aureus*, and *E. coli*, being ineffective against biofilm formation by *L. monocytogens*. As described for gallic acid, ferulic acid also demonstrated higher potential to decrease biomass and viability of gram-negatives, especially for *P. aeruginosa*.

Several reports highlight the role of cinnamaldehyde (**221**) as an important agent against bacterial biofilms. Cinnamaldehyde inhibited biofilm growth by *S. pyogenes*, decreasing biomass, thickness, and colony size at substratum, besides disturbing the production of virulence factors associated with the LuxS-based QS system.[197] Moreover, this compound effectively removed the biofilms of MRSA and decreased *sarA* expression.[198] Similar inhibitory action was observed for *L. monocytogenes* biofilm development and disruption on different material surfaces.[199] It was found that these activities are correlated with modulation of genes involved in initial attachment and quorum sensing response.[199] Targeting gram-negatives, cinnamaldehyde prevented and inactivated uropathogenic *E. coli* and *P. mirabilis* biofilms on polystyrene plates and catheters, showing irregular holes in biofilm due to cell loss and organizational disruption.[200] This natural product also prevented biofilm formation by *Vibrio* species, such as *V. anguillarum*, *V. vulnificus*, and *V. cholerae*, for which the prevention of biofilm formation seems to occur based on the inhibition of matrix production and/or bacterial accumulation.[73] Additionally, it has been shown that cinnamaldehyde inhibits AI-2-mediated QS by decreasing the DNA-binding ability to LuxR, therefore affecting several virulence factors and increasing susceptibility to stress. The derivative 4-methoxy-cinnamaldehyde (**222**) induced a significant decrease in *V. anguillarum* biomass, without activity against other *Vibrio* species.[73]

Studying essential oils, Kim and co-workers[74] showed that the main constituent of cinnamon bark oil, cinnamaldehyde, markedly inhibited *P. aeruginosa* biofilm formation. Moreover, cinnamaldehyde and eugenol (**226**) significantly decreased biofilm formation by *E. coli* and reduced the production of fimbriae. The authors observed that the incorporation of cinnamaldehyde to the polymer poly(D,L-lactide-*co*-glycolide) (PLGA) prevented *P. aeruginosa* and *E. coli* biofilm formation, while the incorporation of eugenol in the PGLA was effective only against *E. coli* biofilm.

Eugenol decreased *S. aureus* biomass in early and mature biofilms and interfered with the expression of biofilm- and enterotoxin-related genes.[201] This phenylpropanoid inhibited biofilm development and inactivated *L. monocytogenes* biofilms formed on polystyrene plates and stainless steel coupons.[199] The compound also acts against biofilm formation by the gram-negatives *P. aeruginosa* and *K. pneumoniae* clinical isolates.[75,141] Different authors have reported the antibiofilm activity of eugenol, characterized by damage to cell membrane and subsequent leakage of cell content. However, the structurally related methyl eugenol (**228**) was shown to reduce *P. aeruginosa* biofilm formation without any growth inhibitory effect.[78]

Zingerone (**227**) inhibited *P. aeruginosa* biofilm formation and eradicated established biofilms.[80,202] Biofilms developed in the presence of zingerone did not present a mucilaginous matrix and were more susceptible to ciprofloxacin, when compared to the untreated control.[202] Extracted from fresh ginger oil, 6-gingerol (**234**) was shown to reduce several virulence factors, including biofilm formation, in addition to decreasing mice mortality induced by *P. aeruginosa*.[81] The compound was also active when biofilm was evaluated under static conditions and in a continuous drip-flow reactor. DNA microarray analysis revealed that several QS related genes were down-regulated in biofilm cells treated with 6-gingerol, such as those involved in the production of rhamnolipid, elastase, pyocyanin, and in the synthesis of the MexGHI-OpmD efflux pump.[81]

Curcumin (**237**) has been consistently investigated due to its potential against bacterial virulence. This compound was shown to inhibit biofilm formation by gram-negative strains, such as *E. coli*, *P. aeruginosa*, and *K. pneumoniae* clinical isolates.[82,141,159] The potential of curcumin against the virulence of the uropathogens *E. coli*, *P. aeruginosa*, *P. mirabilis*, and *S. marcescens* was highlighted by Packiavathy and co-workers.[84] The compound was effective in preventing biofilm formation as well as in disturbing mature biofilms of these uropathogens (except *P. aeruginosa*). Increasing concentrations of curcumin supplemented with commercial antibiotics enhanced bacterial susceptibility, indicating a synergistic effect between these compounds.[84] Curcumin also inhibited biofilm produced by *Vibrio* species, such as *V. harveyi*, *V. parahemolyticus*, and *V. vulnificus*, and was able to disintegrate mature biofilms, reducing the production of EPS components, including alginate.[83] In vivo challenging experiments with *Artemia nauplii* showed that curcumin enhanced the survival rate of *Artemia* by decreasing the virulence of *V. harveyi*.[83] Regarding the action of curcumin on gram-positive bacteria, antibiofilm activity was only described against *S. mutans*.[203]

3.7.8. Quinones. The anthraquinones emodin (**241**) and hypericin (**242**) reduced *S. mutans* biofilm formation on hydroxyapatite, possibly by causing membrane perturbation, while no effect was observed on glucosyltransferase activity.[204] Studying plants used in traditional Chinese medicine, Ding and co-workers[86] showed that the anthraquinones emodin and chrysophanol (**244**) and the naphthoquinone shikonin (**247**) inhibited biofilm formation on a glass surface by the gram-negatives *P. aeruginosa* and *S. maltophilia*, when emodin was the most potent compound. Emodin was shown to act as a QSI, justifying its activity against *P. aeruginosa* biofilm formation; however, its mode of action against *S. maltophilia* was unclear.[86]

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A novel quinone (**243**), isolated from *Aegle marmelos*, exhibited a significant dose-dependent inhibition of biofilm formation of *E. coli*, *Salmonella typhi*, and *P. aeruginosa* and was nontoxic to mammalian cells.[205] Thymoquinone (**245**), purified from *Nigella sativa*, prevented biofilm formation by *S. aureus*, *S. epidermidis*, and *E. faecalis*, reducing the metabolic activity of cells embedded in a biofilm.[206]

3.7.9. Simple Phenols. Screening phenolic compounds against *S. aureus* virulence factors, Lee and co-workers[207] observed that resorcinol (**248**) significantly inhibited biofilm formation and human blood hemolysis induced by *S. aureus*. Acylphloroglucinol derivatives (**249**−**251**) isolated from *Hypericum punctatum* and *Hypericum densiflorum* strongly inhibited biofilms produced by *S. aureus* and *S. epidermidis*, although the detailed mechanism of action of acylphloroglucinols remains to be determined.[208] Another acylphloroglucinol derivative named panduratin A (**252**) is a secondary metabolite isolated from the species *Kaempferia pandurata* and had a dose-dependent effect in preventing and reducing a multispecies oral biofilm composed of *S. mutans* and *S. sanguis* in vitro, but the underlying mechanisms of this inhibition remain unclear.[209] Also, 7-epiclusianone (**254**), isolated from *Rheedia brasiliensis*, was found to decrease *S. mutans* biofilm formation through the reduction of the extracellular-insoluble polysaccharides biomass.[210−212] The compound also reduced the development of caries in rat models, without affecting bacterial viability.[211] The polyprenylated phloroglucinol hyperforin (**255**), the major constituent of *Hypericum perforatum*, was effective in the reduction of *S. aureus* biofilms, though it should be highlighted that clinical isolates were less sensitive to the compound, when compared with their ATCC counterpart strains.[213]

Dihydroxybenzofurane (**256**), purified from *Krameria lappacea*, was able to inhibit biofilm accumulation and disrupted mature biofilms of the gram-positives *S. aureus* and *S. epidermidis*.[142]

3.7.10. Stilbenoids. Lee and co-workers[207] reported the activity of stilbene-related compounds against *S. aureus* virulence, emphasizing the inhibition of biofilm formation by *trans*-stilbene (**258**), dicinnamyl (**259**), and oxyresveratrol (**261**). The authors found that *trans*-stilbene prolonged *C. elegans* survival, indicating that this compound reduces *S. aureus* virulence. *trans*-Stilbene-treated-bacteria presented down-regulation in *icaA* and *icaD* expression, which explains the mechanism through which it inhibits biofilm production by *S. aureus*.

Resveratrol (**260**) showed marked biofilm-eradicating activity against *P. acnes* biofilms;[175] however, as described above, most reports published to date have discussed its activity against gram-negative biofilms. Cho and co-workers[214] showed that resveratrol and ε-viniferin (**262**) inhibited biofilm formation by *P. aeruginosa* and *E. coli* in a dose-dependent fashion, without antimicrobial activity. Resveratrol also inhibited biofilm formation by *V. cholerae* in a concentration-dependent manner and demonstrated binding affinity with AphB (*V. cholerae* virulence activator) protein, indicating a putative target by docking analysis.[215] Screening 498 plant extracts against enterohemorrhagic *E. coli* (EHEC), Lee and co-workers[216] detected the antibiofilm activity of *Carex dimorpholepis* extract and established resveratrol as one of the constituents of this extract. Resveratrol significantly reduced the formation of biofilm by EHEC and diminished the expression of several genes, such as curli genes (*csgA* and *csgB*), flagellar motility genes (*fimA*, *fimH*, *flhD*, *fliA*, *motB*, *qseB*, and *qse*C), and AI-2 quorum-sensing genes (*lsrA*, *luxS*, and *luxR*), as well as those related to fimbriae production. The authors also suggested that this compound could interfere with AI-2 signaling and reduce the formation of biofilm by EHEC by down-regulating *qseBC* and *flhDC*.[216]

The antibiofilm activity of six oligostilbenoids, namely, resveratrol, ε-viniferin, suffruticosol A (**263**), suffruticosol B (**264**), vitisin A (**265**), and vitisin B (**266**), was investigated against enterohemorrhagic *E. coli* and *P. aeruginosa*, and vitisin B was shown to be the most active of these six oligomers.[217] Also, transcriptional and phenotypic assays were used to investigate the molecular mechanism responsible for *E. coli* biofilm inhibition. Gene expression of the *csg* operon, which is involved in curli formation, was markedly repressed by ε-viniferin, suffruticosol B, and vitisin B, while suffruticosol A and vitisin A modified the expression of these genes less significantly. By evaluating QS and motility genes in *E. coli*, it was found that vitisin B induced the expression of four motility genes (*fliA*, *flhD*, *motB*, and *qseB*), whereas the other oligomers did not. These oligostilbenoids reduced fimbriae production, suggesting that biofilm inhibition was caused at least in part by this reduction.

3.7.11. Tannins. Ellagic acid (**268**) is generated from hydrolysis of tannins such as ellagitannin. Ellagic acid showed greater antibiofilm activity against *S. aureus* and *E. coli*, for which biofilms were reduced regarding the surface area covered as well as thickness.[218,219] Besides the possible damage to the cell membrane, ellagic acid and other tannins can precipitate vital proteins involved in the formation of biofilms, such as adhesins, resulting in a marked variation of surface charge and thereby interfering with cell−substratum interactions and biofilm development. Additionally, the study by Huber and co-workers[55] showed that biofilm formation by *B. cepacia* was reduced after treatment with ellagic acid. The structurally related tannin 3-*O*-methyl ellagic acid (**269**), isolated from *Anethum graveolens*, also reduced pigment production by *C. violaceum* and significantly interfered with *S. marcescens* QS, as revealed by the modulation of prodigiosin pigment, biofilm, and protease production in a concentration-dependent manner.[91]

The hydrolyzable tannin found in the bark and leaves of *H. virginiana*, hamamelitannin (**270**), decreased *S. aureus* and *S. epidermidis* biofilm formation on polystyrene, in a concentration-dependent manner.[92] Additionally, in vivo studies in rats showed a strong inhibition of infection when bacteria were treated with hamamelitannin before its administration and when grafts were coated with hamamelitannin. These effects were associated with inhibition of the *agr* QS system by this tannin.[92] The gallotannin 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (**271**), isolated from *Eustigma oblongifolium*, strongly inhibited biofilm formation by *S. aureus*, independently of growth mechanisms.[220] This gallotannin prevented initial attachment of bacteria to solid surfaces and the synthesis of polysaccharide intercellular adhesion, in addition to being nontoxic to humans cells.[220]

Tannic acid (**272**) has been shown to be active against gram-positive biofilms, such as *S. aureus* and *S. mutans*, without inhibiting bacterial growth.[170,179,221] Payne and co-workers[221] presented evidence that the effects of tannic acid on *S. aureus* biofilms was dependent on the putative transglycosylase *IsaA*, as proven by the assays with different mutants. Lee and co-workers[176] tested the antibiofilm activity of 498 plant extracts against *S. aureus*. From this screening, the most active extract, from *A. japonica*, presented tannic acid in its composition. This

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compound inhibited biofilm formation by three *S. aureus* strains in a dose-dependent manner and repressed the expression of genes linked to biofilm formation and other virulence-regulatory genes, including *icaA*, *icaD*, *agrA*, *sigB*, and *sarA*.[176] Considering the literature reports about tannic acid and gram-negative strains, the compound was found to be active against *P. aeruginosa* and *E. coli* biofilms.[160,218] In this sense, using *C. dimorpholepis* extract, Lee and co-workers[216] observed that tannic acid prevents EHEC biofilm. Tannic acid was also able to inhibit biofilm formation by *C. violaceum*, but when it was tested in synergism with antibiotics, the result was an enhancement of biofilm formation.[190]

Artini and co-workers[142] isolated proanthocyanidin A-2 (**274**) from *Aesculus hippocastanum*, which impaired biofilm formation by *S. aureus* and *S. epidermidis* strains. This activity seems to be associated with iron uptake and synthesis of peptidoglycan, since it down-regulated iron-binding protein and other proteins, such as penicillin-binding proteins.[142] B-type linked proanthocyanidins (**279**), isolated from *Pityrocarpa moniliformis*, prevented biofilm adhesion without killing gram-positive cells of *S. epidermidis*, *S. aureus*, and *E. faecalis*. This condensed tannin displayed antibiofilm activity both in solution and when immobilized on a surface and was nontoxic to mammalian cells.[222] Other condensed tannins (**280**−**281**), purified from *Anadenanthera colubrina* and *Commiphora leptophloeos*, strongly prevented biofilm formation by *P. aeruginosa* through bacteriostatic properties.[223]

A-type proanthocyanidin oligomers (**282**), from *Vaccinium macrocarpon*, popularly known as cranberry, were highly effective in inhibiting the synthesis of EPS and impair the accumulation of *S. mutans* biofilms.[224] Topical treatments down-regulated several virulence genes involved in the adhesion of *S. mutans*, acid stress tolerance, and glycolysis, as well as incidence of dental caries in vivo.[225,226] Cranberry oligomers also significantly inhibited biofilm formation of *P. aeruginosa* and caused a reduction of preformed biofilms, rescuing human embryonic kidney cells from lysis by *P. aeruginosa* and attenuating virulence in vivo using a *Galleria mellonella* larvae infection model.[227]

3.7.12. Xanthones. α-Mangostin (**284**), a xanthone purified from *G. mangostana*, effectively reduced the accumulation of *S. mutans* biomass and disrupted its structural integrity, facilitating its mechanical removal. This effect can be, at least in part, explained by inhibition of key enzymatic systems associated with synthesis of extracellular glucans and acidogenicity.[228] On the basis of the results of in silico docking for QSI, five top ranking compounds, including mangiferin (**285**), effectively disrupted biofilm architecture and reduced biofilm formation by *P. aeruginosa*.[183]

3.8. Steroids and Derivatives

Isolated from *Citrus* spp., β-sitosterol-3-*O*-glucopyranoside (**286**) was shown to be the most potent inhibitor of *E. coli* biofilm formation and motility. The proposed mechanism of action involves *rssAB*- and *hns*-mediated repression of the flagellar master operon *flhDC*.[155]

3.9. Terpenoids and Derivatives

Essential oils and their derivatives are known to exhibit different biological activities. In this sense, the potential of use of terpenes as agents in the treatment of infectious diseases and their safety regarding human and animal health has been the focus of several investigations. The monoterpene derivatives thymol (**289**) and its isomer carvacrol (**290**) are frequent compounds in essential oils and, at subinhibitory concentrations, can disrupt biofilm organization. Both compounds presented antibiofilm activity and potential to eradicate preformed *Staphylococcus* spp. biofilms.[96,229,230] Thymol and carvacrol were also active at lower concentrations against *Pseudomonas* spp. and *L. monocytogenes* biofilms,[199,230,231] while carvacrol was effective against *C. violaceum* and *S. typhimurium* biofilms.[199,230,231,96] Menthol (**300**) strongly interfered with QS-regulated virulence factors of *P. aeruginosa*, as observed in a concentration-dependent way, decreasing biofilm formation and EPS production.[100] Studying major components of clary sage, juniper, lemon, and marjoram essential oils, such as terpinene-4-ol (**291**), limonene (**302**), linalool (**305**), and α-pinene (**306**), Kerekes and co-workers[97] found that these compounds inhibited biofilm formation by *E. coli* and *Bacillus cereus*. Except for limonene, all components proved to be better inhibitors than the parent oils. Terpinene-4-ol also had inhibitory effect against *P. putida* biofilm. Because these components have the cell wall and cytoplasmic membrane/or proteins of membrane as main targets, it may be expected that the loss of cell membrane integrity leads to cell death and thus to loss of the ability to attach to surfaces and form biofilms.[97]

Phytol (**310**), an acyclic monounsaturated diterpene alcohol, reduced *P. aeruginosa* biofilm formation, exhibiting higher activity than both positive controls used, streptomycin and ampicillin.[232] Targeting gram-positive biofilms, the abietane-type diterpene dehydroabietic acid (**312**) combines high antibiofilm efficacy with low mammal cytotoxicity and can be obtained from various plants sources.[233] Dehydroabietic acid inhibited biofilm formation by *S. aureus* independently of bacteriostatic properties, and unlike typical antibiotics, the concentrations required to significantly reduce viability and biomass of existing biofilms were only 2−4 times as high as those required by antibiotics drugs.[233] The diterpenes kaurenoic acid (**313**), *ent*-trachyloban-19-oic acid (**314**), and the sesquiterpene xanthorrizol (**317**) displayed anticariogenic potential, reducing *S. mutans* biofilms and other associated virulence factors.[234−236] Through the partial inhibition of several virulence factors of *S. mutans*, kaurenoic acid, isolated from *Aralia continentalis*, altered bacterial attachment and biofilm formation on hydroxyapatite beads and inhibited acid production.[234] *ent*-Trachyloban-19-oic acid and xanthorrizol were isolated using bioassay-guided fractionation from *Iostephane heterophylla* and interfered with established *S. mutans* biofilms.[236] Moreover, the coating of xanthorrizol on polystyrene surface resulted in significant antiplaque activity, reducing adherent bacteria, when compared to chlorhexidine, which currently is the standard principle for oral antibacterial activity.[235]

The casbane diterpene (**315**), originally isolated from *Croton nepetaefolius*, was also found to significantly interfere with biofilm formation.[237] In a preliminary evaluation, Carneiro and co-workers[237] demonstrated that biofilm formation disturbance of *S. aureus* and *Pseudomonas fluorescens* was linked to the ability of the compound to inhibit planktonic growth, although this diterpene acted against *E. coli*, *P. aeruginosa*, and *K. pneumoniae* without affecting planktonic growth. The fusicoccane-type diterpenoid anadensin (**316**) and two aromadendrane-type sesquiterpenoids (**326**, **327**), isolated from *Porella chilensis*, inhibited biofilm formation by *P. aeruginosa* and interfered with the QS process, slightly disturbing bacterial growth.[238]

Farnesol (**318**), an acyclic sesquiterpene alcohol, was shown to inhibit *S. aureus* biofilm formation by affecting cell

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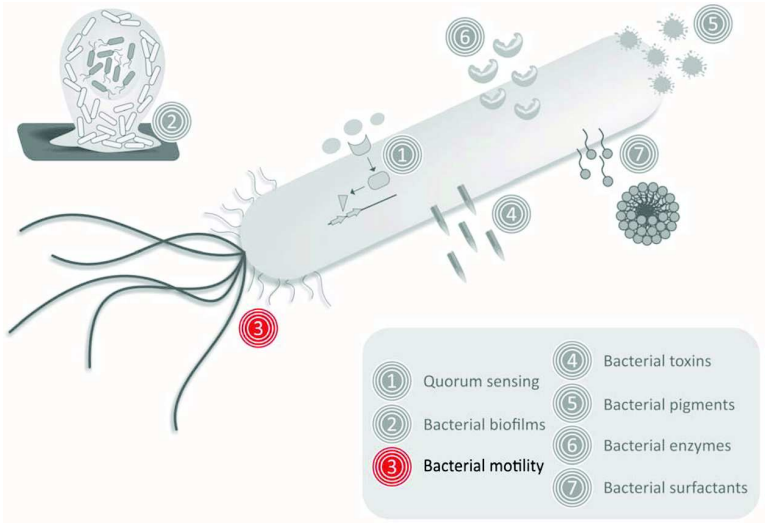

Figure 4. Motility-related structures as a bacterial target for antivirulence compounds.

membrane integrity and demonstrated a synergistic effect with gentamicin.[239] Jeon and co-workers[240] demonstrated that farnesol reduces biomass accumulation and prevents ecological shifts toward *S. mutans* dominance within mixed-species biofilms, through disrupting the membrane function and the physiology of this bacterium. Lee and co-workers[241] screened the antibiofilm activity of 83 essential oils and their common constituents farnesol, *cis*-nerolidol (**320**), and valencene (**322**), which were found to markedly inhibit *S. aureus* biofilm formation.

The sesquiterpene viridiflorol (**328**) and the triterpenoids (**365**, **367**−**371**) were isolated from the liverwort *Lepidozia chordulifera* and evaluated against bacterial biofilms.[238] The compounds shoreic acid (**367**), eichlerialactone (**368**), cabraleone (**369**), cabraleadiol (**370**), 3-β-hydroxy-nordammaran-20-one (**371**), and viridiflorol significantly decreased *P. aeruginosa* biofilm formation, while the compounds taraxerol (**365**), among others (**328**, **367**, **369**, **370**), reduced *S. aureus* biofilm formation. A computer-assisted study of these triterpenoid compounds was carried out for a better understanding of the structure−activity relationships. Among the analyzed triterpenoids, those that have an adammarane skeleton were the most potent inhibitors of *P. aeruginosa* biofilm formation. Also, it seems that the THF ring on C-17 bonded to an isopropylol group plays an important inhibitory role against *S. aureus* biofilm.[242] Six sesquiterpene lactones isolated from *Centratherum punctatum* displayed antibiofilm potential against *P. aeruginosa*, three of which belong to the goyazensolide (**329**−**331**) and three to the isogoyazensolide (**332**−**334**) classes of compounds, in an action mechanism that may be linked to interference in the QS.[102] Cartagena and co-workers[243] also studied the effects of terpene lactones on *P. aeruginosa* biofilm production, since the chemical structures are similar to the lactone moiety present in *N*-acyl homoserine lactones. Nine sesquiterpene lactones (**335**−**343**), isolated from *Acanthospermum hispidum* and *Enydra anagalli*, strongly inhibited biofilm formation when incorporated into bacterial cultures of *P. aeruginosa*.[243]

Considering nor-triterpenes, Vikram and co-workers[244] showed that citrus limonoids, such as isolimonic acid (**344**), ichangin (**345**), isoobacunoic acid (**346**), isoobacunoic acid glucoside (**347**), and deacetyl nomilinic acid glucoside (**348**), inhibited EHEC biofilm formation, with isolimonic acid being the most potent inhibitor. The inhibitory activity of isolimonic acid was lost in a Δ*qseBC* mutant and the expression of *qseA* in Δ*qseA* restored the repressive effect. Thus, these results indicate the existence of a model according to which isolimonic acid modulates biofilm and the type III secretion system (TTSS) in a manner that depends on QseBC and QseA. It was also shown that isolimonic acid and ichangin seem to affect biofilm formation and TTSS by repressing the locus of enterocyte effacement (LEE) and the flagellar operon.[244] Five other limonoids (**349**−**353**) were purified from *Citrus paradise* and demonstrated a concentration-dependent inhibitory effect against *V. harveyi* and *E. coli* biofilm formation.[245] Considering this series of nor-triterpenes, obacunone (**350**) was the most effective compound against biofilms produced by both bacteria, indicating that the suppression of biofilm formation probably does not depend on growth inhibitory properties and is likely to affect QS signaling.[245]

The triterpene 3β,6β,16β-trihydroxylup-20(29)-ene (**354**), isolated from *Combretum leprosum*, inhibited biofilm biomass from *S. mutans* and *Streptococcus mitis*, reducing the number of biofilm-entrapped cells. The proposed mechanism is likely to be associated with a decrease in cell viability.[246] The pentacyclic triterpene acids asiatic acid (**356**) and corosolic acid (**357**) exhibited positive interactions with tobramycin, by reducing the tolerance of *P. aeruginosa* biofilm to antibiotics.[247] Ursolic acid (**358**), a triterpene closely related to asiatic and corosolic acids, did not have a significant statistical interaction with tobramycin, despite inhibiting *P. aeruginosa* biofilm formation.[247,248] Moreover, ursolic acid inhibited biofilm formation by five different *E. coli* strains, *P. aeruginosa*, and *V. harveyi*.[249] The analysis of *E. coli* biofilm structure confirmed that biofilm development was inhibited by ursolic acid, with a substantial decrease in biomass, extent of surface covered, and thickness. In addition, it was noted that biofilm formation was not affected by this triterpene in a paralyzed strain of *E. coli* (with a *motAB* mutation), suggesting that the removal of the *motAB* gene is sufficient to counteract the ability of ursolic acid to inhibit biofilms. The suggested mechanism of action considered the induction of chemotaxis and motility genes by this molecule during biofilm

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development, which may destabilize the biofilm and prevent biofilm maturation.[249]

Hu and co-workers[248] also reported the potential of naturally occurring ursolic acid derivatives from *Diospyros dendo* as gram-negative biofilm inhibitors. In that study, considering the four triterpenes purified (**359**−**362**), the compound 3*β*-*O*-*cis*-*p*-coumaroyl-20*β*-hydroxy-12-ursen-28-oic acid (**359**) presented the best activity against *P. aeruginosa* biofilms.[248] Celastrol (**363**), a triterpenoid derivative, demonstrated a potent biofilm eradication activity against staphylococcal biofilms, reducing the amount of biofilm cells and destructuring the biofilm matrix, in addition to a synergy with gentamicin.[196]

4. PLANT-DERIVED NATURAL PRODUCTS AGAINST BACTERIAL MOTILITY

4.1. Overview

Bacterial adhesion is an important initiation step in bacterial colonization and persistence, for both pathogens and commensals, and represents a survival strategy in harsh environments. Bacteria express structural cell-surface components or appendages such as capsule, fimbriae or pili, and several surface proteins known as adhesins. Typically, these adhesive structures are not expressed at the same time as the flagellum, so that movement and attachment occur at different times. The switch of bacteria from motile to sessile lifestyles and vice versa is closely linked with chemotaxis, the ability to orientate along certain chemical gradients, controlling the direction of movement and the extent of colonization. Cell−cell signaling (such as QS systems), motility, and chemotaxis interact in complex ways, enabling bacteria to detect and pursue nutrients in order to reach new niches for colonization (Figure 4).[250,251]

The best known type of motility is related to the use of a specialized rotating organelle, the flagellum. Swimming and swarming are types of flagella-dependent bacterial motility and can contribute to the virulence of pathogens through host colonization and other forms of complex colonial behavior, including the formation of biofilms. Swimming motility is a mode of bacterial movement powered by rotating flagella but, unlike swarming motility, which is the movement of a group of bacteria, it takes place as individual cells moving in liquid environments. Twitching motility is a flagella-independent form of bacterial translocation powered by the extension and retraction of type IV pili located at one or both poles of the cell. Another form of motility is sliding, a passive form of surface spreading that does not require an active motor but instead relies on surfactants to reduce surface tension, enabling the colony to spread away from the origin driven by the expansive forces of a growing colony.[251−253]

Bacterial motility is often intimately linked with virulence by complex regulatory networks, since it is required for a successful infection, as demonstrated by a number of mutant nonmotile strains. Motility-related structures affect the pathways toward host colonization and subsequent invasion, promoting early biofilm formation and virulence factor secretions and triggering adaptive and innate immune defenses.[250] Importantly in this context, c-di-GMP signaling seems to play an important role in modulating a series of molecular processes as a breaking point, whether bacteria remain in a planktonic motile or in a sessile lifestyle.[129]

Altough motility inhibition would not be selective for pathogens, also affecting commensals, the interference in this target could decrease the efficiency of colonization and secretion or expression of virulence factors, which are required for successful infections.[254−258]

Considering the plant-derived natural products active against the virulence factor motility, the classes of active products discussed below were found.

4.2. Alkaloids and Derivatives

The flagellar function of *E. coli* was assessed in the presence of subinhibitory concentrations of the indole alkaloid reserpine (**9**) and the piperidine alkaloid piperine (**11**) by quantifying bacterial swimming and swarming motilities.[259] Both compounds decreased bacterial motility, revealing a decrease in the expression of the flagellar gene (*fliC*) and motility genes (*motA* and *motB*). Interestingly, both alkaloids increased biofilm formation along with an increased expression of adhesin genes (*fimA*, *papA*, *uvrY*) and piperine increased penetration of ciprofloxacin and azithromycin in *E. coli* biofilms, enhancing the ability of these antibiotics to disperse pre-established biofilms.[259] Gradual concentrations of piperine also decreased the swarming movement of *H. pylori*, due to the suppression of the biosynthetic regulator gene *flhA* and flagellar hook gene *flgE*.[260] Monte and co-workers[29] demonstrated that indole-3-carbinol (**3**) decreased the swarming and swimming motilities of *E. coli*, while it was not able to change the sliding motility of *S. aureus*.

The swarming motility of *P. aeruginosa* was inhibited with caffeine (**24**), allowing the observation of a bacterial colony with short and undefined tendrils.[30] The authors found that motility limitation occurs due to the anti-QS properties of caffeine, as described above, and attributed a possible relationship between swarming inhibition and rhamnolipid production by *P. aeruginosa*.[30] Confirming this finding, caffeine isolated from the leguminosae *T. foenum-graceum* strongly interfered with QS-regulated virulence factors of *P. aeruginosa*, as observed in a concentration-dependent drop in swarming motility.[31]

4.3. Fatty Acids and Derivatives

Using a qualitative test, Inoue and co-workers[261] demonstrated that fatty acids such as 11-methyldodecanoic acid (**26**) were partly efficient to inhibit *P. aeruginosa* swarming motility, while the fatty acids vaccenic acid (**31**) and oleic acid (**32**) induced the complete inhibition of motility, without inhibiting bacterial growth. Liaw and co-workers[262] investigated the effect of lauric acid (**27**), myristic acid (**28**), palmitic acid (**29**), and stearic acid (**30**) on *P. mirabilis* virulence factors. All these compounds significantly inhibited swarming motility. Myristic acid, lauric acid, and palmitic acid were not able to inhibit swarming in a *rsbA*-defective mutant, indicating that these fatty acids exert their inhibitory effect through an RsbA-dependent pathway. In contrast, stearic acid reduced swarming of *rsbA*-defective mutant, indicating that the compound inhibited *P. mirabilis* swarming through an RsbA-independent pathway. *rsbA* is a gene that encodes a histidine-containing phosphotransmitter of the bacterial two-component signaling system. The data obtained by qRT-PCR also showed that myristic acid inhibits the expression of *flhDC*. The authors indicated that fatty acids or their derivatives may act as intracellular signals to regulate swarming and virulence factor expression through either an RsbA-dependent or RsbA-independent pathway.[262]

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4.4. Organosulfurs and Derivatives

The action of allylisothiocyanate (**34**) and 2-phenylethylisothiocyanate (**36**) against *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* motilities was investigated by Borges and co-workers.[146] These organosulfurs promoted total inhibition of the swimming, swarming, and twitching motilities of *P. aeruginosa*, while both molecules inhibited the swarming and swimming of *E. coli*. It was observed also that *L. monocytogenes* swimming motility and *S. aureus* colony spreading were reduced by the two compounds. The authors suggested that this effect is linked to changes in bacterial cell surface properties.

4.5. Other Aliphatic Compounds

No compound found.

4.6. Other Cyclic Compounds

A nonhalogenated furanone found in a variety of fruits, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (**61**), substantially decreased QS-related virulence factors of *P. aeruginosa*, including swarming and swimming motilities, while the effect on twitching motility was only marginal.[42]

4.7. Phenolics and Derivatives

4.7.1. Anthocyanins. No compound found.

4.7.2. Coumarins. *P. aeruginosa* motility, a multicellular virulence marker, was evaluated by Gutierrez-Barranquero and co-workers[44] through the measurement of dendrite length, when it was noted that coumarin (**66**) treatment reduced swarming. Monte and co-workers[29] demonstrated that umbelliferon (7-hydroxycoumarin) (**67**) was able to promote a decrease in the swimming and swarming motilities of *E. coli* and the sliding motility of *S. aureus*. Lee and co-workers[153] observed that coumarin and umbelliferone reduced *E. coli* swarming motility, but not swimming motility, of the bacterium. Supporting these data, the authors observed a reduction in fimbriae production generated by the repression of curli (*csgA* and *csgB*) and motility (*flhD* and *motB*) genes, for both compounds.[153] The furanocoumarins bergamottin (**75**) and imperatorin (**76**), isolated from *Citrus* spp., demonstrated significant inhibition of *E. coli* motility halos, without affecting cell viability.[155]

4.7.3. Flavonoids. Epigallocatechin gallate (**94**) was found to block the swarming motility of *P. aeruginosa*, and a similar effect was observed for *B. cepacia*.[55,263] Also, according to Castillo and co-workers,[54] this flavonol was able to considerably decrease *C. jejuni* motility in a dose-dependent manner.

Naringenin (**97**), a flavonone present in grapefruit and related species, influenced *S. typhimurium* motility and invasiveness, without affecting bacterial growth.[264] It was suggested that naringenin affected the flagellar genes by down-regulating the *flhDC*, possibly via *grpE* and fimbriae genes *fimZ* and *fimA*.[264] Additionally, the exposure of *Y. enterocolitica* to the glycosylated derivative naringin (**98**) inhibited swimming motility and inducted the transcription of *yenR* (a gene involved in the synthesis of AHLs) and *flhDC* and *fliA* (members of the flagellar regulon system).[57] In a study carried out by Annapoorani and co-workers,[183] several natural compounds were subjected to docking studies for *P. aeruginosa* QSI. This analysis allowed the identification of naringin and the flavonol morin (**148**) as potential inhibitors. The activity of both compounds regarding the reduction of swarming motility was experimentally confirmed.[183]

The flavone luteolin (**125**) decreased *E. coli* bacterial surface hydrophobicity and swimming motility, indicating that this metabolite contributes to the reduction of bacterial adhesion, invasion, and biofilm formation.[172] Three flavones, heptamethoxyflavone (**133**), nobiletin (**134**), and sinensitin (**135**), isolated from *Citrus* spp. also were shown to inhibit *E. coli* motility, without affecting cell viability, and nobiletin was identified as the most potent inhibitor.[155] The flavonol quercetin (**144**) reduced twitching motility and protrusions of *P. aeruginosa* colonies.[180]

4.7.4. Lignans. No compound found.

4.7.5. Phenolic Acids. Borges and co-workers[188] studied the ability of gallic acid (**196**), at subinhibitory concentrations, to interfere with the three types of bacterial motilities: swimming/colony spreading, swarming, and twitching. This phenolic acid caused a significant decrease in swimming, swarming, and twitching motilities of *P. aeruginosa* and in swimming and swarming of *E. coli* and a complete inhibition of *L. monocytogenes* swimming motility.[188] Moreover, gallic acid was found to inhibit the swarming motility of *P. aeruginosa* by O'May and co-workers.[263] Also, Dusane and co-workers[190] recently demonstrated its activity on *C. violaceum* swimming motility.

In a study conducted by Bandara and co-workers,[70] salicylic acid (**198**), derived from the metabolism of salicin, reduced the twitching and swimming motilities of an invasive phenotype of *P. aeruginosa*. Monte and co-workers[29] demonstrated that salicylic acid was also able to promote a decrease in the swimming of *E. coli* and the sliding motility of *S. aureus* but not the swarming motility of the gram-negative strain. Lee and co-workers[193] showed that ginkgolic acid (**206**) reduced *E. coli* swarming motility at the same time that it induced swimming motility, suggesting that swarming rather than swimming motility influences enterohemorrhagic *E. coli* biofilm formation.

4.7.6. Phenylethanoids. No compound found.

4.7.7. Phenylpropanoids. Ferulic acid (**219**), at subinhibitory concentrations, significantly decreased the swimming, swarming, and twitching motilities of *P. aeruginosa* and the swimming and swarming of *E. coli* and led to a total inhibition of *L. monocytogenes* swimming and *S. aureus* colony spreading.[188] During a screening of herbal extracts, eugenol (**226**) was identified as a potential QSI, being able to decrease several virulence factors of *P. aeruginosa*, including swarming motility.[75] A similar inhibition profile was reported by Kim and co-workers,[74] in which eugenol and cinnamaldeyde (**221**) impaired *P. aeruginosa* swarming motility. Cinnamaldehyde also was effective in reducing *L. monocytogenes* motility, as manifested by significant down-regulation of the expression of many virulence genes, especially the expression of *motB* and *motA* genes. The phenylpropanoids chlorogenic acid (**230**) and rosmaniric acid (**231**) were identified as *P. aeruginosa* QS inhibitors by docking analysis, and they decreased swarming motility in vitro.[183]

Zingerone (**235**), one of the major components of dry ginger root and found in many herbal spices, is capable of reducing all motility phenotypes (swimming, swarming, and twitching motilities) of *P. aeruginosa*.[80,202] In the study conducted by Packiavathy and co-workers,[84] curcumin (**237**) inhibited the swimming and swarming motilities of the uropathogens *E. coli*, *P. aeruginosa*, *P. mirabilis*, and S. *marcescens*. Similarly, the swimming and swarming motilities of *V. harveyi*, *V. parahemolyticus*, and *V. vulnificus* decreased after curcumin treatment.[83]

4.7.8. Quinones. 10′(*Z*),13′(*E*)-Heptadecadienylhydroquinone (**246**), isolated from the sap of *Rhus succedanea*, inhibited

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the swarming migration of *P. mirabilis* in a dose-dependent manner, even at a concentration that was not toxic for human urothelial cells. This effect was observed against the wild-type and *rppA* mutant, but not the *rcsB* mutant, indicating that quinone avoids swarming through a RcsB-dependent pathway, a two-component signaling pathway that negatively regulates swarming and virulence factor expression. Additionally, the compound increased *P. mirabilis* polymyxin susceptibility, which is intrinsically highly resistant to this antimicrobial.[87]

4.7.9. Simple Phenols. No compound found.

4.7.10. Stilbenoids. Wang and co-workers[89] demonstrated that resveratrol (**260**) is able to inhibit *P. mirabilis* swarming and the expression of other virulence factors in a dose-dependent manner, affording complete inhibition of the swarming phenotype. Whereas swarming of wild type *P. mirabilis* was inhibited by resveratrol, the motility of the *rsbA*-defective mutant was not, indicating an RsbA-dependent pathway, without involving bacterial cell growth. Additionally, this metabolite also inhibited the ability of *P. mirabilis* to invade human urothelial cells.

High levels of resveratrol were identified in the extract of *Carex dimorpholepis*. The compound reduces the swimming and swarming motilities of *E. coli*. By transcriptional analysis, the authors reported a decrease in the expression of key motility genes (*flhD*, *fliA*, *fimA*, *fimH*, *motB*, *qseB*, and *qseC*), as well as genes related to curli production.[216]

4.7.11. Tannins. Tannic acid (**272**), a hydrolyzable tannin, was found to block the swarming motility of *P. aeruginosa* and the swimming motility of *C. violaceum*, while its precursor unit ellagic acid (**268**) has been shown to reduce the ability of *B. cepacia* to swarm.[55,190,263] The swarming and swimming motilities of *S. typhimurium* were evaluated in the presence of subinhibitory concentrations of the hydrolyzable tannin punicalagin (**273**), the main active compound in pomegranate peel.[94] Punicalagin slightly reduced both motilities at low concentrations and completely abolished them when high doses were administered.[94] Punicalagin and A-type proanthocyanidin oligomers (**282**) from cranberry also limited the swarming motility of *P. aeruginosa*, considering both the distance covered and the complexity of the swarming pattern, but these tannins did not block swimming or twitching motilities.[227,265]

4.7.12. Xanthones. The computer-based discovery of putative QSI of *P. aeruginosa* allowed identifying mangiferin (**285**) as an agent that reduced flagellar- and pili-mediated motility (swarming), thus inhibiting biofilm formation.[183]

4.8. Steroids and Derivatives

Vikram and co-workers[155] showed that β-sitosterol-3-*O*-glycopiranoside (**286**), isolated from *Citrus* spp., inhibited *E. coli* motility without affecting cell viability. The authors demonstrated that the mechanism was linked to *rssAB*- and *hns*-mediated repression of the flagellar master operon *flhDC*.

4.9. Terpenoids and Derivatives

The monoterpenes thymol (**289**) and carvacrol (**290**) were effective in restricting *L. monocytogenes* motility.[266] Both compounds significantly down-regulated the expression of many virulence genes, presenting maximal inhibitory effect on the transcription of *lmo1666* and *intB* (which play a role in *L. monocytogenes* adhesion and invasion of host tissue), as evidenced by a reduced adhesion and invasion of Caco-2 and HBMEC cells.[266] Burt and co-workers[267] showed that *E. coli* incubated with carvacrol was nonmotile, as determined by the hanging-drop technique. Carvacrol-treated cells were observed to lack flagella and appeared longer and smoother than nontreated cells, which correlated with a down-regulation of a protein identified as flagellin. Additionally, the findings indicated that carvacrol must be present while cells are dividing in order to produce an inhibitory effect on flagellin, since treatment with carvacrol in the exponential phase of bacterial growth did not cause a significant decrease in flagellin amounts.[267] Also, van Alphen and co-workers[268] reported the antimotility effect of carvacrol on *C. jejuni*, in light of the virtually abolished motility and invasion of eukaryotic cells, without affecting bacterial growth.

Menthol (**300**) strongly interfered with QS-regulated virulence factors of *P. aeruginosa*, as observed in a concentration-dependent decrease in swarming motility.[100] Several monoterpenes were evaluated by Echeverrigaray and co-workers,[269] including α-terpineol (**291**), citral (**293**), citronellol (**294**), citronellal (**295**), geraniol (**296**), pulegone (**298**), terpinene-4-ol (**303**), and 1,8-cineol (**304**), concerning inhibition of *P. mirabilis* motility. Of all the compounds evaluated, these eight monoterpenes that effectively inhibited swarming are oxygenated, indicating the importance of the hydroxyl group for *P. mirabilis* swarming inhibition.[269]

Subinhibitory concentrations of phytol (**310**), an acyclic diterpenoid, effectively reduced *P. aeruginosa* twitching and flagella motility. Bacteria-treated cells were unable to produce a twitching zone and presented almost round, smooth, and regular colony edges.[232]

5. PLANT-DERIVED NATURAL PRODUCTS AGAINST BACTERIAL TOXINS

5.1. Overview

According to the chemical classification, bacterial toxins can be divided into two distinct groups, (i) lipopolysaccharides, which are chemical constituents of the outer cell membrane structure of gram-negative bacteria and, therefore, known as endotoxins, and (ii) proteins, which are secreted from a wide range of bacteria and diffuse extracellularly, being called exotoxins. Briefly, exotoxins can be grouped into three categories, namely, cytolytic toxins, AB toxins, and superantigens.

Pore-forming toxins are a type of cytolytic toxin produced by both gram-negative and gram-positive bacteria. They exhibit similar properties and are secreted as water-soluble monomers that interact with host membrane components. Once in contact with the target host cell membrane, the toxin oligomerizes into a prepore structure that undergoes a conformational change, facilitating insertion into the lipid bilayer.[270,271] *S. aureus* secretes pore-forming toxins, also called hemolysins or leukotoxins, which have been widely recognized for lysing red and/or white blood cells, in order to promote pathogenesis. Interestingly α-toxin (α-hemolysin, α-HL) is released by 95% of *S. aureus* strains and is the prototype of pore-forming cytotoxins.[272,273] Other bicomponent toxins produced by *S. aureus*, which are structurally similar to α-toxin, include Panton−Valentine leukocidin (PVL), the leukocidins LukED and LukAB, and two γ-toxins, or γ-hemolysins (HlgAB, HlgCB).[274] Similarly, by secreting a pore-forming toxin called listeriolysin O (LLO), the gram-positive bacilli *L. monocytogenes* gains access to the host cell cytosol and escapes from phagosomes in order to develop the intracellular infectious cycle.[275] Pneumolysin (PLY) is a cholesterol-dependent cytolysin produced by virtually all clinical isolates of *Streptoccoccus pneumoniae*. PLY is a key virulence factor that

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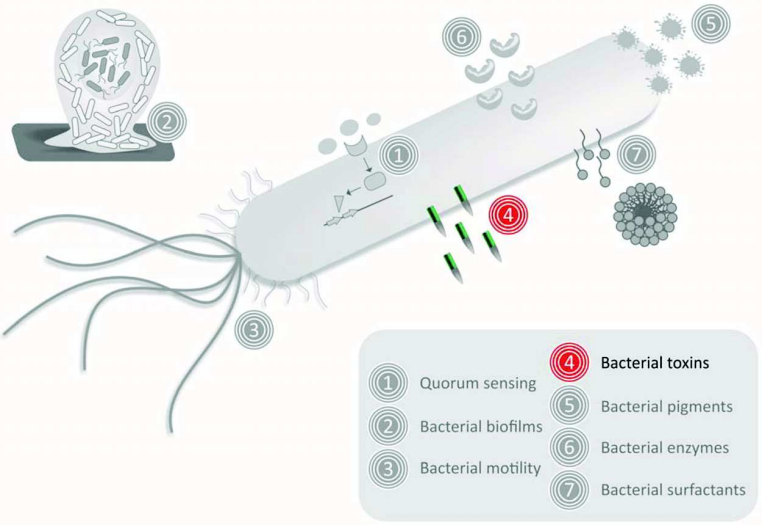

Figure 5. Bacterial toxins as target for antivirulence compounds.

contributes to the pathogenesis of pneumococcal diseases, promoting the apoptosis of several eukaryotic cells and releasing pro-inflammatory molecules.[276] The gram-negative bacilli *Proteus* spp. produce two types of hemolysins, HpmA and HlyA.[277] HpmA belongs to the pore-forming-toxins family and is cytolytic to a broad array of cell types from different host species.[278]

Phenol-soluble modulins (PSMs) are a family of small and amphipathic non-pore-forming peptides produced by *S. aureus* and *S. epidermidis*, which include three peptides termed α-PSM, β-PSMβ, and γ-PSM, the last of which is the long-known δ-hemolysin (Hld). PSMs differ from other cytolytic *S. aureus* toxins, such as α-toxin or two-component leukocidins, by targeting almost every eukaryotic cytoplasmic membrane.[279]

Superantigenic toxins target the host immune system, causing massive T- lymphocytes activation, cytokine releasing, and systemic shock. As some of the most potent bioactive toxins ever discovered, they stimulate human T-cells at femtomolar concentrations. The best studied superantigens (SAgs) are the family of staphylococcal enterotoxins (SEs) and streptococcal pyrogenic exotoxins (SPEs) secreted by the gram-positives *S. aureus* and *Streptococcus pyogenes*.[280] Among several SAgs from *S. aureus* described so far, we highlight (i) the toxic shock syndrome toxin 1 (TSST-1), which is the major causative toxin of toxic shock syndrome, a rare but potentially fatal toxin-mediated febrile illness, classically associated with, but not limited to, tampon use, and (ii) the virulent staphylococcal enterotoxin A and B (SEA and SEB), which elicit proinflammatory cytokines that can induce fever, hypotension, multiorgan failure, and lethal shock.[273,281] When derived by ingestion, SEs are reported to induce emesis and diarrhea, one of the most prevalent forms of food poisoning throughout the world.[282]

Toxins are appropriate targets in the search for new drugs to fight infections, because they are considered a major factor of microbial virulence and play a very important role in the establishment of infection, triggering a myriad of cellular processes in the host. The growing understanding of bacterial toxin structure and of their action pathways has greatly aided in the exploration of antitoxin therapies (Figure 5).[283]

Different therapeutic strategies have been investigated to inhibit toxins, among which are monoclonal antibodies, small-molecule inhibitors, and polymers.[284] The inhibition of various effects caused by toxins can be achieved through the prevention of pore formation/assembly, pore blockage, and interruption of toxin binding to the cell surface or toxin trafficking obstruction.[285−287] Despite the differences in toxins, in some cases traits such as functional similarity in perforating cellular membranes and the inherent need to attach to the cellular membrane are characteristics that a number of bacterial toxins share. The potential of bacterial toxins as a target to neutralize infections has been evidenced by the use of antibodies, which presented high efficacy in animal studies and clinical trials.[288] Interestingly, a synergistic effect was demonstrated using different protective monoclonal antibodies, since they target different epitopes of a toxin molecule.[289] However, one of the obstacles in this treatment is the high cost associated with the antibody production process.[285]

Moreover, toxin effects can be indirectly hindered through systems that modulate the transport of proteins across to plasma membrane (bacterial secretion systems) and global regulators (QS systems).[290] By targeting QS, besides inhibition of toxin expression, a number of other virulence factors could also be blocked, as discussed in section 2.1. Finally, toxin−antitoxin (TA) systems appear as a recent promising alternative in the development of new antimicrobials. TA systems are usually composed of two genes. The toxin gene encodes a toxin protein that has a toxic effect on the cells, whereas the antitoxin can be either RNA or protein, which neutralizes the toxicity of its cognate toxin.[291] Interestingly, TA genes have no human homologues and appear to be present in the most important bacterial pathogens.[292] The strategies involving TA systems have been the subject of important research and include the inhibition of transcription of the TA pair, affecting the autoregulation and impairing the synthesis of the TA; the inhibition of translation of the antitoxin by antisense molecules (in this case the study of molecules in preclinical and clinical evaluation has proved to be promising); and the promotion of proteases that cleave the antitoxin.[291,292]

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Considering the plant-derived natural products active against the virulence factor toxins, the classes of active products so far reported are discussed below.

5.2. Alkaloids and Derivatives

Several studies have looked into the ability of a variety of plant-derived indoles to inhibit hemolysis caused by *P. aeruginosa*. Though the precursor compound indole (**1**) was the most potent, its derivatives 3-indolylacetonitrile (**2**), indole-3-carbinol (**3**), indole-3-acetaldehyde (**4**), indole-3-acetamide (**6**), and isatin (**8**) exhibited different levels of efficacy to prevent hemolysis induced by the bacterium.[135]

Mitchell and co-workers[32] discovered that tomatidine (**12**), a steroidal alkaloid found in tomatoes, inhibits the hemolytic activity of several *S. aureus* strains. Importantly, the ability of *S. aureus* to lyse red blood cells is regulated by the QS-dependent *agr* system, and it has been confirmed that the expression of *hld* gene (encoding the δ-toxin and a fragment of RNAIII, the effector of the *agr* system) decreased with different concentrations of tomatidine.[32] In mice challenged with TSST-1, a toxin produced by *S. aureus*, anisodamine (**23**) significantly decreased serum proinflammatory cytokine levels and prevented TSST-1-induced death.[293] Moreover, this tropanic alkaloid showed no cytotoxicity to human monocytes.[293]

At subinhibitory concentrations, capsaicin (**25**), the main amide-derived alkaloid found in chili (genus *Capsicum*), substantially decreased the production of α-toxin by community-associated MRSA, as reported by Qiu and co-workers.[34] No immunoreactive α-toxin antigen could be detected in the supernatant of the tested strain, and the transcriptional levels of the *hla* gene fell 9-fold when *S. aureus* was cultured with the highest concentration of capsaicin. The addition of capsaicin prevented α-toxin-mediated human alveolar cell injury in coculture with *S. aureus*, and in vivo experiments indicated that capsaicin protected mice from MRSA pneumonia.

5.3. Fatty Acids and Derivatives

Lauric acid (**27**), myristic acid (**28**), and palmitic acid (**29**) inhibited the hemolysin activity of wild-type *P. mirabilis*, but not the *rsbA*-defective mutant, indicating that these fatty acids could act through an RsbA-dependent pathway. In contrast, stearic acid (**30**) inhibited the hemolysin activity of the wild-type strain and the *rsbA*-defective mutant, suggesting that this compound, as far as swarming modulation is concerned, negatively regulates hemolysin expression in *P. mirabilis* through an RsbA-independent pathway.[262]

5.4. Organosulfurs and derivatives

The major biologically active component of garlic, allicin (**37**), was effective to neutralize PLY, a toxin produced by *S. pneumoniae* that is toxic to essentially all human cell types.[294] This organosulfur decreased PLY hemolytic activity in a concentration-dependent manner, reaching a complete inhibition when using lysate or intact *S. pneumonia* cells. The inhibitory effect of allicin was restored by addition of a reducing agent, suggesting that it likely inhibits PLY by binding to the cysteinyl residue in the binding site.[294] Allicin, at subinhibitory concentrations, also was shown to reduce *S. aureus* hemolytic activity by decreasing the production of α-toxin in both MSSA and MRSA, in a dose-dependent manner. Moreover, the transcription of the *hla* gene in *S. aureus* was inhibited by allicin by about 7-fold.[36]

5.5. Other Aliphatic Compounds

No compound found.

5.6. Other Cyclic Compounds

The addition of phenylethyl alcohol (**58**) to three strains of *S. aureus* cultures led to a dose-dependent increase of biomass while resulting in lower concentrations of TSST-1 in the medium.[295]

5.7. Phenolics and Derivatives

5.7.1. Anthocyanins. No compound found.

5.7.2. Coumarins. No compound found.

5.7.3. Flavonoids. At subinhibitory concentrations the chalcone phloretin (**82**), which is abundant in apples, minimized the hemolytic activity induced by *S. aureus*.[177] A similar phenotype was observed in the presence of licochalcone A (**88**) and licochalcone E (**89**) isolated from *Glycyrrhiza glabra* that, at subinhibitory concentrations, markedly inhibited the *hla* gene and reduced α-toxin secretion and hemolysis.[51,52] Besides the effect on α-toxin secretion, licochalcone A significantly decreased the secretion of two major enterotoxins (SEA and SEB) in a dose-dependent manner by both MSSA and MRSA.[296]Consistent with these results the authors showed that the transcriptional level of *sea* and *seb* genes was reduced in a dose-dependent manner.[296]

A series of papers have been published investigating the antihemolytic activity of flavonoids, placing this class as a promising group of natural products targeting toxins. Shah and co-workers[297] demonstrated that the flavanol epicatechin gallate (**87**) reduces the secretion of α-toxin in culture supernatants of five MRSA clinical isolates. The authors speculate that these effects could be partly explained through the epicatechin gallate-mediated inhibition of α-toxin combined with induction of secreted proteases.[297] Likewise, subinhibitory concentrations of the flavanone farrerol (**101**), a traditional Chinese medicine, repressed hemolysis and α-toxin production of MSSA and MRSA strains. As expected, farrerol markedly decreased the transcription of *hla* in a dose-dependent manner, while it was not cytotoxic to the mouse macrophage cell line.[66]

The effect of the flavanones naringenin (**97**), liquiritigenin (**98**), and pinocembrin (**104**), of the flavones apigenin (**123**) and chrysin (**124**), of the flavolignan silibinin (**154**), and of the isoflavone puerarin (**166**) on α-HL expression was examined using different *S. aureus* strains.[58−60,62,63,170] At subinhibitory concentrations, the hemolytic activity in vitro was reduced in a dose-dependent manner, down to complete inhibition. Consistent with this result, the relative expression levels of the *hla* gene were gradually inhibited by increasing concentrations of naringenin, apigenin, chrysin, silibinin, and puerarin, as well as injury caused by α-toxin. Additionally, liquiritigenin, pinocembrin, apigenin, chrysin, and silibinin alleviated the lung injury caused by staphylococcal pneumonia.

Lee and co-workers[177,176] and Cho and co-workers[170] showed that several flavones or flavonols markedly reduced *S. aureus* hemolysis activity, including 6-hydroxyflavone (**121**), 6-aminoflavone (**122**), apigenin (**123**), fisetin (**149**), kaempferol (**150**), and genistein (**156**), while flavone (**120**), luteolin (**125**), and quercetin (**144**) have been shown to abolish hemolysis. Real-time qRT-PCR revealed that flavone clearly repressed the transcription of the *hla* gene by 11-fold and of the *sae* gene by 4-fold (a global regulator inducing *hla*), supporting the reduction of hemolysis in *S. aureus* cells.[177] Similarly, Qiu and co-workers[65] investigated luteolin and verified that this

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flavone substantially decreases *S. aureus* α-toxin production, inhibiting the expression of the *hla* gene by as much as 7 times.

Interestingly, the flavanone cyrtominetin (**102**), the flavone baicalin (**127**), the *O*-methylated flavone oroxylin A (**128**), and the flavonol morin (**148**) repressed the hemolytic activity of purified α-toxin, without anti-*S. aureus* activity.[298−301] It was also revealed that the production and secretion of α-toxin are not affected by these compounds; rather, research concluded that they bind directly to the toxin. All these compounds inhibited the self-assembly of the heptameric transmembrane pore, thereby decreasing the lytic activity of α-toxin. Additionally, baicalin, oroxylin A, and morin protected human alveolar epithelial cells against α-toxin-mediated injury when coculture experiments with *S. aureus* and human cells were performed. Also, morin and baicalin had protective effect against mortality induced by *S. aureus* pneumonia in a mouse infection model.[300,301] Likewise, Qiu and co-workers[302] studied how oroxylin A and the structurally related compounds oroxylin A 7-*O*-glucoronide (**129**) and oroxylin B (**130**) inhibit the hemolytic activity caused by α-toxin. It was shown that these three ligands may strongly interact with both sides of the binding cavity through van der Waals and electrostatic interactions between the ligand and α-toxin, restricting the conformational transition from monomer to oligomer. Another flavone studied by Qiu and co-workers[300] was wogonoside (**136**), which prevented hemolysis induced by *S. aureus* α-toxin by binding to the toxin and avoiding heptamer formation; however, its activity was lower than that of baicalin.

A potent antagonist of the *L. monocytogenes* toxin, called listeriolysin O, which mediated hemolysis, has been described. Wang and co-workers[303] demonstrated that incubation of fisetin at concentrations that were high enough to prevent hemolysis did not affect the expression and stability of listeriolysin O or *L. monocytogenes* growth. Molecular modeling and mutational analysis revealed that the flavonol directly anchors to specific sites on listeriolysin O, leading to the blockage of cholesterol binding (which is critical for oligomerization) and inhibiting its hemolytic activity. Importantly, fisetin effectively inhibited both tissue culture and mice infection models, facilitating the elimination of *L. monocytogenes* by macrophages by inhibiting escape from the phagosome.[303] A recent publication revealed that several flavonoids, including narigerin, chrysin, baicalin, myricetin (**147**), and morin, could inhibit the hemolytic activity of listeriolysin O more effectively than fisetin.[304] On the basis of computer algorithms, an overlay of the five modeling structures revealed that they bound to listeriolysin O following the same binding mode and that their mechanism of inhibition is consistent with those reported for fisetin. Moreover, it was shown that inhibitors having a double bond on the 4*H*-chromen-4-one moiety, i.e., flavones and flavonol, had highly increased biochemical potency when compared to the flavonone naringerin, which has a single bond.[304]

In addition to the activity reported for gram-positive toxins, flavonoids also presented substantial ability to avoid hemolysis induced by *P. aeruginosa* in a computational analysis that indicated that morin and naringin are active compounds.[183]

5.7.4. Lignans. Subinhibitory concentrations of magnolol (**192**), the main phenolic component of *Magnolia officinalis*, inhibited the transcription of *hla* in *S. aureus* in a dose-dependent manner. This reduced α-toxin secretion and, thus, reduced hemolysis by MSSA and MRSA.[305]

5.7.5. Phenolic Acids. The hemolytic activity of an α-toxin produced by different *S. aureus* strains cultured with graded subinhibitory concentrations of gallic acid (**196**) was investigated by Luis and co-workers,[187] and a slight activity was reported.

5.7.6. Phenylethanoids. 4-Hydroxytyrosol (**209**), commonly found in olives, was shown to inactivate the superantigenic activity of the SEA enterotoxin secreted by *S. aureus* in mice splenocytes.[306]

5.7.7. Phenylpropanoids. In a medium supplemented with subinhibitory concentrations of caffeic acid (**218**), the hemolytic activity of culture supernatants of *S. aureus* diminished, when the inhibition was shown to be strain-dependent.[187] Qiu and co-workers[76] showed that eugenol (**226**), the main component of clove oil, decreased the hemolytic activity of *S. aureus* culture supernatants in a dose-dependent manner and significantly reduced the production of SEA and SEB enterotoxins and TSST-1, consistent with the repressed transcription levels of the *S. aureus sea*, *seb*, *tst*, and *hla* genes. The structurally related compound eugenyl acetate (**227**) was reported to inhibit hemolysin activity by *S. aureus* and this effect was possibly related to the anti-QS activity.[77] Moreover, Li and co-workers[307] showed that the hemolysis of *S. aureus* culture treated with chlorogenic acid (**230**) was significantly decreased. Additionally, reduced binding to fibrinogen and decreased production of SEA were also observed after treatment with chlorogenic acid. The authors presumed that the limited secretion of toxins may partly depend on the down-regulation of the *agr* gene.[307]

Listeriolysin O activity in the supernatant of *L. monocytogenes* culture grown in the presence of subinhibitory concentrations of cinnamaldehyde (**221**) was quantified by hemolysis. The phenylpropanoid was found to decrease the production of hemolysin significantly and modulated the expression of virulence genes such as *hly* (that encodes listeriolysin production) and *prfA* (a transcriptional activator that positively controls the expression of genes coding for motility, adhesion, invasion, and production of hemolysin).[266] Considering *P. aeruginosa* toxins, computational discovery of putative QSI pointed out chlorogenic acid and rosmarinic acid (**231**) as possible active compounds that, in vitro, induced a substantial decrease in hemolysis.[183] Furthermore, three other phenylpropanoids, namely, cinnamaldehyde, eugenol, and zingerone (**235**), were highly effective in suppressing hemolysin production in a dose-dependent manner.[74,80]

5.7.8. Quinones. It has been reported that the expression of virulence factors in *P. mirabilis* is regulated coordinately with swarming behavior. Since 10′(*Z*),13′(*E*)-heptadecadienylhydroquinone (**246**), isolated from the sap of *R. succedanea*, inhibited *P. mirabilis* swarming motility, Liu and co-workers[87] also studied its effect on hemolysin activity. At a concentration that was not toxic for human urothelial cells, the quinone inhibited hemolysis and its transcription gene *hpmA*. The authors suggested that hemolysis suppression was mediated through the RcsB-dependent pathway.

5.7.9. Simple Phenols. Resorcinol (**248**), a precursor of stilbenoids backbone, inhibited *S. aureus* hemolytic activity, as demonstrated by Lee and co-workers.[207] The most promising activities were observed for stilbenes, as described below.

5.7.10. Stilbenoids. A screening investigation using stilbene-related compounds was performed by Lee and co-workers,[207] and showed that *cis*-stilbene (**257**), *trans*-stilbene (**258**), resveratrol (**260**), ε-viniferin (**262**), vitisin A (**265**), and

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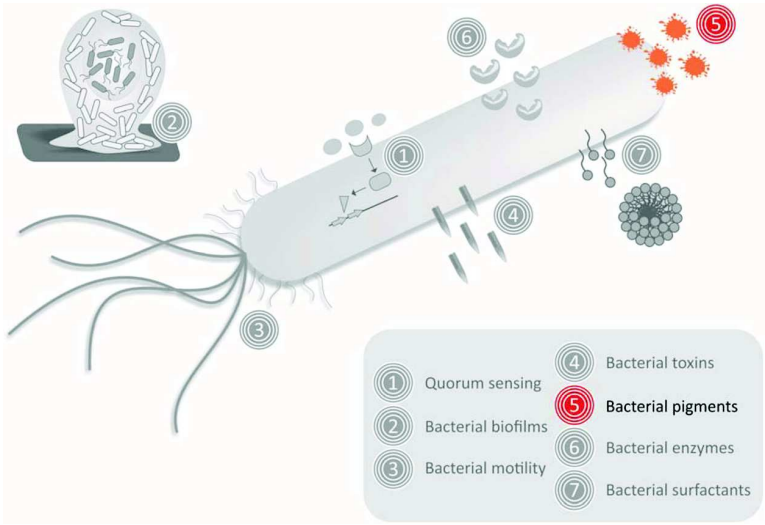

Figure 6. Pigment production and secretion as a bacterial target for antivirulence compounds.

vitisin B (**266**) prevented human blood hemolysis by *S. aureus*. Hemolysis data suggested that the stilbene backbone plays an important role in the reduction of hemolysis by *S. aureus*. *trans*-Stilbene and resveratrol increased the survival of *C. elegans* exposed to the bacteria, while *trans*-stilbene repressed genes associated with virulence, such as *hla* and *agr*.

Studying compounds responsible for the properties of red wines, Cho and co-workers[170] also demonstrated a marked reduction in *S. aureus* hemolysis activity by resveratrol. Moreover, this stilbenoid inhibited wild-type *P. mirabilis* hemolysis, but not in the *rsbA*-defective mutant, indicating that resveratrol could inhibit the expression of hemolysin and other virulence factors in *P. mirabilis* through an RsbA-dependent pathway.[89]

5.7.11. Tannins. Structure-based virtual screening allowed Kiran and co-workers[92] to identify 2,5-di-*O*-galloyl-D-hamamelose, or hamamelitannin (**270**), as a QSI that was able to reduce the amount of δ-hemolysin produced by MRSA. The effect of hamamelitannin on staphylococcal QS was experimentally confirmed, explaining the reduction in bacterial toxin production.

5.7.12. Xanthones. Mangiferin (**285**), a xanthonoid found in mangos, considerably decreased hemolysis caused by *P. aeruginosa* in a study that analyzed different bacterial virulence factors. Interestingly, this metabolite was shown to inhibit the QS system in a computational evaluation conducted by Annapoorani and co-workers.[183]

5.8. Steroids and Derivatives

No compound found.

5.9. Terpenoids and Derivatives

Subinhibitory concentrations of thymol (**289**), a monoterpene phenol derived from cymene and primarily found in thyme, oregano, and tangerine peel, were shown to inhibit the secretion of α-toxin and SEA and SEB enterotoxins in both MSSA and MRSA isolates.[99] Quantitative RT-PCR analysis supported these results, indicating that thymol diminishes the transcription of the *hla*, *sea*, and *seb* genes by several times.[99] In addition, Souza and co-workers[308] showed that subinhibitory concentrations of thymol and of its isomer carvacrol (**290**) completely inhibited the production of enterotoxins by *S. aureus*. These monoterpenes also were reported to reduce *L. monocytogenes* hemolysin production, repressing genes implicated in this virulence factor, such as *hly* and *prfA*.[266] Menthol (**300**), a monocyclic terpene alcohol that occurs naturally in plants of the *Mentha* genus and is the major component of peppermint oil, was shown to effectively inhibit a variety of staphylococcal toxins, such as α-toxin, SEA, SEB, and TSST-1, without interfering with *S. aureus* growth.[98] The decrease in toxin production was not associated with increased protease secretion induced by menthol; rather, the relative expression levels of the toxin-encoding genes (*hla*, *sea*, *seb*, and *tst*) were lower by several times.[98] McNamara and co-workers[295] showed that various compounds in volatile oils containing an isoprene unit inhibited TSST-1 production by *S. aureus* without affecting bacterial growth significantly. The best results were obtained for terpineol (**291**) and linalool (**305**), followed by menthol, thymol, and *p*-menthane-1,8-diol (**301**).[295]

Echeverrigaray and co-workers[269] evaluated the effect of monoterpenes on *P. mirabilis* hemolysin activity and observed that particularly oxygenated terpenoids such as citral (**293**), citronellol (**294**), and geraniol (**296**) reduced *P. mirabilis* hemolysis when they were used at subinhibitory concentrations.

Screening essential oils and their common constituents such as farnesol (**318**) and *cis*-nerolidol (**320**), Lee and co-workers[241] reported that these sesquiterpenes almost abolished the hemolytic activity of *S. aureus*. Additionally, *cis*-nerolidol also prolonged *C. elegans* survival in the presence of the pathogen.[241] The sesquiterpenes α-cyperone (**323**) and isoalantolactone (**324**) and the triperpenoid derivative glycyrrhetinic acid (**364**) also were shown to markedly inhibit *S. aureus* α-toxin production, using distinct strains and subinhibitory concentrations.[103−105] At the highest concentration tested, α-toxin in supernatants was almost undetectable, and the transcription level of *hla* gene was several times lower. Moreover, when human lung cells were cocultured with *S. aureus* in the presence of increasing concentrations of the compounds, lung cells were protected from injury induced by α-toxin. In a mice model of *S. aureus*-induced pneumonia, animals treated with isoalantolactone or glycyrrhetinic acid were protected against illness due to the low accumulation of cellular infiltrates, as indicated by histopathologic analysis of

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lungs.[103,105] In a study that evaluated red wine components, the pentacyclic triterpene betulinic acid (**355**) also exhibited a notable decrease in *S. aureus* hemolysis activity.[170]

6. PLANT-DERIVED NATURAL PRODUCTS AGAINST BACTERIAL PIGMENTS

6.1. Overview

Recent advances in microbial pigment biochemistry and the genetic basis of pigment production have revealed that many pigments provide a survival advantage for the pathogen in the host environment by interfering with host immune clearance mechanisms or by exhibiting pro-inflammatory or cytotoxic properties (Figure 6).[309] Here we emphasize some pigments produced by *S. aureus* and *P. aeruginosa* that are likely to promote bacterial virulence.

Most *S. aureus* strains are able to produce a membrane-bound golden carotenoid pigment called staphyloxanthin. The postulated biosynthesis of staphyloxanthin involves the metabolism of isoprenoids, resembling that of cholesterol biosynthesis.[310] Staphyloxanthin is a typical bacterial secondary metabolite, i.e., it is not required for the growth and reproduction of *S. aureus*, but it plays a role in bacterial virulence.[310] In fact, this pigment acts as antioxidant by enabling the detoxification of ROS generated by the host's immune system, such as the oxygen radical (O_2^-) and hydrogen peroxide (H_2O_2), and promotes resistance to neutrophil-based killing.[311−313] Blocking staphyloxanthin biosynthesis is a potentially attractive therapeutic target to prevent bacterial infection. The first step in staphyloxanthin biosynthesis is catalyzed by *S. aureus* dehydrosqualene synthase (CrtM). Cholesterol-lowering drugs prescribed to human patients against squalene synthase could also be active against CrtM, since staphyloxanthin production is remarkably similar to that of human cholesterol synthesis, though the only difference is observed in the last step of the pathway.[311,314] Phosphonosulfonates like BPH-652 are examples of this type of compound. Interestingly, this agent already progressed into early human clinical trials as a cholesterol-lowering agent, presenting low toxicity and no effect on the growth of the different human cell lines. An advantage to be mentioned is that phosphonosulfonates are some of several antivirulence drugs that are repurposed from existing drugs, which reduces development time and costs.[311,315] Staphyloxanthin inhibition is an example of narrow-spectrum therapy that renders *S. aureus* more susceptible to clearance by the host's innate immune defenses.[312,314] This therapeutic approach affords substantial advantages, because it causes only minimal disturbances to normal microbial flora and resistance development.

Pseudomonas species secrete two important pigments, pyoverdines and pyocyanins. Both pigments modulate iron, a crucial requirement for the growth of *Pseudomonas*. Pyoverdine is the generic name given to a vast family of fluorescent green-yellowish pigments that are synthesized by bacteria when subjected to iron starvation conditions as a means to acquire iron from the extracellular medium. Therefore, pyoverdines act as primary siderophores, being not only powerful iron(III) scavengers but efficient iron(III) transporters as well.[316] Considering the pigment pyocyanin, it is known that nearly 90−95% of all isolates of *P. aeruginosa* produce this type of pigment, blue-green in color and called "'blue pus'" (from pyocyaneus), that is a known evolutionarily conserved virulence factor.[317] This phenazine-derived pigment confers a greenish hue to the sputum of cystic fibrosis patients with chronic lung infection.[309] Pyocyanin is a redox-active secondary metabolite abundantly produced in low-iron media, and plays an important role in iron metabolism, reducing and releasing it from transferrin.[317] In addition to its role in the uptake of iron, pyocyanin also serves as a signaling molecule in *P. aeruginosa*, controlling a limited set of genes, including efflux pump genes (*mexGHI-opmD*) and the putative monooxygenase gene, which are involved in efflux and redox processes. Pyocyanin biosynthesis in *P. aeruginosa* is under the regulation of the Las/Rhl and PQS systems and is influenced by AmpR.[27,318,319] Pyocyanin has been proved to cause a wide spectrum of cell damage, such as inhibition of cell respiration and ciliary function, epidermal cell growth, and prostacyclin release, besides the disruption of calcium homeostasis.[320] Additionally, some works provide strong support to the notion that the full virulence of *P. aeruginosa* during airway infections requires the synthesis of this pigment.[320] The fact that the role played by pyocyanin is more extensive than previously thought makes it a good target, because its inhibition may have consequences not only regarding the acquisition of iron but also *P. aeruginosa* signalization-associated processes.[321] As for other factors regulated by the QS system, phenazines may be inhibited via the bacterial communication system. However, a recent study showed that pyocyanin inhibition may be mediated by a pathway that does not depend on LasR/RhlR, suggesting a post-transcriptional mechanism of regulation of this virulence factor.[321] In this way, the advancements in the understanding of the complete signaling integration network of pathogen will eventually afford the identification of the mechanistic targets that may be useful in the treatment of *P. aeruginosa* infections. In the search for compounds that are able to inhibit this pigment, authors focus their efforts on enzymes able to efficiently degrade this molecule with consequences for the cell−cell signaling of *P. aeruginosa* and for the prevention of cytotoxic effects of pyocyanin.[27] However, to date no agents with such features are available, pointing to the need of more in-depth investigations in this field of research.

Considering the plant-derived natural products active against virulence factor pigments, the following classes of active products have been reported in the literature.

6.2. Alkaloids and Derivatives

3-Indolylacetonitrile (**2**), a potent indole derivative from plants, modulated the *P. aeruginosa* PQS QS system, thereby decreasing the production of pyocyanin and pyoverdine pigments.[134] Caffeine (**24**), isolated from the leguminosae *T. foenum-graceum*, strongly interfered with *P. aeruginosa* QS-regulated virulence factors, as observed in a concentration-dependent manner, causing a drop in pyocyanin production.[31]

6.3. Fatty Acids and Derivatives

No compound found.

6.4. Organosulfurs and Derivatives

Sulforaphane (**41**) and erucin (**42**), two natural isothiocyanates, were found to strongly inhibit pyocyanin production by *P. aeruginosa* as a consequence of interference in the QS regulatory network.[39]

6.5. Other Aliphatic Compounds

Marine bacteria, including *Vibrio* species, produce melanin-like pigments, also known as pyomelanins.[322] Melanogenesis in *V. cholerae* is enhanced under stress conditions, whether in aquatic environments or even human hosts, suggesting that melanin

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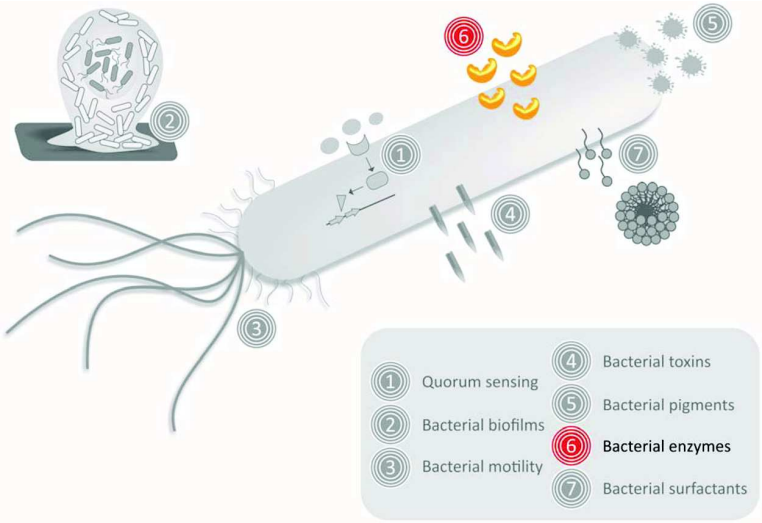

Figure 7. Enzyme production and secretion as a bacterial target for antivirulence compounds.

production plays a specific role in the survival of this bacterium in adverse circumstances.[323] The aliphatic ketone *trans*-3-decen-2-one (**54**) and the aliphatic aldehyde *trans*-2-nonenal (**48**) decreased pigment production by *V. anguillarum*.[40] These findings were consistent with AI-2-mediated QS modulation, since pigment production in this bacterium is, at least partly, controlled by the AI-2 QS system.[40] Using two bioreporter strains, Ahmad and co-workers[41] showed that *cis*-3-nonen-1-ol (**57**), a common volatile compound present in essential oils, decreases pyocyanin production by *P. aeruginosa* and modulates the QS system.

6.6. Other Cyclic Compounds

4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (**61**), a nonhalogenated furanone detected in a variety of fruits, significantly affected virulence factors of *P. aeruginosa*, including a substantial reduction in the production of pyocyanin.[42]

6.7. Phenolics and Derivatives

6.7.1. Anthocyanins. No compound found.

6.7.2. Coumarins. Gutierrez-Barranquero and co-workers[44] analyzed the potential of coumarin (**66**) to inhibit QS signaling in a range of pathogenic organisms and observed the suppression of phenazine production in *P. aeruginosa* in a concentration-dependent manner.

6.7.3. Flavonoids. The flavonol catechin (**90**) was able to reduce the production of pyocyanin by *P. aeruginosa*, exhibiting higher inhibitory activity than the positive controls used, streptomycin and ampicillin.[180] The flavanones naringenin (**97**) and eriodictyol (**105**) and the flavanonol taxifolin (**119**) had limited impact on the growth of *P. aeruginosa* and led to significant reduction in pyocyanin pigment production, as described by Vandeputte and co-workers.[49] The authors showed that naringenin decreases the expression of QS-related genes involved in the production of pyocyanin (*phzA1*), explaining the inhibition rates observed.[49]

Qualitative and quantitative analysis clearly indicated that flavone (**120**) reduced production of the pigment staphyloxanthin by 10-fold, compared with nontreated *S. aureus*, without inhibiting bacterial growth.[177] Consequently, flavone reduced susceptibility of *S. aureus* to H_2O_2 by 100 times, while the structurally related compounds chrysin and 6-hydroxyflavone had no or only a very mild effect on survival rate.

6.7.4. Lignans. No compound found.

6.7.5. Phenolic Acids. Salicylic acid (**198**) and its derivatives acetyl salicylic acid (**200**), methyl salicylate (**201**), salicylamide (**202**), and benzoic acid (**203**) reduced, apart from other virulence factors, pyocyanin formation by *P. aeruginosa*.[192] The authors highlighted the observation that salicylic acid down-regulates genes related to QS, which are implicated with the synthesis of several virulence mediators.[192] Moreover, malabaricone C (**208**), from *M. cinnamomea* barks, inhibited the production of pyocyanin by *P. aeruginosa*, possibly due to its interference in QS circuits, without exhibiting adverse effects on bacteria viability.[72]

6.7.6. Phenylethanoids. No compound found.

6.7.7. Phenylpropanoids. Several studies carried out in the search for natural compounds as potential QSI observed that cinnamaldehyde (**221**), eugenol (**226**), isoeugenol (**227**), 6-gingerol (**234**), zingerone (**235**), and curcumin (**237**) were able to decrease several *P. aeruginosa* virulence factors, including pyocyanin production, in a dose-dependent manner.[41,71,75,80,82] Cinnamaldehyde, which was shown to inhibit AI-2-mediated QS, also decreased pigment production of the gram-negative *V. anguillarum*,[40,73] while curcumin reduced the production of prodigiosin, a QS-controlled pigment from *S. marcescens*.[40,73,84]

Eugenyl acetate (**229**) promoted a concentration-dependent decrease in the production of the pigments pyocyanin and pyoverdine by *P. aeruginosa*, by 9.4 and 3.7 times, respectively. Furthermore, this compound completely prevented *S. aureus* staphyloxantin synthesis, possibly due to its anti-QS action.[77]

6.7.8. Quinones. No compound found.

6.7.9. Simple Phenols. Subinhibitory concentrations of rhodomyrtone (**253**), a member of the acylphloroglucinols isolated from *Rhodomyrtus tomentosa* leaves, changed the pigmentation of *S. aureus*, as reported by Leejae and co-workers.[324] The amount of intermediate compounds in the biosynthetic pathway of staphyloxanthin was reduced by up to 6 times. Less carotenoid pigment to act as an antioxidant scavenger led to a dose-dependent increase in the susceptibility

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of *S. aureus* to H_2O_2, singlet oxygen, and human whole blood killing.

6.7.10. Stilbenoids. No compound found.

6.7.11. Tannins. No compound found.

6.7.12. Xanthones. No compound found.

6.8. Steroids and Derivatives

No compound found.

6.9. Terpenoids and Derivatives

Ahmad and co-workers[41] designed a study to document the effect of 29 common volatiles occurring in essential oils on QS-related pigment production by *P. aeruginosa*. The authors highlighted the activity of the monoterpenes α-phellandrene (**287**), *p*-cimene (**288**), thymol (**289**), carvacol (**290**), α-terpineol (**291**), thujone (**292**), citral (**293**), geraniol (**296**), menthone (**299**), linalool (**305**), camphene (**307**), and camphor (**308**); of the (−)-enatiomers of carvone (**297**), limonene (**302**), and borneol (**309**); and of the sesquiterpenes nerolidol (**320**) and nerol (**321**). Menthol (**300**) also was demonstrated to be active by Husain and co-workers,[100] who suggested that this monoterpene interferes with PQS signaling and then reduces pigment secretion.

In addition to the sesquiterpene farnesol (**318**), other long-chain isoprenoid derivatives with related structures, such as farnesyl acetate (**319**) and the diterpenes phytol (**310**) and geranyllinalool (**311**), significantly decreased the production of pyocyanin by *P. aeruginosa*. Their activities were also associated with interference in the bacterial QS system.[101,232]

7. PLANT-DERIVED NATURAL PRODUCTS AGAINST BACTERIAL ENZYMES

7.1. Overview

When considering bacterial enzymes as targets for antivirulence therapies, it is ideal that such specific proteins be strongly linked to pathogenesis. The target needs to have sufficient availability of detailed biochemical and structural information because it is an important prerequisite when considering the sites for inhibition. In this section, we have compiled some of the enzymes with a role in virulence that can be considered promising as a suitable targets for pharmacological inhibition (Figure 7).

Sortases are a family of gram-positive membrane-bound cysteine transpeptidases responsible for catalyzing and anchoring surface proteins to the peptidoglycan cell wall layer of staphylococci, enterococci, and streptococci, known as sortase A and B. It has been shown that sortases play a role in modulating the surface properties during critical virulence mechanisms, such as bacterial adhesion and invasion of host tissues, biofilm formation, and immune evasion by inhibition of opsonization and phagocytosis.[325] Development of sortase inhibitors has received considerable attention, and candidate inhibitors have been investigated.[326,327] Because sortases are at the center of a pathway that controls multiple virulence factors in gram-positive bacteria, their inhibition could effectively target a large number of virulence factors simultaneously. Additionally, sortases are an exclusively bacterial family of enzymes. The closest functional homologues of sortases in humans are likely to be structurally unrelated cysteine proteases, such as calpains, caspases, and cathepsins, which sortase-specific inhibitors should not affect.[327] Gotz[328] showed that a *srtA* mutant is less virulent in a murine septic arthritis model and presents decreased ability to reach target organs and to induce an inflammatory response. Although several sortase inhibitors have been identified in silico and evaluated in vitro, only a few studies about sortase inhibitors have been carried out in vivo.[327]

Coagulase is secreted by the majority of clinically relevant *S. aureus* isolates. *S. aureus* secretes two coagulases, coagulase (Coa) and von-Willebrand factor binding protein (vWbp). These proteins are associated with clumping factor (ClfA) and promote agglutination. ClfA is a sortase-anchored surface protein and has been linked with bacterial binding to fibrinogen.[329] During host infection, coagulase conformationally activates the central coagulation zymogen, prothrombin, thereby triggering the cleavage of fibrinogen to fibrin. Some authors have reported important contributions of this enzyme to the pathogenesis of staphylococci by generating mutants that do not display virulence phenotypes in endocarditis, skin abscess, and mastitis models in mice. In addition, the mutants in coagulase genes showed virulence defects in three mouse models for staphylococcal disease, including blood survival, lethal bacteremia, and renal abscess formation.[330,331] Thrombin inhibitors, such as argatroban, dabigatran, and dabigatran etexilate, which have been approved by the FDA for the treatment of diseases associated with clotting disorders, act by inhibiting the proteolytically active Coa−prothrombin complex.[329,332] Although the role of coagulases in the establishment of disease by *S. aureus* has not been completely elucidated, the use of thrombin inhibitors in combination with the ClfA-specific monoclonal antibody provides a therapeutic strategy to disrupt this virulence factor and prevent the pathogenesis of *S. aureus*.[329]

Degradative proteases are important in many cellular processes of almost every pathogenic bacterial species.[333] Most strains of the opportunistic pathogen *P. aeruginosa* produce various proteases with broad substrate specificities, like elastase (LasB) and alkaline protease (AprA). Among exoproteases, LasB is a zinc metalloprotease encoded by the *lasB* gene. This protease damages tissues by breaching the endothelial and epithelial barriers and attacking intercellular tight junctions and is able to degrade various plasma proteins, such as immunoglobulins and coagulation and complement factors. There is evidence of the role of elastase in localized chronic infections, as in experimental pseudomonas keratitis, pneumonia, and burn infections, for instance.[334−336] AprA is a another zinc metalloprotease known to interfere with several components of the host immune system, as in complement proteins or cytokines, such as IFN-γ and TNF-α, for example.[337] In *S. epidermidis*, Clp protease degrades the negative regulator Spx, a transcription factor that represses biofilm formation and the production of genes that facilitate primary attachment to the uroepithelium.[333] Protease inhibitors may prove to be valuable to combat bacterial virulence-associated traits. Despite their essential physiological roles, their conservation among bacterial species, and their apparent druggability, currently there are no approved agents that target this enzyme class. This may be due in part to some problems described in the literature in studies about protease inhibitors. The most important of these issues is specificity. Promiscuous inhibitors that target broad classes of proteases have been described more frequently than specific compounds. Moreover, since homologues of each of the bacterial proteases exist in human mitochondria, protease modulators may result in considerable toxicity in eukaryotes. Additionally, many protease inhibitors present poor pharmacological properties and often lack oral bioavailability. These agents present reduced entry

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through the bacterial cell wall and can be degraded by peptidases in the host and bacterial cytoplasm. The full exploitation of the possibilities of these enzymes as attractive therapeutic targets demands a more in-depth understanding of the biochemistry and physiology of these proteins.[333]

Urease, the first protein to be identified as a nickel enzyme, is also considered an important target against certain bacterial species. Urease is reported to be involved in pathologies induced by *Helicobacter pylori* and *Proteus* species. The enzyme is considering one of the *H. pylori* virulence factors, allowing bacteria to survive in the low pH environment of the stomach during colonization, creating a neutralizing environment for protection. Thus, *H. pylori* urease plays an important role in various gastric-related diseases, especially in pathogenesis of gastric and peptic ulcers. Urinary stones, consisting of ammonium phosphate, struvite, and carbonate apatite, result from complicated urinary tract infections that are caused by urease-producing bacteria, primarily *P. mirabilis*. Since the bacteria are protected inside the stone structure, antibiotic treatment is usually unsuccessful.[338] Interestingly, humans do not produce urease, and no human nickel enzymes are known, making urease a potential therapeutic target.[339] A common ancestor for this enzyme has been suggested due to the high similarity of the amino acid sequence among ureases of multiple origins.[340] The pathway of urease maturation, which is complex, is a potential target for chemical intervention. Additionally, inhibitors of the mature enzyme, including nickel chelators and substrate analogues, are promising targets.[339] However, while the effect of urease inhibitors on bacteria survival has been extensively studied in vitro, little is known about the role of these compounds in vivo. In addition, various compounds described in the literature display only a moderate inhibitory activity, the translation to clinical applications.

Sialidases (neuraminidases) catalyze the removal of sialic acid residues from glycoconjugates on the host cell surface, which are used by many pathogenic and nonpathogenic sialidase-producing bacteria as a carbon and energy source. The physiological significance of the enzyme has been linked with the pathogenicity of bacterial infections, highlighting its involvement in biofilm formation[341,342] and its role as receptor for bacterial attachment to airway epithelial cells.[343] Sialidase activity has been observed in bacteria isolated from a variety of infections, particularly typical human pathogens, including *S. pneumoniae*, *P. aeruginosa*, *V. cholerae*, and *Clostridium perfringens*.[344] Current improvements in the search and development of selective inhibitors against bacterial and human sialidases have been modest.[345] Sialidase inhibitors have mainly been exploited in the treatment of viral infections. The discovery of zanamivir and oseltamivir highlights the value of sialidase inhibitors in combating several influenza viruses. Despite the use of these compounds, none has shown efficacy in an in vivo model. The questions of resistance development and possible side effects remain yet to be elucidated.[346,347] Moreover, further investigation is necessary in the attempt to find new sialidase inhibitors and/or new applications for existing inhibitors, such as, for example, in the treatment of bacterial infections.

Considering the plant-derived natural products active against the virulence factor enzymes, the known classes of active products are discussed below.

7.2. Alkaloids and Derivatives

The isoquinoline alkaloid berberine (**14**), isolated from the rizhomes of *Coptis chinensis*, showed strong inhibitory activity against *S. aureus* sortase, at subinhibitory concentrations, being about 5 times more potent than the synthetic inhibitor *p*-hydroxymecuribenzoic acid (pHMB) that was used as a positive control.[167] The authors observed that a structurally related alkaloid, palmatine (**17**), also was active against sortase, but to a lesser extent than berberine. Interestingly, a difference in sortase inhibition was observed when berberine was tested against chloride or sulfate salts, and the first one presented the best sortase IC_{50} value.[348] Seven isoquinoline alkaloids, berberine, palmatine, coptisine (**18**), pseudodehydrocoridaline (**19**), jatrorrhizine (**20**), dehydrocorybubine (**21**), and pseudocopitisine (**22**), isolated from the *Corydalis turtschaninovii* rhizome, displayed significant levels of *C. perfringens* neuraminidase inhibitory activity in a dose-dependent fashion. On the basis of the kinetic studies and molecular simulations, the quaternary isoquinoline alkaloids berberine and palmatine showed reversible noncompetitive behavior in the ligand−receptor interaction.[167]

Caffeine (**24**), isolated from the leguminosae *T. foenum-graceum*, strongly interfered with QS-regulated virulence factors of *P. aeruginosa*, inhibiting the LasIR-encoded protease and elastase activities in a concentration-dependent manner.[31]

7.3. Fatty Acids and Derivatives

No compound found.

7.4. Organosulfurs and Derivatives

Allicin (**37**), a compound found in garlic extracts, decreased urease activity of both prelysed and intact cells of *P. mirabilis* in a concentration-dependent manner.[149] It is likely that the compound acts via a mechanism involving the formation of a disulfide bond with the reactive cysteine residue at the urease active site.[149] Diallyl trisulfide (**40**) is a sulfide compound also found in garlic and in other *Allium* species that significantly reduced the urease activity of susceptible and drug-resistant *H. pylori* strains.[349]

7.5. Other Aliphatic Compounds

The aliphatic aldehyde *trans*-2-nonenal (**48**) and the aliphatic ketone *trans*-3-decen-2-one (**54**) were found to decrease protease activity in *V. anguillarum* and *V. cholera*. It has been hypothesized that the chemical structure of cinnamaldehyde analogs may act as LuxR ligands, and protease inhibition has been attributed to the interference in AI-2 mediated QS. These compounds significantly increased survival after infection of *C. elegans* with different *Vibrio* species.[40]

7.6. Other Cyclic Compounds

A nonhalogenated furanone found in a variety of fruits and considered safe for consumption, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (**61**), importantly affected *P. aeruginosa* virulence factors, including a remarkable reduction in the production of LasA protease.[42]

7.7. Phenolics and Derivatives

7.7.1. Anthocyanins. No compound found.

7.7.2. Coumarins. The impact of coumarin (**66**) on QS-associated protease activity was investigated against *S. aureus*, *P. aeruginosa*, *V. anguillarum*, *S. maltophilia*, and *B. cepacia*. The compound, which is found in foods and cosmetics potentially without risks for human health, was able to inhibit protease activity in *S. maltophilia* and *B. cepacia*.[44]

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7.7.3. Flavonoids. *trans*-Chalcone (**77**), the precursor molecule of many flavonoids, was able to block *S. mutans* sortase A in vitro, as demonstrated by Wallock-Richards and co-workers.[156] The chalcone is likely to covalently modify the enzyme by forming a Michael addition aduct with the active site cysteine, which was identified as essential for optimal enzyme activity.[156] Shah and co-workers[297] showed that the flavonol epicatechin gallate (**92**) decreased the levels of coagulase activity in culture supernatants of MRSA clinical strains. The authors suggested that this effect could be partly explained by the prevention of protein secretion as a result of the changes induced by the flavonol in the physical nature of the bacterial cytoplasmic membrane.[297] The flavanone kurarinol (**110**), isolated from *Sophora flavescens*, also was active against enzymes from *S. aureus*, exhibiting potent inhibitory activity against *S. aureus* sortase A.[350] The researchers also showed that the A-type side chain has a hydroxyl group at C-8, which is required for potent inhibitory activity.[350]

Using recombinant sortase A and B from *S. aureus*, Kang and co-workers[351] reported that the flavonol quercetin (**144**), isolated from *Rhus verniciflua* barks, was responsible for the strong sortase A inhibitory activity. Moreover, flavonols structurally related to quercetin, such as myricetin (**147**) and morin (**148**), which did not alter *S. aureus* growth, also exhibit important sortase inhibitory activity. Morin was found to be the most active, especially against sortase B. In addition, these flavonols reduced the ability of the bacterium to form clumps with fibrinogen, in a dose-dependent manner.[351] Among 27 compounds screened, Liu and co-workers[352] showed that the flavonol quercitrin (**146**) was able to remarkably inhibit the catalytic activity of the recombinant sortase A from *S. aureus*. Quercitrin did not interfere with *S. aureus* growth, at the tested concentrations, and reduced bacterial adherence to fibrinogen-coated and fibronectin-coated surfaces, which is consistent, since sortase seems to be indispensable for *S. aureus* adherence to host matrices. By using molecular dynamics simulation, the authors indicated the specific binding sites of quercitrin to the active cavity of the enzyme.[352] Kaempferol-3-rutinoside (**151**) and isorhamnetin 3-*O*-β-D-rutinoside (**152**), two flavonol glycosides isolated from *Sophora japonica* flowers, exhibited inhibitory activity against sortase A from *S. mutans*, at subinhibitory concentrations.[353] The second compound showed the most potent activity, presenting IC_{50} value lower than that of the positive controls used.[353]

The potential activity of the chalcone *trans*-benzylideneacetophenone (**81**), of the flavanols catechin (**90**) and epicatechin (**91**), and of the flavanones naringenin (**97**) and eriodictyol (**105**), as well as of the flavanonol taxifolin (**119**), in the production of *P. aeruginosa* QS-controlled virulence factors was evaluated.[49,53] All these compounds were found to have inhibitory effect on elastase production by *P. aeruginosa*, besides repressing the expression of QS-related genes, without affecting bacterial growth.[49,53] In the study carried out by Annapoorani and co-workers,[183] several natural compounds were subjected to docking analysis against LasR and RhlR receptor proteins of *P. aeruginosa.* The authors identified the flavanone naringin (**98**) and the flavonol morin (**148**) as QS inhibitors. Also, the evaluation of QS-controlled virulence factors of this bacterium revealed a reduction of protease and elastase production. A protein degradation assay demonstrated that the compounds tested did not degrade the virulence enzymes, suggesting that they possibly act as an antagonist of LasR and RhlR signals.[183]

A series of flavonoids have been reported to act against bacterial neuraminidase enzymes. Four flavanones, namely, amoradicin (**106**), amorisin (**107**), isoamoritin (**109**), and amoricin (**110**), isolated from *Amorpha fruticosa*, also inhibited *C. perfringens* neuraminidase activity by noncompetitive kinetics, reducing biofilm formation.[167] Similar results were observed for nine isoflavones (**157**−**165**) isolated from the roots of *Flemingia philippinensis*, which displayed significant levels of *C. perfringens* neuraminidase inhibitory activity in a dose-dependent manner and were found to exhibit non-competitive kinetics.[354] Nguyen and co-workers[355] performed a neuraminidase inhibitor screening using 15 pterocarpan derivatives (**167**−**181**) isolated as active compounds from *Erythrina abyssinica*. All these isoflavonoids exhibited significant inhibitory effects on *C. perfringens* and *V. cholerae* neuraminidases. Kinetic studies developed for *C. perfringens* neuraminidase showed that various constituents (**169**, **171**−**174**, **176**, **179**−**181**) have noncompetitive inhibition modes, with stronger activity than positive controls. In contrast, two other compounds (**170** and **175**) behaved as competitive inhibitors and were less effective than the positive control.[355] Six other pterocarpans, namely, bicolosin A (**182**), bicolosin B (**183**), bicolosin C (**184**), erithrabyssin II (**185**), lespebuergine (**186**), and 1-methoxyerithrabyssin II (**187**), isolated from the root bark of *Lespedeza bicolor*, displayed significant levels of *C. perfringens* neuraminidase inhibitory activity in a dose-dependent fashion and were found to exhibit noncompetitive kinetics.[356] The rotenoids amorphigine (**188**), dalbinol (**189**), and 6-ketodehydro-amorphigenin (**190**), from *A. fruticosa*, also inhibited *C. perfringens* neuraminidase activity, apart from reducing biofilm formation.[167] The isoprenylated flavone artocarpin (**137**) exhibited inhibitory action in a dose-dependent manner against pneumococcal neuramidase.[174] Low micromolar concentrations of artocarpin effectively blocked the catalytic activity of all *S. pneumoniae* strains in three different types of neuramidase inhibition assays.[174]

Baicalin (**127**) and scutellarin (**138**) are the main bioactive components of *Scutellaria baicalensis*, which has been extensively incorporated into formulations used in the treatment of *H. pylori*-related gastrointestinal disorders in traditional Chinese medicine. Recently, Yu and co-workers[357] showed that these flavones effectively suppressed bacterial urease in a dose-dependent and time-independent manner. Scutellarin was demonstrated to be the best inhibitor, compared to acetohydroxamic acid, a well-known *H. pylori* urease inhibitor. Kinetic analysis revealed that this enzyme follows the Michaelis−Menten kinetic, and the authors demonstrated that both compounds act as noncompetitive inhibitors, targeting sulfhydryl groups of the specific cysteine residues of the active site of *H. pylori* urease.

7.7.4. Lignans. Using the metabolites of *Pulsatilla koreana*, the activity of the lignans (7*S*,8*S*)-dihydrodehydrodiconiferyl alcohol (**194**) and its glucoside (7*S*,8*S*)-dihydrodehydrodiconiferyl alcohol 9′-*O*-β-D-glucopyranoside (**195**) was analyzed by Lee and co-workers,[358] in a study showing that *S. mutans* sortase A inhibition values are similar to those induced by the positive control, pHMB.

7.7.5. Phenolic Acids. The data obtained by Prithiviraj and co-workers[192] showed that salicylic acid (**198**) and its derivatives acetyl salicylic acid (**200**), methyl salicylate (**201**), salicylamide (**202**), and benzoic acid (**203**) reduced the activity of both elastase and protease, suggesting that these compounds, especially salicylic acid, interfered with the production of

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several virulence-related exoenzymes by *P. aeruginosa*. The same phenomenon was observed for salicylic acid, together with the inhibition of acute *P. aeruginosa* cytotoxicity in transformed human corneal epithelial cells.[70] In addition, protocatechuic acid (**204**) was shown to be a mild inhibitor of urease activity from susceptible and drug-resistant *H. pylori*.[349]

7.7.6. Phenylethanoids. Yang and co-workers[353] isolated a new maltol derivative from flowers of *S. japonica*, which was called maltol-3-O-(4′-*O*-*cis*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-*β*-glucopyranoside (**214**). The authors also isolated its isomer, maltol-3-*O*-(4′-*O*-*cis*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-*β*-glucopyranoside (**215**). Using recombinant sortase A derived from *S. mutans*, they observed that both compounds inhibited sortase A activity at concentrations that did not interfere with bacterial growth. Maltol-3-*O*-(4′-*O*-*cis*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-*β*-glucopyranoside was the most active, presenting IC_{50} values lower than those of the positive controls used. It was expected that inhibitors of sortase A would block protein anchoring mediated by this enzyme and prevent the adherence and aggregation ability of *S. mutans* cells. Maltol-3-*O*-(4′-*O*-*cis*-*p*-coumaroyl-6′-*O*-(3-hydroxy-3-methylglutaroyl))-*β*-glucopyranoside significantly reduced the saliva-induced aggregation of *S. mutans* in a dose-dependent manner, similarly to the *srtA S. mutans* knockout.

7.7.7. Phenylpropanoids. Caffeic acid (**218**) and rosmarinic acid (**231**), isolated from *P. koreana* roots, significantly inhibited the action of sortase A from *S. mutans*, which modulates the surface properties and cariogenicity process.[358] Among other compounds isolated in the study, rosmarinic acid presented the lowest IC_{50} value.[358] The inhibition of a purified *S. mutans* sortase A by curcumin (**237**), at subinhibitory concentrations, was reported by Hu and co-workers[203] and Hu and co-workers.[359] Similarly, curcumin, isolated from *C. longa*, exhibited *S. aureus* sortase A inhibitory activity, being more potent than its structurally related compounds demethoxycurcumin (**238**) and bisdemethoxycurcumin (**239**).[360] It is expected that sortase inhibitors should reduce bacterial fibronectin-binding protein. Similarly, the treatment of *S. aureus* with curcumin significantly reduced the capacity of the bacteria to adhere to fibronectin-coated surfaces in a dose-dependent manner, comparable to the behavior of untreated sortase knockout mutants.[360]

Protease production is one of the important pathogenic characteristics of *P. aeruginosa*. In the study carried out by Annapoorani and co-workers,[183] a virtual screening for LasR and RhlR receptor inhibitors identified rosmarinic acid and chlorogenic acid (**230**). The authors observed the in vitro reduction of protease and elastase production, which was not related to degradation of the virulence enzymes, suggesting the LasR and RhlR antagonist action of the QSI compounds.[183] In addition, Rudrappa and Bais[82] showed that curcumin decreased the production of protease and elastase by *P. aeruginosa* by approximately 2 times. Eugenol (**226**), eugenyl acetate (**228**), 6-gingerol (**234**), and zingerone (**235**) were identified as QSI inhibitors that successfully repressed exoprotease production by *P. aeruginosa*.[75,77,80,81] The anti-QS activity demonstrated by the compounds was associated with the protease inhibitory effect, apart from attenuation of other virulence factors.[75,77,80,81]

Inhibition of protease production by *Vibrio* strains was demonstrated for cinnamaldehyde (**221**) and its derivative 4-methoxycinnamaldehyde (**222**) as well as curcumin, based on their ability to interfere with QS-dependent virulence factors.[40,73,83] Cinnamaldehyde also completely inhibited phospholipase activity, which is critical for attachment to and invasion of enterocytes during *L. monocytogenes* infection.[266]

Upadhyay and co-workers[266] investigated diarylheptanoid katsumadain A (**240**) and observed that the compound exhibited inhibitory action in a dose-dependent manner against pneumococcal neuramidase. At low micromolar concentrations, katsumadain A effectively blocked the neuramidase catalytic activity of all *S. pneumoniae* strains tested, reducing pneumococcal adhesion to human lung cells.[174]

7.7.8. Quinones. No compound found.

7.7.9. Simple Phenols. No compound found.

7.7.10. Stilbenoids. Urease, which is responsible for the formation of bladder and kidney stones at later stages of *P. mirabilis* infection, was inhibited by resveratrol (**260**).[89] This stilbenoid repressed the wild-type *P. mirabilis*, but not that of the *rsbA*-defectetive mutant, indicating that urease inhibition occurred through an RsbA-dependent pathway.

Yuk and co-workers[361] showed that neuraminidase inhibitory properties of the seeds of *Paeonia lactiflora* against *C. perfringens* are due to four oligostilbene compounds, namely, *ε*-vineferin (**262**), suffruticosol A (**263**), suffruticosol B (**264**), and *trans*-gnetin H (**267**). All compounds displayed noncompetitive inhibition, and *trans*-gnetin H was shown to be the most potent.

7.7.11. Tannins. Pastene and co-workers[362] purified two catechin-derived procyanidins, procyanidin B3 (**276**) and procyanidin C2 (**278**), from *Peumus boldus*, a tree native to Chile and used in folk medicine for hepatic and gastrointestinal illness. These procyanidins inhibited *H. pylori* urease to a greater extent than epicatechin-derived procyandins, such as procyanidin B2 (**275**) and procyanidin C1 (**277**).

7.7.12. Xanthones. No compound found.

7.8. Steroids and Derivatives

β-Sitosterol-3-*O*-glucopyranoside (**286**) was isolated from *Frittilaria verticillata* bulbs through a guided fractionation bioassay. At subinhibitory concentrations, the steroid provided potent inhibition of recombinant sortase from *S. aureus*. Moreover, the authors demonstrated that the activity reported depends on the glucopyranoside side chain moiety, since sitosterol was found to be inactive.[363]

7.9. Terpenoids and Derivatives

Sublethal concentrations of the monoterpenes tymol (**289**) and of its isomer carvacrol (**290**) strongly inhibited the enzymatic activity of coagulase and lipase produced by *S. aureus*.[308] It was speculated that the suppression of enzymatic activities may result either from the direct interactions of the compounds with the enzymes or the prevention in protein secretion.[308] Moreover, Upadhyay and co-workers[266] revealed that carvacrol and thymol completely inhibited *L. monocytogenes* phospholipase activity, as revealed by the reduction in *plcA* and *plcB* gene expression, which is critical for invasion of enterocytes during infection. The sesquiterpene patchouli alcohol (**325**), isolated from *Pogostemon cablin*, limited *H. pylori* urease activity in a dose-dependent manner, acting as noncompetitive urease inhibitor.[364]

Several terpenes were described to limit *P. aeruginosa* QS-mediated virulence factors. The monoterpene menthol (**300**) strongly interfered with the transcriptional activation of LasB signaling, as observed in a concentration-dependent decrease in protease, elastase, and chitinase activities.[100] Six sesquiterpene lactones (**329**−**334**) also presented the ability to decrease

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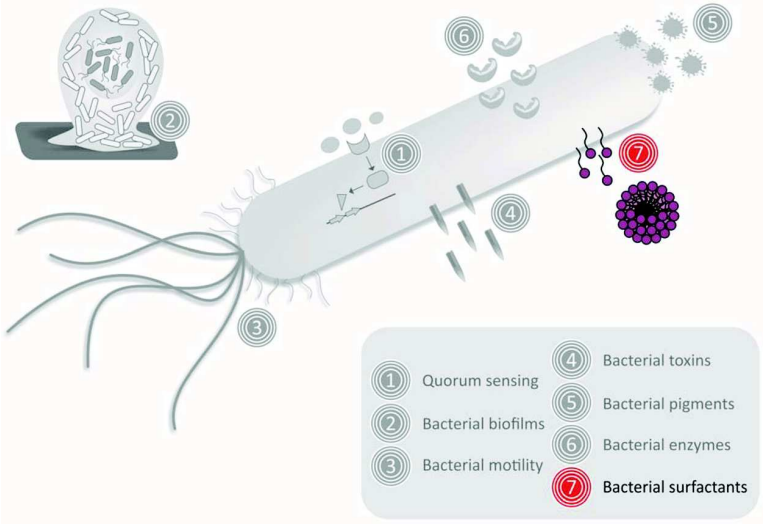

Figure 8. Surfactant production and secretion as a bacterial target for antivirulence compounds.

elastase activity.[102] Likewise, the sesquiterpene viridiflorol (**328**) and the triterpenoids betulinic acid (**355**), ursolic acid (**358**), taraxerol (**365**), oleanolic acid (**366**), shoreic acid (**367**), eichlerialactone (**368**), cabraleone (**369**), cabraleadiol (**370**), and 3-β-hydroxynordammaran-20-one (**371**), from the liverwort *L. chordulifera*, were able to significantly decrease elastase activity, higlighting the activity of the triterpene acids betulinic and ursolic acids.[242]

8. PLANT-DERIVED NATURAL PRODUCTS AGAINST BACTERIAL SURFACTANTS

8.1. Overview

Among the various categories of glycolipid biosurfactants, rhamnolipids are an important extracellular virulence factor, playing a major role in *P. aeruginosa* pathogenesis. Rhamnolipids are heterogeneous glycolipid molecules composed of the β-hydroxy fatty acid connected to a rhamnose sugar molecule by the carboxyl radical. A wide variety of microorganisms produce rhamnolipids, which may vary in length and branch size. Some bacteria are known to produce only monorhamnolipids, while others produce mono- and dirhamnolipids.[365]

The possible mechanism by which rhamnolipids promote their effects remains incompletely understood. Studies have shown that, under some conditions, microorganisms produce surfactants as a defense mechanism. Rhamnolipids have been implicated in the emergence of deleterious effects on mucociliary clearance and phagocytosis by macrophages. Bacteria have long been known to secrete biosurfactants that alter surface properties such as wettability and charge.[366] These glycolipids are also important in swarming and twitching motilities and in biofilm formation.[367] Rhamnolipids are required for maintaining the open channels in the matured mushroom-shaped biofilm, and these open channels could serve as a tunnel to distribute nutrients and oxygen between bacterial colonies.[368]

Rhamnolipid expression is regulated directly or indirectly by *lasI-lasR*, *rhlI-rhlR*, and *P. aeruginosa* quinolone signal (PQS) systems.[369] Studies have demonstrated that the *P. aeruginosa rhlA* mutant is deficient in the synthesis of rhamnolipids and is not able to form microcolonies and mushroom-shaped structures in the initial and later phases of biofilm formation.[370,371] Since rhamnolipids are controlled by the QS system, compounds able to modulate these circuits would consequently interfere with rhamnolipid synthesis. There are no studies in the literature showing compounds able to decrease rhamnolipid production by blocking enzymes directly involved in its biosynthetic cascade (at a post-transcriptional level). It is important to highlight that surfactants could be considered incipient as virulence targets, probably due to the chemical diversity of surfactants combined with their poorly understood function in virulence, posing considerable challenges to researchers. Taken together, ideally the inhibition of surfactants by a QS-independent way is likely to be an attractive target, since it will not promote a strong disturbance in bacterial fitness and would concomitantly modulate biofilm development and motility (Figure 8).

Considering the plant-derived natural products active against the virulence factor surfactants, the following classes of active products were found:

8.2. Alkaloids and Derivatives

No compound found.

8.3. Fatty Acids and Derivatives

No compound found.

8.4. Organosulfurs and Derivatives

Ajoene (**38**), a lipid-soluble allyl sulfide derived from allicin and present in garlic extract, was investigated as a *P. aeruginosa* antivirulence compound.[38] The increasing concentrations of ajoene inversely correlated with rhamnolipid concentrations in treated samples, indicating the ability of the compound to attenuate QS-controlled virulence factors.[38] In another study, the isothiocyanate iberin (**39**), produced by *Armoracia rusticana*, showed a steadily decreasing rate of rhamnolipid synthesis.[37] It was observed that iberin is able to completely block and expressively down-regulate genes involved in rhamnolipid production (*rhlA* and *rhlB*). According the authors, these findings are coherent with the generally acknowledged model of QS-regulated production of rhamnolipids. Although iberin displayed a strong reductive effect on rhamnolipid production in vitro, it is therefore curious that it was not able to significantly attenuate bacterial populations in the foreign-body infection model.[37]

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8.5. Other Aliphatic Compounds

No compound found.

8.6. Other Cyclic Compounds

Inhibitory effects of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (**61**) on AHL-mediated *P. aeruginosa* virulence factors were emphasized by Choi and co-workers[42] in a study that demonstrated considerable reduction in rhamnolipd production and gene expression.

8.7. Phenolics and Derivatives

8.7.1. Anthocyanins. No compound found.
8.7.2. Coumarins. No compound found.
8.7.3. Flavonoids. No compound found.
8.7.4. Lignans. No compound found.
8.7.5. Phenolic acids. No compound found.
8.7.6. Phenylethanoids. No compound found.
8.7.7. Phenylpropanoids. 6-Gingerol (**234**), zingerone (**235**), and curcumin (**237**) were experimentally found to attenuate *P. aeruginosa* QS-dependent virulence factors and to successfully repress rhamnolipd production.[80,81,84]
8.7.8. Quinones. No compound found.
8.7.9. Simple phenols. No compound found.
8.7.10. Stilbenoids. No compound found.
8.7.11. Tannins. No compound found.
8.7.12. Xanthones. No compound found.

8.8. Steroids and Derivatives

No compound found.

8.9. Terpenoids and Derivatives

No compound found.

9. CONCLUSIONS AND PERSPECTIVES

The information compiled and presented herein demonstrates that prototypes of new drugs may potentially be discovered from plant metabolites in order to fight bacterial infections. The diversity of classes of drug scaffolds endowed with antivirulence action comprises an alternative against pathogens that are becoming increasingly resistant to several traditional antibacterials.

Of the 235 original articles reviewed and 371 chemical structures (Figure 9) analyzed, which were classified into eight major plant-derived classes (Figure 10A), we highlight the class of phenolic compounds that covered the highest number of

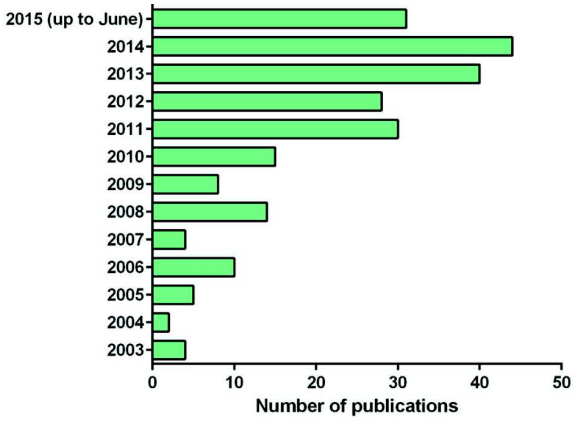

Figure 9. Number of publications fitting the selection criteria adopted in the present study, by year of publication.

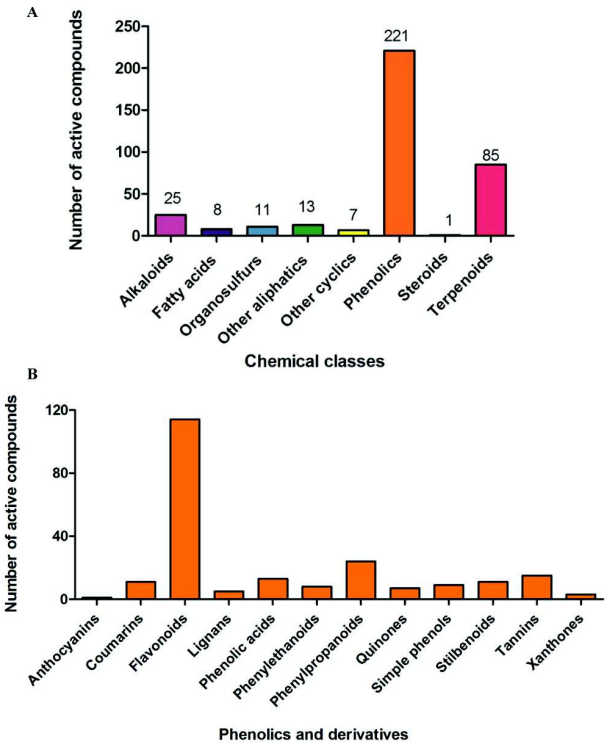

Figure 10. (A) Number of plant-derived compounds compiled in this study and classified according to chemical class. (B) Number and classification of phenolic compounds covered in the review.

bioactive compounds, accounting for 221 structures, particularly flavonoids (Figure 10B). Several reports suggest that phenolics have multiple mechanisms of action, including their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, to cause cytoplasmic membrane damage and/or a reduction in membrane fluidity, and to inhibit nucleic acid synthesis, cell wall synthesis, and energy metabolism, which explains the bacteriostatic or bactericidal effects observed against a wide array of bacteria.[373,374] In addition to the direct and synergic well-known antibacterial activity, we have shown mounting evidence that phenolics may present a tight and site-specific modulation in various bacterial virulence targets, as demonstrated by their interference in enzymes, toxins, and signal receptors.

Biofilm formation was the most investigated antivirulence bacterial target (Figure 11), possibly due to its substantial impact on medicine and public health in recent decades, which prompted scientific efforts toward more in-depth knowledge about this bacterial lifestyle process. Furthermore, the fact that experimental procedures are easily carried out, typically involving the use of simple phenotypic assays in initial analysis, is likely to have enlarged the number of literature reports. A positive hit for a biofilm target may include an additional effect against related virulence factors, such as QS and/or motility.

The ideal target is the one which the bacteria will hardly overcome. In this sense, the idea of how drugs will disturb essential bacterial functions, including growth, survival, and crucial social interactions, becomes important. Quorum sensing has been believed to be a leading target, although the complexity of the system enables distinct cascades to bypass the inhibited pathway. Since the QS system coordinates several functions in the cell, the inhibition of such a target will cause

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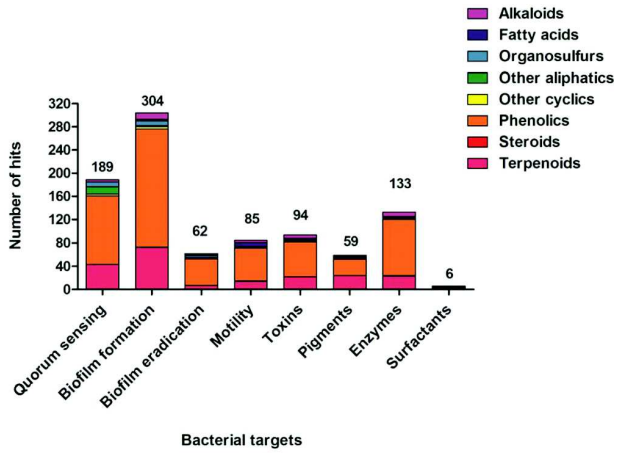

Figure 11. Number of hits for compounds active against each bacterial target.

important disturbances in bacterial fitness, requiring adaptation efforts that may generate, in the long term, a new resistance mechanism. On the other hand, it is expected that blocking deleterious effects in the host through antivirulence compounds that effectively inhibit secreted bacterial components, such as enzymes and toxins, will promote a milder disturbance on bacterial fitness, hindering resistance development, as schematically proposed in Figure 12.

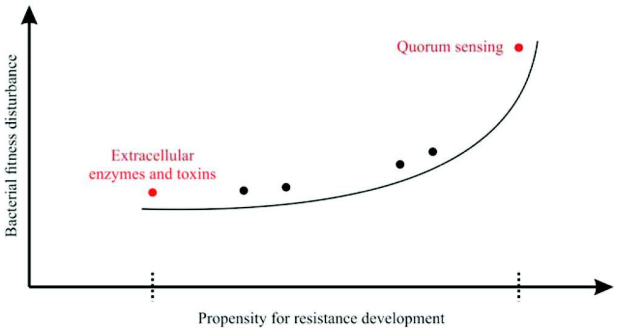

Figure 12. Proposed scheme for bacterial fitness disturbance degree caused by inhibition of virulence targets.

An optimally designed antivirulence agent should contemplate some important features, namely, (i) not to disrupt bacterial growth or viability, decreasing the pressure for resistance development; (ii) to be highly specific to and hinder a bacterial pathway required for virulence during different infection steps; (iii) to be efficient against distinct pathogens, by targeting an indispensable and evolutionarily conserved virulence trait; (iv) to be nontoxic to host and active at low concentrations; and (v) to be affordable for chemical synthesis with high yield, in addition to being open for optimization.

Antivirulence is a very attractive but incipient concept; therefore, further research is required to demonstrate the clinical efficacy of some of the promising compounds identified so far, without underestimating their in vivo potential. From a stricter point of view, more in-depth studies are required on bioavailability and pharmacodynamics. Also, it is increasingly important to understand pharmacological synergies between traditional antibacterials and antivirulence agents, to make authentic progress in this field of research. The main challenge in translating these molecules into real intervention strategies relies on the fact that procedures applied to classical antimicrobial drugs are useless, and in fact, there are no standardized antivirulence methods to validate these findings. Additional challenges include much faster and more accurate diagnosis, both essential for the application of antivirulence inhibitors presenting a narrow spectrum with pathogen-specific identity. Although the applicability of this approach in clinical settings remains to be clarified, the promising prototypes here addressed raise hopes concerning the development of antivirulence drugs, opening a path toward anti-infective therapeutics.

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Notes

The authors declare no competing financial interest.

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Karine Rigon Zimmer is a pharmacist who graduated from Pontifical Catholic University of Rio Grande do Sul (Porto Alegre, Brazil), in 2002. She holds an M.Sc. in Molecular Interactions and Human Pathologies/Therapeutic Research, from University of Picardie Jules Verne (Amiens, France, 2007), and a Ph.D. in Molecular and Cellular Biology, from Federal University of Rio Grande do Sul (UFRGS) (Porto Alegre, Brazil, 2012). Her Ph.D. thesis received honorable mention in the Brazilian national award "CAPES Theses Award" in the field of biological sciences. In 2013, she completed a postdoctoral fellowship at UFRGS under the supervision of Prof. Alexandre José Macedo. From 2013 to 2015 she worked as an assistant professor at Federal University of Rio Grande (Rio Grande, Brazil) in biochemistry. Currently, she is working as an assistant professor in the department of Basic and Health Sciences at the Federal University of Health Sciences of Porto Alegre (Porto Alegre, Brazil) in the field of microbiology. Her expertise involves purification of antivirulence compounds and evaluation of their mechanism of action. Her research interest focuses on the search for natural and synthetic compounds with particular emphasis on the modulation of pathogenic biofilms.

Alexandre José Macedo is a chemist who graduated from Federal University of Rio Grande do Sul (UFRGS) (Porto Alegre, Brazil), in 1999. He holds a M.Sc. in Molecular and Cellular Biology (UFRGS, 2002), and received his Ph.D. in Natural Sciences, with an emphasis on microbiology, at Technical University of Braunschweig and Helmholtz Centre for Infection Research (Braunschweig, Germany), in 2006, under the guidance of Prof. Dr. Kenneth Nigel Timmis and Dr. Wolf-Rainer Abraham. Since 2007 he has held the professor position at the Faculty of Pharmacy and Biotechnology Centre of UFRGS, leading the research group "Biofilms and Microbial Diversity". His research interests are focused on the search for natural products able to inhibit bacterial adhesion and to design anti-infective surfaces.

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SILVA LN, DA HORA GCA, SOARES TA, BOJER MS, INGMER H, MACEDO AJ, TRENTIN DS. **MYRICETIN PROTECTS *Galleria mellonella* FROM *Staphylococcus aureus* INFECTION VIA INHIBITION OF SEVERAL VIRULENCE FACTORS.**

Myricetin Protects *Galleria mellonella* From *Staphylococcus aureus* Infection Via Inhibition Of Several Virulence Factors

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ABSTRACT

Staphylococcus aureus is an opportunistic pathogen related to a variety of life-threatening diseases. The bursting of antimicrobial resistance has restrained clinically useful antibacterials options against *S. aureus* infections. Hence, interfering with the repertoire of *S. aureus* virulence factors, comprises an alternative approach to control infectious processes. In this study, we report that myricetin (Myr), a common flavonol compound derived from vegetables, fruits, nuts, berries and tea, has no antibacterial activity, but can remarkably decrease the production of several *S. aureus* virulence factors, including bacterial adhesion, biofilm formation, hemolysis and staphyloxanthin production. To explore the mechanism by which Myr inhibits *S. aureus* virulence, while its glycosylated form does not, we verified the relative expression levels of virulence related genes and employed molecular dynamics simulations with pivotal enzymes in pathogenesis process. Furthermore, Myr conferred a significant degree of protection against staphylococcal infection in *Galleria mellonella* model. Thus, the results of the present work reveal the potential of Myr as an alternative multi-target antivirulence candidate to control pathogenicity of *S. aureus*.

Introduction

Staphylococcus aureus is an important human opportunistic pathogen involved in a wide range of clinical infections. This bacterium is frequently associated with bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary and device-related infections[1]. Nowadays, the current usage of bactericidal compounds has led to the emergence of several strains resistant against clinically useful antibacterials like methicillin and vancomycin, making treatment of *S. aureus* infections a major challenge, especially in hospital settings[2,3]. Moreover, the rapid increasing of multidrug resistant microorganisms and the relative absence of new antimicrobials coming to market contribute to the low number of effective agents for some life-threatening infections[4].

The pathogenicity of *S. aureus* is related with secretion of an impressive collection of virulence factors such as exotoxins and enzymes - hemolysin, enterotoxins and coagulase[5], biofilm formation[6], staphyloxanthin pigment production[7] and bacterial quorum sensing[8]. The α-hemolysin (Hla), most aptly referred to as α-toxin based on its broad range of cellular specificity, is secreted from most pathogenic strains of *S. aureus* as a 33.2 kDa water-soluble monomer[9,10]. Through the binding to the host membrane, the monomer oligomerizes to form a 232.4 kDa membrane-inserted heptamer. This pore-forming toxin has been reported as an important protein that mediates tissue damages promoted by *S. aureus*. Other virulence factors are covalently anchored to peptidoglycan by sortase enzymes, a group of widely distributed cysteine transpeptidases in gram-positive bacteria, known as sortases[11]. Sortases, particularly SrtA, are essential for the functional assembly of different surface proteins and seem to play an expressive role in the establishment of staphylococcal bacterial infections both systemic and localized[12,13].

Hence, alternative therapeutic strategies such as antivirulence compounds have attracted great attention. Unlike antibacterials that aim to inhibit cell growth, antivirulence therapies are based preferably on the inhibition of bacterial virulence, since virulence factors display an important pathological role in bacterial colonization and invasion and are not essential for survival[14,15]. In this regard antivirulence therapies present a number of advantages since, potentially, must (i) produce a mild evolutionary pressure for development of resistance, (ii) provide an increased repertoire of pharmacological targets and (iii) generate antimicrobial agents with new mechanisms action. Plants represent rich sources of bioactive molecules and thus, are being explored for discovery and development of novel antivirulence agents. Among them, phenolic compounds such as flavonoids deserve special attention regarding its potential to control bacterial virulence[16].

Myricetin (Myr), 3,5,7,3′,4′,5′-hexahydroxyflavone (Fig. 1a), is a commonly flavonoid ingested through human diets such as fruits, vegetables, tea, berries and red wine. This flavonol has been proven to possess various beneficial pharmacological properties, including anti-oxidative and cytoprotective effects, anti-carcinogenic actions, antiviral properties, antiplatelet activity, anti-inflammation and anti-hyperlipidemia[17,18]. In this study, we examined the effect of Myr and of its glycoside myricitrin (Myr-gly - Fig. 2a) on several virulence factors produced by *S. aureus* and the capacity of the flavonol to protect the host during staphylococcal infection using the *in vivo Galleria mellonella* model.

Results

Initial adhesion and biofilm development in presence of Myr and Myr-gly

To investigate whether Myr and Myr-gly hinder biofilm formation, we compared *S. aureus* biofilm formation in the absence or presence of increasing concentrations of both compounds. Myr significantly inhibited *S. aureus* adhesion when tested in the early times of incubation (Fig. 1D-E) and the formation of biofilms in a dose-dependent manner (Fig. 2F-G), without affecting bacterial growth (Fig. 2B-C). SEM images show the decreasing of biofilm development on hydrophobic polystyrene surface according higher exposure to Myr when compared to untreated *S. aureus* biofilms. These images correlated with the dose-response curve showing that Myr inhibited biofilm formation and kept most cells in the planktonic state (Fig. 2H-M). However, the same inhibition profile was not observed when biofilms were exposed to Myr-gly. In fact, Myr-gly did not affect *S. aureus* bacterial growth (Fig. 2A-B) and also was not able to avoid initial bacterial adhesion neither biofilm development (Fig. 2D-E e 3F-G). As expected, SEM images of biofilms treated with Myr-gly presented no difference from the control (Fig. 2H-M), Additionally, microscopy evaluations also confirm that both compounds did not affect cell morphology, as better visualized in images inserts, supporting, once again, that their action is not related to cell death.

When tested against gram-negative strains, both compounds were not active to prevent biofilms by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains, indicating that Myr action is specific for gram-positives (Supplementary Figure S1).

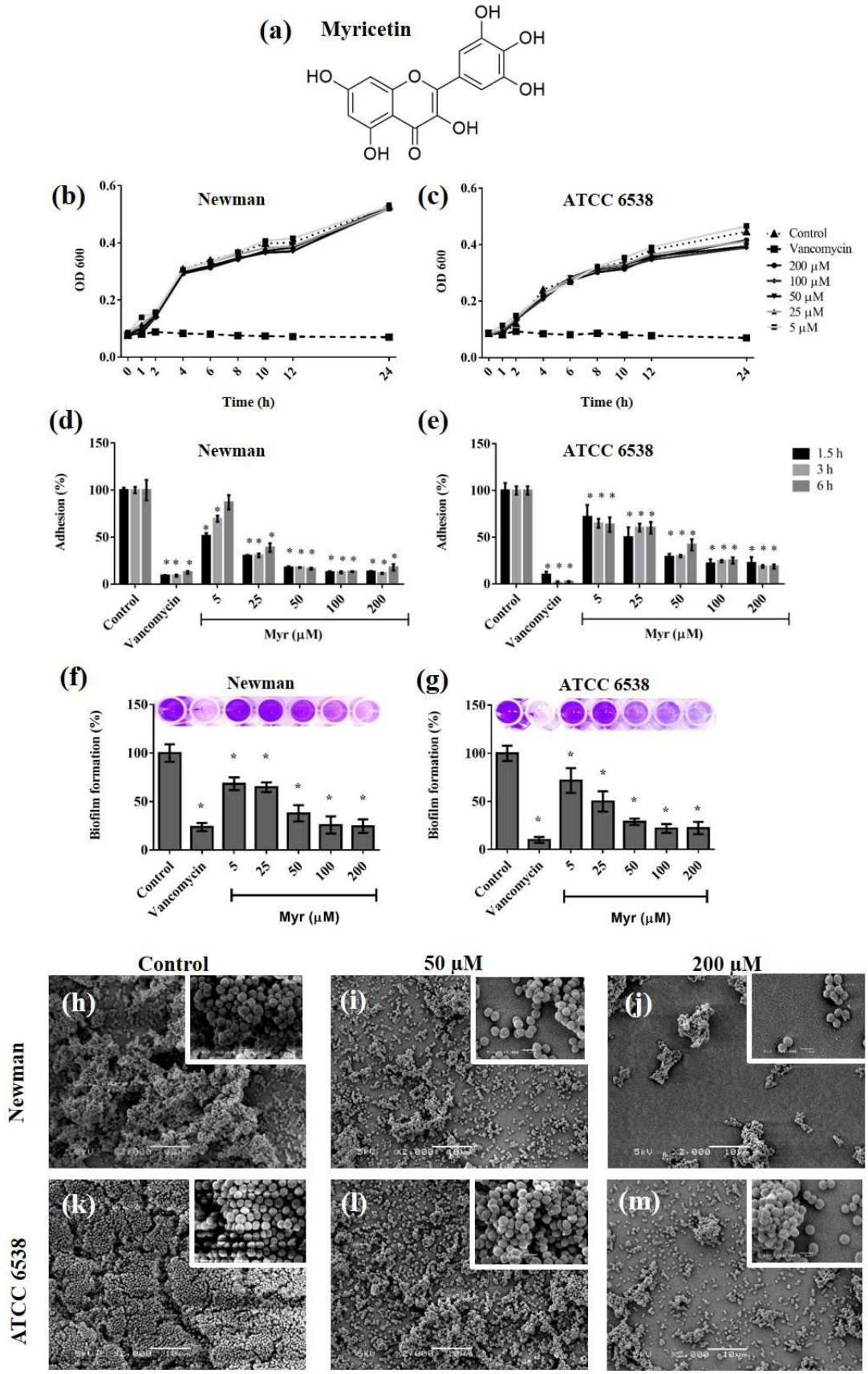

(a) Myricetin
OH
OH
HO
O
OH
OH
OH
O
(b) Newman
OD 600
Time (h)
(c) ATCC 6538
Control
Vancomycin
200 µM
100 µM
50 µM
25 µM
5 µM
(d) Newman
Adhesion (%)
Control
Vancomycin
Myr (µM)
(e) ATCC 6538
1.5 h
3 h
6 h
(f) Newman
Biofilm formation (%)
(g) ATCC 6538
Control
50 µM
200 µM
(h)
(i)
(j)
(k)
(l)
(m)
Newman
ATCC 6538

Figure 1 Effects of Myricetin (Myr) on *S. aureus* growth, initial adhesion and biofilm formation. (a) Chemical structure of the Myr consisting of 3-hydroxyflavone backbone and 6 hydroxyl groups. **(b-c)** Growth kinetics at different concentrations of Myr (0, 5, 25, 50, 100 and 200 µM) during 24h against *S. aureus* Newman and ATCC 6538. **(d-e)** Initial adherence of *S. aureus* Newman and ATCC 6538 treated with Myr during 1:30, 3 and 6 h. **(f-g)** Dose-response curve of biofilm formation tested against *S. aureus* Newman and ATCC 6538 in a 96-well polystyrene microtiter plate in the presence of the Myr. * represents statistically significant differences (*p* value <0.01). Photos of crystal violet assay: increasing violet color indicates higher biofilm formation. **(h-m)** Scanning electron microscopy (SEM) images of biofilms developed on Permanox slides: first line shows *S. aureus* Newman treated with 0, 50 and 200 µM of Myr (h-j), and second line presents *S. aureus* ATCC 6538 treated with the same concentrations (k-m). Scale bars: 10µm (inserts in the images present 1µm).

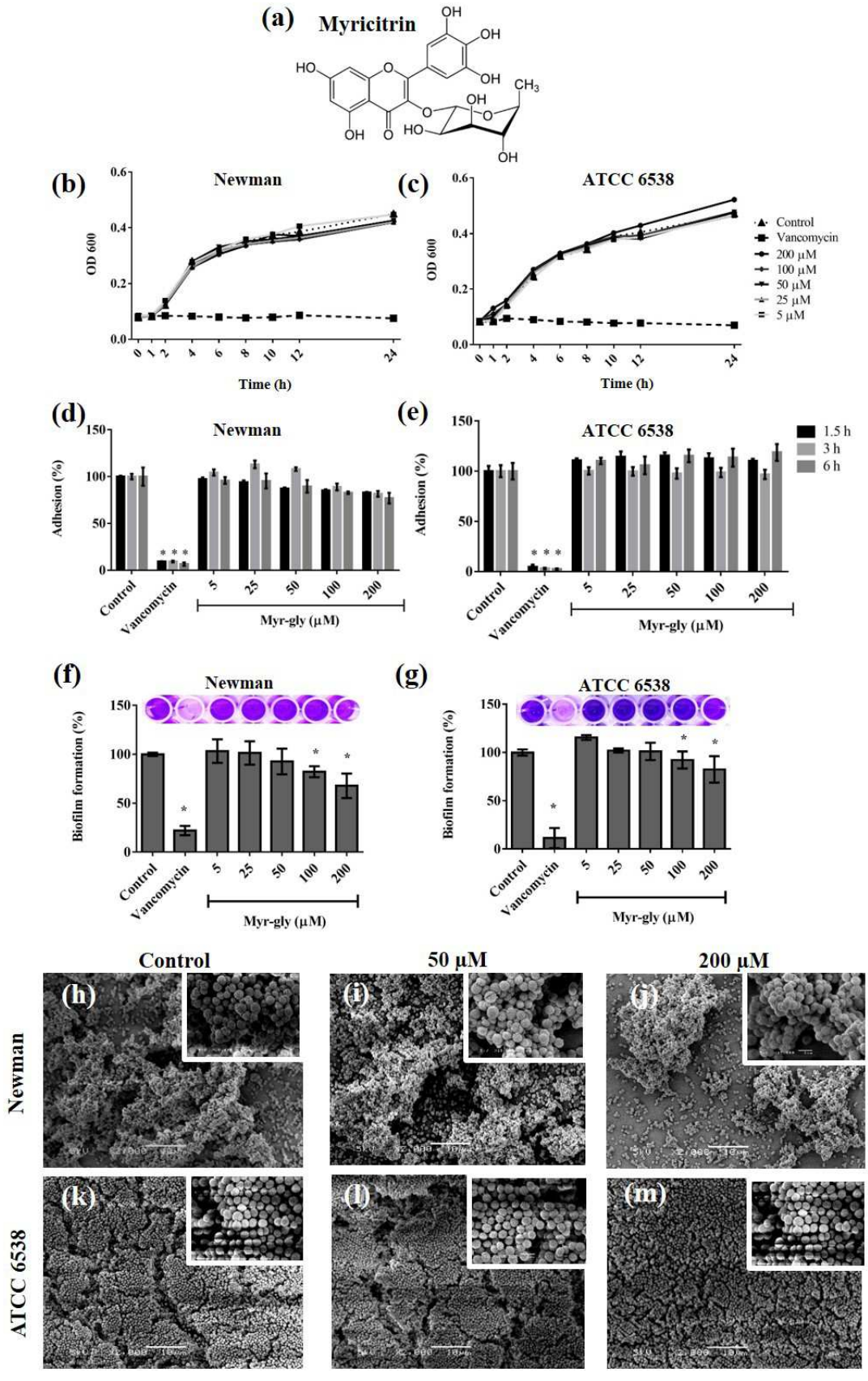

(a)
Myricitrin
(b)
Newman
(c)
ATCC 6538
OD 600
Time (h)
Control
Vancomycin
200 μM
100 μM
50 μM
25 μM
5 μM
(d)
Newman
(e)
ATCC 6538
Adhesion (%)
1.5 h
3 h
6 h
Myr-gly (μM)
(f)
Newman
(g)
ATCC 6538
Biofilm formation (%)
Control
50 μM
200 μM
(h)
(i)
(j)
(k)
(l)
(m)
Newman
ATCC 6538

Figure 2 Effects of Myricitrin (Myr-gly) on *S. aureus* growth, initial adhesion and biofilm formation. (a) Chemical structure of the flavonol Myr-gly, corresponding to derivative 3-O-rhamnoside of myricetin. **(b-c)** Growth kinetics at different concentrations of Myr-gly (0, 5, 25, 50, 100 and 200 µM) during 24h against *S. aureus* Newman and ATCC 6538. **(d-e)** Initial adherence of *S. aureus* Newman and ATCC 6538 treated with Myr during 1:30, 3 and 6 h. **(f-g)** Dose-response curve of biofilm formation tested against *S. aureus* Newman and ATCC 6538 in a 96-well polystyrene microtiter plate in the presence of the Myr-gly. * represents statistically significant differences (*p*-value <0.01). Photos of crystal violet assay: increasing violet color indicates higher biofilm formation. **(h-m)** Scanning electron microscopy (SEM) images of biofilms developed on Permanox slides: first line shows *S. aureus* Newman treated with 0, 50 and 200 µM of Myr-gly (h-j), and second line presents *S. aureus* ATCC 6538 treated with the same concentrations (k-m). Scales bar: 10µm (inserts in the images present 1µm).

Adherence to cell-matrix protein and microbial surface hydrophobicity index in presence of Myr

An active sortase enzyme is indispensable for the adherence of *S. aureus* to host cell matrices and establishment of an infection. Proteins such as protein A, clumping factor proteins, and fibronectin-binding proteins are attached to the cell wall by this enzyme. The clumping-inhibitory activity of Myr was investigated against *S. aureus* strains. The treatment reduced the capacity of the bacteria to form clumps with fibrinogen in a dose-dependent manner and *S. aureus* treated with 200 µM of Myr exhibited a threefold decrease in the level of fibrinogen cell clumping (Fig. 3a-b).

The hydrophobic character of bacterial surface also has been reported to play an important role in microbial attachment, not only to host cell matrices but also to abiotic surfaces. Thus, cell surface hydrophobicity is relevant to adhesion property of biofilm-producing bacteria, since it knows that hydrophobic cells adhered to a greater extent than hydrophilic cells. Myr was able to reduce the hydrophobicity of the *S. aureus* surface, decreasing by at least one fold its hydrophobic nature compared with untreated controls (Fig. 3e-f).

Structural model for the complex Myr and SrtA

The NMR-derived structure of SrtA is covalently bound to an analog of the LPXTG sequence, its natural substrate[19]. The substrate LPXTG binds to SrtA through a large groove that leads into the active site. The groove floor is formed by residues in strands 4 and 7 (groove floor), whereas the groove walls are formed by surface loops connecting strand 6 to strand 7 (β6/β7 loop), strand 7 to strand 8 (β7/β8 loop), strand 3 to strand 4 (β3/β4 loop), and strand 2 to helix H1 (β2//H1 loop) (Fig. 3g). The NMR-derived structure of SrtA differs significantly from the crystal structure of the non-covalent complex between SrtA and the LPXTG peptide (PDB ID 1T2W)[20], particularly in loops β6/β7 and β7/β8 and in the bound conformation of the LPXTG peptide[19]. The loop β6/β7 has been shown to be essential for catalysis, since amino acid mutations in this region impair enzyme activity and alter specific substrate recognition. Furthermore, the NMR spectroscopy data shows that substrate binding induces a structural transition involving loop β6/β7 which transitions from a structurally disordered and open conformation to an ordered and closed conformation. This substantial structural change was not observed in the crystal structure of the SrtA non-covalently bound to LPXTG[19]. Molecular docking calculations were performed for Myr using the solution structure of

SrtA[19]. Therefore, it takes into account the conformational change induced by the substrate, and should be representative of the enzyme intermediate state during catalysis. Molecular docking calculations show that Myr binds to the SrtA active site groove similarly to the covalent bound LPXTG analog (Fig. 3h-3i). The resulting lowest energy conformations for the ligand-receptor complex were obtained from extensive sampling of 4.05×10^8 conformations. Remarkably, the 100 lowest energy conformers sampled for Myr are conformationally identical (within a RMSD of 2 Å) (Fig. 3i).

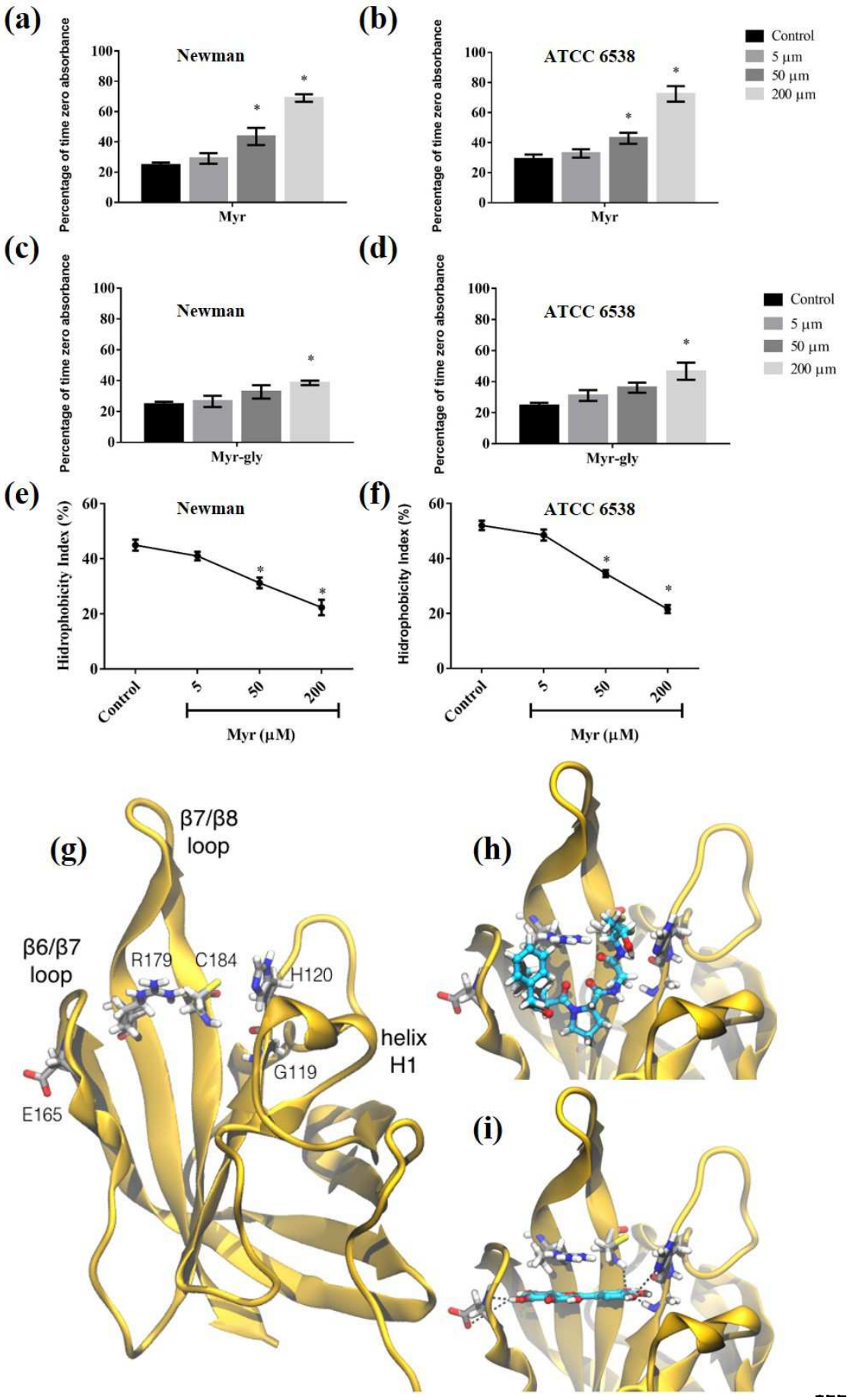

(a)
Newman
Percentage of time zero absorbance
Myr
(b)
ATCC 6538
Control
5 µm
50 µm
200 µm
(c)
Newman
Myr-gly
(d)
ATCC 6538
(e)
Newman
Hidrophobicity Index (%)
Control
5
50
200
Myr (µM)
(f)
ATCC 6538
(g)
β7/β8 loop
β6/β7 loop
R179
C184
H120
G119
helix H1
E165
(h)
(i)

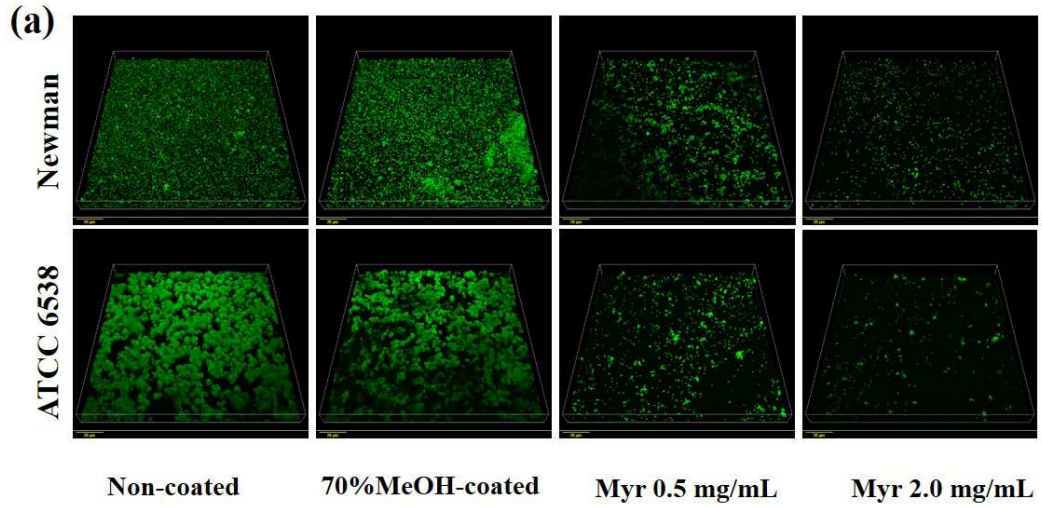
Figure 3 Effects of Myr on *S. aureus* clumping, cell surface hydrophobicity and molecular dynamics simulation sortase A (SrtA). (a-d) Inhibitory activity of flavonols on the ability of *S. aureus* Newman (a and c) and ATCC 6538 (b and d) to clump with fibrinogen. **(e-f)** Hydrophobicity index of *S. aureus* Newman and ATCC 6538 cells after treatment with Myr, measured according the microbial adhesion to hydrocarbon test. * represents statistically significant differences (*p*-value <0.01) **(g-i)** Computational analysis of SrtA: (g) Overall fold of SrtA highlighting regions undergoing large substrate-induced conformational changes; (h) Detail of the LPXTG sequence analog covalently bound to SrtA; (i) Lowest energy conformation obtained for Myr through molecular docking calculations. Receptor residues are represented in gray and ligands in cyan.

Prototype *Green*-coated surface with Myr and surface wettability

Research efforts to evolve a new generation of biomedical materials have been focused in the development of bioactive surface coatings in order to hinder infections associated to biomaterials. Due to its low immunogenicity and toxicity, natural products with recognized therapeutic efficacy and safety, particularly polyphenol-based coatings, represent a *green* alternative to pharmaceuticals[21-23].

Since Myr prevented bacterial adhesion without killing cells, we developed a prototype of natural product-coated surface, using spin-coating technique. This bioinspired surfaces were strongly resistant to bacterial adhesion, presenting only few cell clusters or just single attached cells and then prevented biofilm formation. Non-coated and methanol-treated surfaces enabled bacterial adherence and accumulation, allowing a robust biofilm formation (Fig. 4a). The counting of colonies, after biofilms were scraped from surfaces, also evidenced a drop on adhered cells (Fig. 4b). A log

reduction was observed for both bacteria tested, ranging from almost 2-3 log CFU/cm^2 reduction for *S. aureus* 6538, and 1-1.5 log CFU/cm^2 for *S. aureus* Newman. Moreover, surface characterization indicated that these prototype surfaces had a more hydrophilic character than the non-coated and 70% MeOH-treated Permanox surfaces, presenting a WCA of about 76°, 95° and 92°, respectively (Fig. 4c).



(b)	Non-coated	70%MeOH-coated	Myr 0.5 mg/mL	Myr 2.0 mg/mL
	log CFU/cm^2			
Newman	6.96 ± 0.1	7.16 ± 0.2	6.05 ± 0.1 *	5.65 ± 0.1 *
ATCC 6538	7.7 ± 0.1	7.96 ± 0.2	5.85 ± 0.3 *	4.99 ± 0.8 *

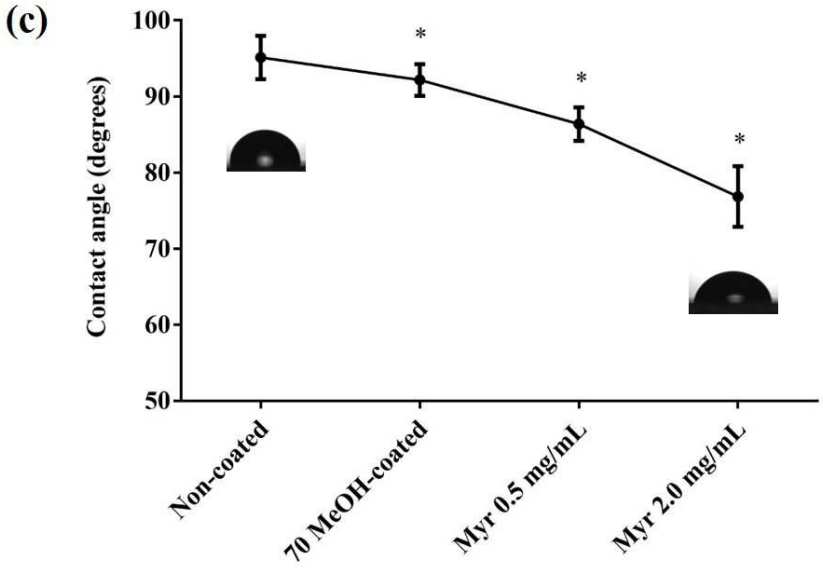


Figure 4 Effects of Myr when coated in a material surface. (a) Confocal images of *S. aureus* biofilm formation on Permanox coated with Myr (bars indicate 20 μm) and **(b)** quantitative data of adhered cells. **(b)** Water contact angle of material surfaces. * represents statistically significant differences (*p*-value <0.01).

Staphyloxanthin production and hydrogen peroxide resistance in presence of Myr and Myr-gly

The golden pigment staphyloxanthin could be visually identified in the cell pellets of *S. aureus* strains. Cell pellets recovery from Myr treatment clearly indicated that staphyloxanthin production was reduced. Quantitative analysis also showed that Myr, differently from Myr-gly, significantly decreased the staphyloxanthin production, when compared with non-treated control (Fig. 5a). Staphyloxanthin acts as an antioxidant by enabling the detoxification of host-immune system-generated ROS such as oxygen radical (O_2^-) and hydrogen peroxide (H_2O_2), therefore, we examined the effect of Myr on the survival rate of *S. aureus* in the presence of H_2O_2. As expected, Myr-treated cells were more susceptible to H_2O_2 than non-treated *S. aureus* (Fig. 5b).

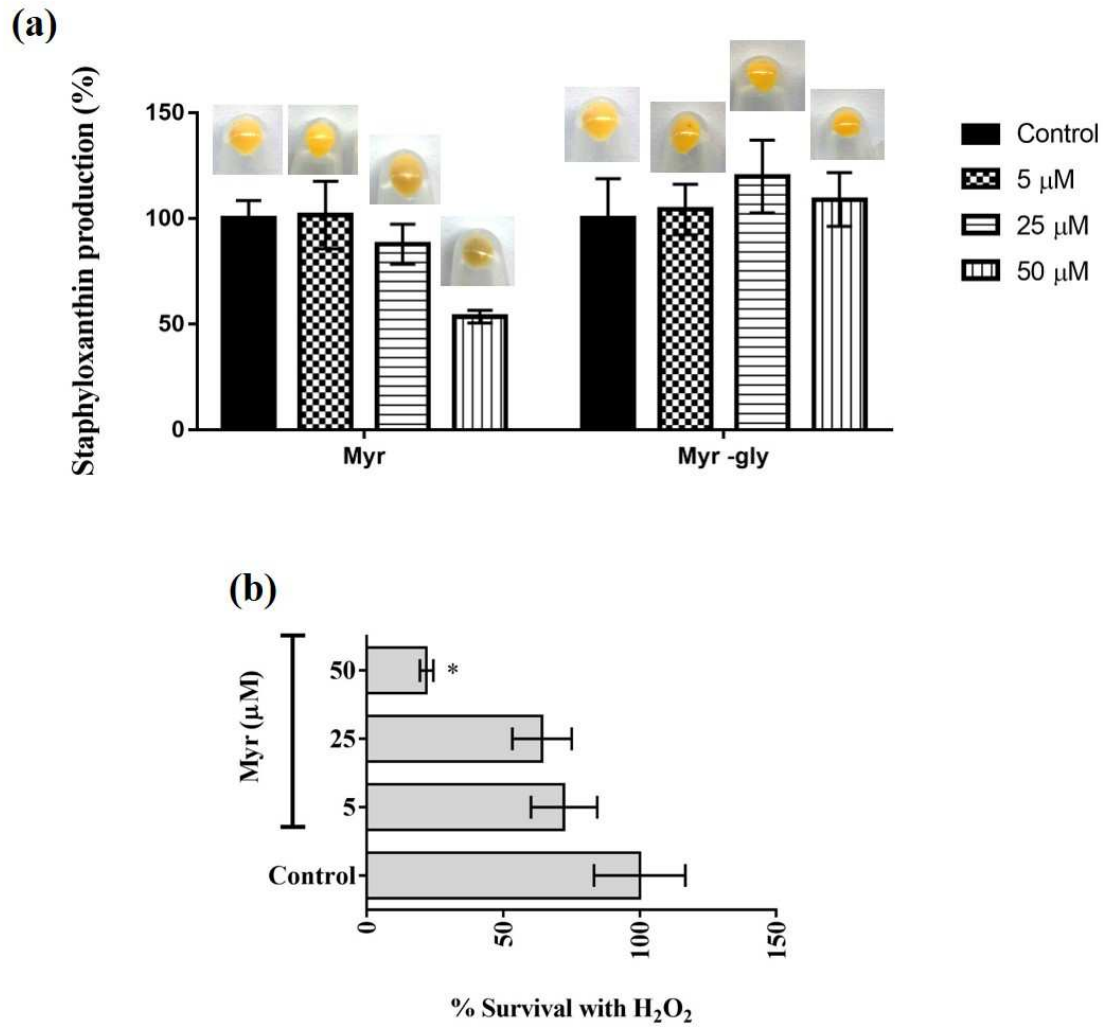


Figure 5 Effects of Myr and Myr-gly on *S. aureus* production of staphyloxanthin and hydrogen peroxide resistance assay. (a) Activity of Myr and Myr-gly on the staphyloxanthin pigment production by *S. aureus* ATCC 6538. **(b)** *S. aureus* ATCC 6538 susceptibility to H_2O_2 after staphyloxanthin reduction induced by Myr. * represents statistically significant differences (*p*-value <0.01) The experiment was done in triplicate and representative images are shown.

Citotoxicity evaluation of Myr and Myr gly to human erythrocytes and their capacity to prevent hemolysis caused by *S. aureus*

Possible cytotoxicity of Myr and Myr-gly was initially assessed using human erythrocytes. In this assay, all tested concentration of Myr and Myr-gly did not cause any damage to the plasma membrane of the erythrocytes, unlike positive control (data not shown). Then, we investigate the effect of *S. aureus* treatments with Myr and Myr-gly on α-hemolysin culture supernatants. The percentage of hemolysis was calculated by comparison with the untreated control, indicating that supernatant of Myr-treated bacteria was repressed the cause hemolysis according the increasing of Myr concentrations (Fig. 6a).

Structural model for the complex Myr-α-HL

α-HL is secreted as a water-soluble monomeric protein, which forms membrane-inserted heptameric pores upon binding to the target bilayer. Our experimental measurements show that Myr binds to the monomeric α-HL, inhibiting the formation of the heptameric pore structure. Therefore, we have performed computational simulations to investigate the structural basis of Myr-induced inhibition of the α-HL oligomerization process. It has been previously shown by the means of X-ray crystallography that conformational transitions among the monomeric and heptameric forms of α-HL trigger oligomerization process[24]. Two regions are major players in this process, namely the prestem and amino latch (Fig. 6b). In monomeric α-HL the prestem region is folded into a three-stranded antiparallel β-sheet with a long connecting loop beside the cap domain whereas the N-terminal amino latch is located at the edge of the β-sheet of the stem region (Fig. 6b). The prestem is fastened to the cap domain with a key hydrogen bond between D45 and Y118[24]. Upon oligomerization, the amino latch is released, disrupting the hydrogen bond D45-Y118. This event initiates the protrusion of the prestem characteristic of the α-HL heptameric form.

Based on the proposed mechanism of pore formation for α-HL, molecular docking calculations were performed for Myr throughout the receptor regions participating in the transition from monomeric to heptameric (pore forming) conformations. Extensive conformational sampling for Myr yielded multiple conformations, which bound to the monomeric X-ray structure of α-HL with similar binding affinities and without a preferential binding site (Fig. 6c). Because X-ray structures represent average geometrical features of 1014-1017 copies of a given molecule at a given time, each copy may occupy distinct structural microstates which are not adequately represented by a single average conformation[25-27]. Hence, MD simulations of the wild-type α-HL was performed to explore distinct microstates which could potentially bind Myr in a more specific manner. Root-mean-square deviation (RMSD) of Cα atoms of the wild-type α-HL from the X-ray structure (4YHD) reached convergence after 20 ns of simulation which was extended to 50 ns (Supplementary Fig. S2). Root-mean-square atom-positional fluctuations (RMSF) of Cα atoms probed the most flexible region to residues G134-I136 (Supplementary Fig. S3). These residues are located in the highly disordered loop in the prestem region for which atomic coordinates are missing the X-ray structure[24]. The present MD simulation also shows that the amino latch region switches between a β-strand (in a four-strand β-sheet) and less ordered conformations where the N-terminal residues interact with the prestem region (Fig. 6d). In this event the hydrogen bond D45-Y118 is disrupted (Supplementary Fig. S4). This rupture has been previously postulated to release the amino latch and initiate the protrusion of the prestem to yield the heptameric form of α-HL[24].

A representative conformation of MD-derived ensemble of structures obtained for the wild-type α-HL was subsequently used for another round of molecular docking calculations using Myr as ligand. The lowest energy conformation obtained for Myr

bound to the MD-derived structure is representative of a cluster of conformers containing 98% of the lowest energy conformers sampled out of 4.05 x 10^8 possibilities. It shows Myr bound to a region of the cap enclosed by the prestem and amino latch regions, in between residues D45 and Y118 (Fig. 6e). The molecular docking calculations suggest that Myr can favorably bind to monomeric α-HL with estimated binding energies of ca. 7 kcal.mol-1 and Ki within the μM range. Myr is anchored in the cleft between the prestem and amino latch regions through hydrogen bonds with surrounding residues, including Y118 (Fig. 6e). Subsequently, the Myr-α-HL complex was submitted to MD simulations to evaluate the effect of ligand binding on the receptor structural dynamics. The RMSD calculated for Cα atoms of the complex with respect to the X-ray structure (4YHD) exhibits longer convergence times with a structural ensemble which diverges more from the crystal structure than the free α-HL (Supplementary Fig. S2). RMSF profiles for free and Myr-bound α-HL are similar but with increased atomic fluctuations for the latter (Supplementary Fig. S3). The most flexible regions occur in the loops containing residues E71-G72, G134-I136 and P160-D162.

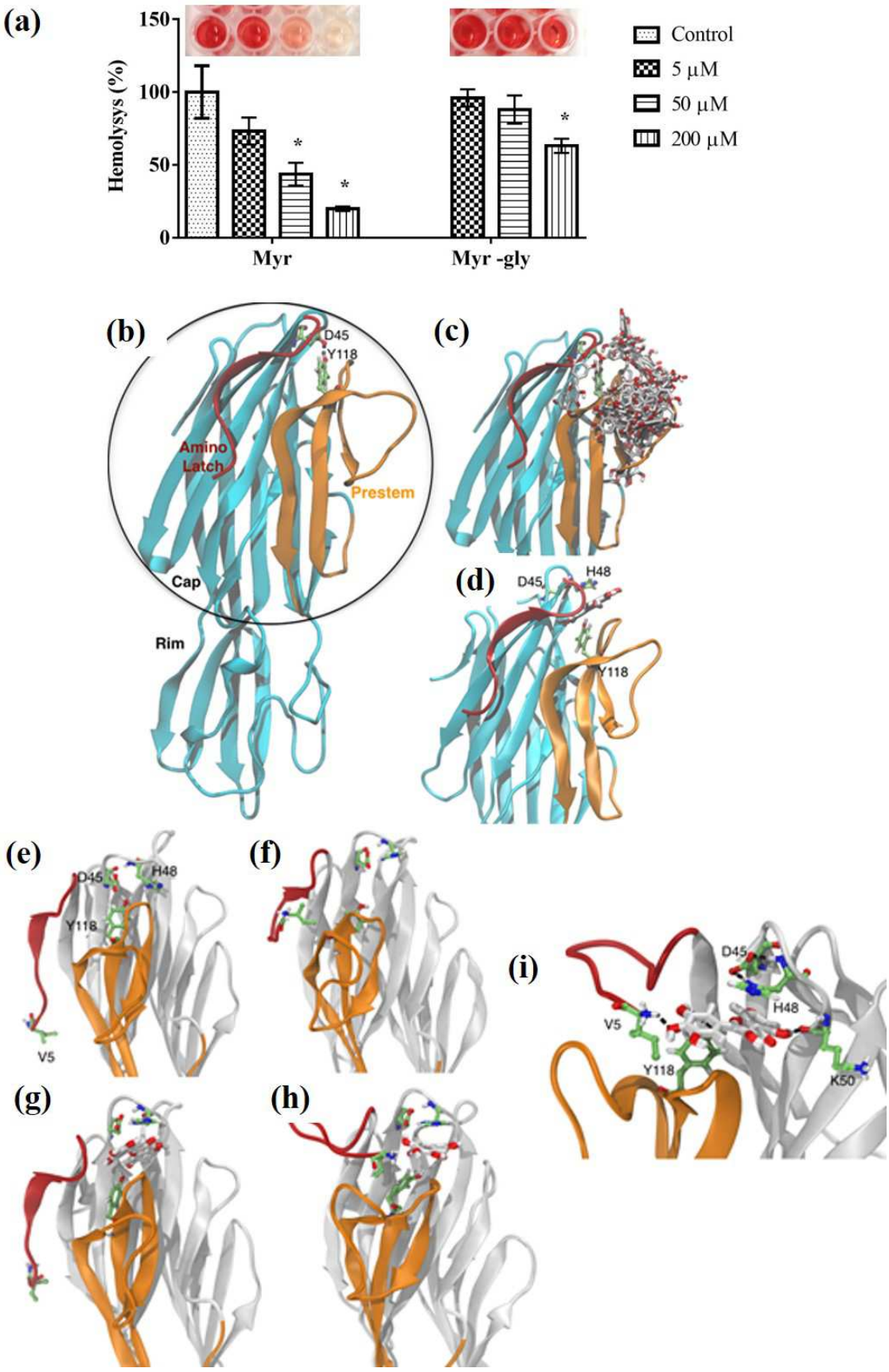

(a)
Hemolysys (%)
150
100
50
0
Myr
Myr -gly
Control
5 μM
50 μM
200 μM
(b)
D45
Y118
Amino Latch
Prestem
Cap
Rim
(c)
(d)
D45
H48
Y118
(e)
D45
H48
Y118
V5
(f)
(g)
(h)
(i)
D45
H48
V5
Y118
K50

Figure 6 Effects of Myr and Myr-gly on hemolysis induced by *S. aureus*, α-hemolysin (Hla) molecular dynamics simulation and docking studies. (a) Hemolysis promoted by supernatants of *S. aureus* ATCC 29213 culture treated with Myr and Myr-gly. * represents statistically significant differences (*p*-value <0.01) The experiment was done in triplicate and representative images are shown. **(b-d)** Cartoon representation of the monomeric structure of α-HL and the lowest energy conformations of Myr obtained from molecular docking calculations for the crystal and MD-derived conformations. (b) Representation of α-HL domains and regions accordingly to ref.[1]. The amino latch is colored in red, and the prestem in orange. The cap domain is represented inside a black circle whereas the rim domain is outside. Lowest energy conformations obtained for (c) the X-ray structure (4YHD) and (d) the MD-generated conformation. Receptor residues are represented by green sticks and Myr by white sticks. **(e-i)** Initial and final conformations of free (e-f) and Myr-bound (g-i) α-HL obtained from MD simulations in explicit solvent. The amino latch is colored in red, and the prestem in orange. Receptor residues are shown in green sticks and Myr in white sticks. Hydrogen bonds are represented by black dashed lines.

Evaluation of Myr toxicity and protection of infection caused by *S. aureus* in *G. mellonella* larvae model

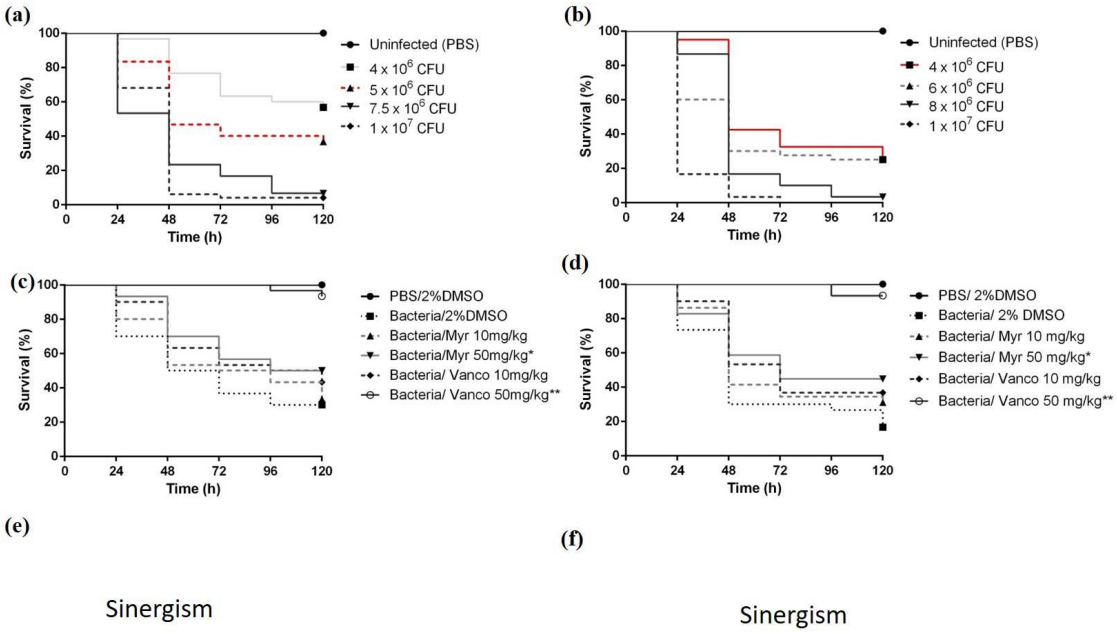
Galleria mellonella larvae has been recently introduced as an alternative model to evaluate *in vivo* toxicity of new antimicrobial agents. This model represents a quick and economical experimental host to be used prior the test using more expensive mammalian models[28]. Myr solutions administered to larvae hemocoel, at concentrations up to 50 mg/kg, did not result in death or visible injury, indicating that the flavonoid was not toxic towards the larvae (data not shown). This data corroborates with our

previous *in vitro* data, where we demonstrated that Myr is not toxic towards human erythrocytes (data not shown).

Regarding the infection model, the curve produced with different *S. aureus* inoculum concentrations indicated that effect of infection on larvae survival is dose dependent, being reduced with increasing inoculum of *S. aureus* (Fig. 7a-b). Although all inoculum led to a reduction in larval survival, inoculum doses of about 5×10^6 and 4×10^6 CFU/larvae were selected for use in further studies, respectively for *S. aureus* Newman and ATCC 6538. This inoculum showed significant differences in larval survival compared with the uninfected control group and a gradual reduction in survival rate covering the whole experiment period (Figure 7a-b, red lines). After infection with *S. aureus*, the groups of larvae treated with Myr or with the positive control vancomycin at 50 mg/kg, demonstrated a higher survival rate, when compared to the negative control group. As expected, the antibacterial vancomycin protected *G. mellonella* from infection in a greater extent than Myr since it is a bactericidal agent and Myr presents antivirulence effects without modulating growth (Figure 7c-d).

Transcriptional profiles of *S. aureus* cells in the presence of Myr or Myr-gly

To investigate the mechanism of Myr antivirulence activity, real-time qRT-PCR was used to determine a differential expression of virulence factor-related genes, such as global regulators (*rna*III, *sar*A, *sig*B and *sae*R), surface proteins (*fnb*A, *fnb*B, *clf*A and *clf*B), sortase proteins (*Srt*A and *Srt*B), polysaccharides production (*ica*A and *ica*R) and hemolysin (*hla*) genes in *S. aureus* cells with and without Myr (Fig 7g). Some difference in expression is seen between Myr and Myr-gly, there seem to be quite significant up-regulation of *srt*B (approx. 15-fold) and *ica*A, *fnb*A and *fnb*B (40-50 fold). For *hla* and *rna*III transcripts in the panel, and we see only minor effects on those.



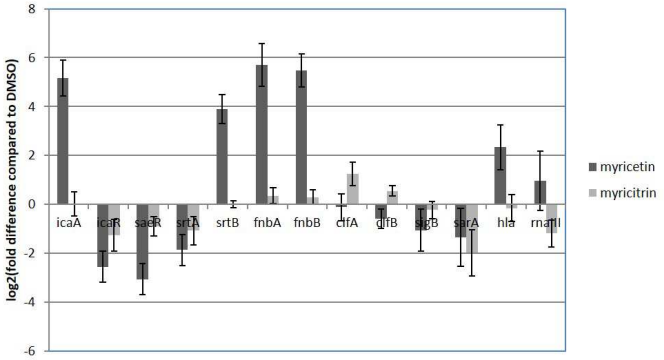


Figure 7 (a-f) Kaplan-Meier survival-curve of infected *G. mellonella* larvae. Survival of *G. mellonella* larvae infected with different concentrations of *S. aureus* Newman (a)

and ATCC 6538 (a). The inoculum selected for further experiments are presented in red. (c-d) Survival of *G. mellonella* larvae infected with *S. aureus* Newman and ATCC 6538, respectively, and treated with two different doses of Myr or vancomycin after 30 min post-infection. * and ** represent statistically significant differences, respectively $p < 0.05$ and $p < 0.01$, in relation to the larvae group that were infected and received PBS as treatment. (g) Transcriptional profile of *S. aureus* virulence genes.

Discussion

The pathogenicity of *S. aureus* is widely due to its ability to produce a large number of virulence determinants during different stages of host colonization and infection. The transcription control of physiologic and virulence genes must be sync with the changing in environmental and nutritional conditions[29]. During initial stages of infection, *S. aureus* preferentially express surface proteins that are required for adhesion to extracellular-matrix molecules. These surface proteins referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), as fibronectin (Fn)-binding proteins (FnBPs) and clumping factor, are important mediators of early biofilm attachment[30]. Our findings demonstrated that Myr impaired *S. aureus* biofilm formation from initial stages of adhesion with no growth inhibitory activity while Myr-glyc was not active. Arita-Morioka, et al. [31] described that Myr inhibited biofilm formation of various *S. aureus* and *Escherichia coli* strains in a dose-dependent manner, also without inhibiting their growth. Although no mechanism was discussed for *S. aureus* inhibition, these authors evidenced that Myr suppresses curli-dependent biofilm formation of *E. coli* by inhibiting cellular functions of molecular chaperone DnaK. Here we show that Myr modulates surface properties of *S. aureus*, such as hydrophobicity and fibrinogen clumping, and that treated-cells display similar

phenotypes to untreated cells on Congo Red Agar (Supplementary Figure S5), suggesting an icaADBC- independent biofilm development mechanism (operon responsible for polysaccharide intercellular adhesin - PIA synthesis).

Indeed, some of the virulence-associated surface proteins are covalently anchored to bacterial cell wall peptidoglycan catalyzed by the transpeptidases sortases. The sortase A (SrtA) isoform plays a critical role in the establishment *S. aureus* infections by modulating the ability of the bacterium to adhere to host tissue[12]. Thus, this enzyme comprises a promising pharmacological target that could therefore effectively reduce bacterial virulence and biofilm formation[32]. The suppression of fibronectin-binding activity by Myr demonstrates the inhibition of sortase activity promoted by this compound and not by its glycosylated form. In order to understand the binding mechanism of Myr to SrtA, molecular dynamics simulations and molecular docking analysis were carried out. Myr adopts a well-defined conformation upon binding to SrtA (Fig. 3i). It is anchored to SrtA via hydrogen bonds to residues G119, H120, E165, C184, and π-stacking interactions with R179 (Fig. 3i). The predicted binding mode of Myr is closely related the binding mode of other previously identified SrtA inhibitors[33]. For instance, the 2-phenyl-2,3-dihydro-1H-perimidine scaffold interacts with Srt-A similarly to Myr, and exhibits a IC50 of 47.2 ± 5.9 μM[33]. These calculations suggest that the SrtA can be a molecular target for inhibition by Myr, consistently with our experimental findings.

Trying to mimic a mouse foreign body infection, Vergara-Irigaray, et al. [34] verified that *S. aureus* defective mutant in fnbAB was significantly less able to colonize a subcutaneous implanted catheter material than the wild-type or icaADBC mutant strains. In case that our observed inhibition could be via protein-mediated fashion, we investigated the anti-infective potential of coatings produced with Myr on polymeric

substrates. The prototype material coated with Myr was capable to strongly prevent *S. aureus* biofilm development. Although spin-coating technique is one of the most common methods for applying uniform thin films to substrates, further studies are needed to evaluate a possible grafted of this polyphenol. Gomez-Florit, et al. [35] recently proposed a bioactive surface based on the covalent immobilization of similar flavonoid quercetin, which extended long-term efficacy of coating and enhance the soft tissue integration, retaining biological activity.

Staphyloxanthin, a golden carotenoid pigment, is another important virulence factor which has been implicated with bacterial survival enhancement in harsh environments and infections. Some *S. aureus* strains present deficiency in production of this pigment, so they can be rapidly killed by reactive oxygen species from host neutrophils and fail to form skin abscesses[7]. We found that Myr inhibited *S. aureus* pigment formation, making the cells more susceptible to H_2O_2 killing. In this sense, Lee, et al. [36] screened a series of plant flavonoids for staphyloxanthin reduction and pointed flavone, which is the backbone compound of flavonoids, as the most potent inhibitor.

In the course of *S. aureus* infection progression, the synthesis and secretion of proteins contribute to the invasion of adjacent tissues through membrane damage. α-Hemolysin is one of the most characterized virulence factor of *S. aureus* and thus, we investigated the effects of Myr on preventing blood hemolysis induced by α-hemolysin. Myr presented a superior anti-hemolytic activity when compared with its glycoside. Since Myr was not able to inhibit the expression of *hla*, as demonstrated by transcriptional profile, we suggest that Myr is able to bind directly to the Hla monomer, preventing heptamer pore formation. Myr remained tightly bound to monomeric α-HL throughout the 50 ns of simulation (Fig. 6F). It was anchored to the receptor via two

highly persistent hydrogen bonds with the carbonyl group of K50 and the hydroxyl group of Y118 (Supplementary Fig. S4). A third hydrogen bond was formed with the N-terminal region of the amino latch (Fig. 6E-F and Supplementary Fig. S4). The latter interaction was brought about by a structural rearrangement of the amino latch, yielding a conformation that resembles an intermediate state between the monomeric and pore protomeric forms of α-HL[24]. The structural rearrangement is similar to that seen in the free α-HL simulation (Fig. 6d); However, while in the latter the amino latch switches reversibly between ordered and less ordered β-strand conformations, in the Myr-α-HL complex it switches irreversibly from the initial β-strand conformation into a loop which remains fastened to myricetin for the remaining of the simulated time (Supplementary Fig. S4).Although Myr hampers the reappearance of the D45-Y118 interaction upon binding, these residues are kept in place via direct (Y118) or indirect (D45) interactions with the ligand. Hence, D45 makes two hydrogen bonds with H50, which in turn interacts with the chromanone rings via π-stacking (Fig. 6f). The present findings suggest that the antivirulence activity of Myr relies on the inhibition of α-HL oligomerization process via stabilization of an intermediate structural state between the monomeric and pore protomeric forms of the protein. Other reports corroborate with our finding, demonstrating that structure related-flavonoids also can bind to Hla and lead to the inhibition of the formation of the heptameric transmembrane pore, which results in a decrease in cell damaged induced by Hla[37-39].

A number of genes have been reported to be involved in different steps of *S. aureus* pathogenesis. Some of these genes were selected for evaluation of their susceptibility to gene expression inhibition by Myr and Myr-gly using a real time RT-PCR approach. The expression of the global *S. aureus* regulators (*rna*III, *sar*A and *sig*B) was not different between treatments, however *sae*R expression was down-

regulated for Myr. SaeR activates the expression of exoproteins involved in adhesion and invasion of host cells and cell wall-associated proteins but, at least, *hla* and *fnb* genes are up-expressed by Myr. Additionally, the operon ica is up-regulated, in contrast with the antibiofilm effect of Myr. The net outcome of the effect of Myr on *ica*A could result from the combined effect of the dynamic changes of gene expression.

S. aureus treated with Myr is not able to clump fibrinogen and has low surface hydrophobicity, suggesting the action on sortase A. We had significant up-regulation in *fnb*A and *fnb*B genes in bacteria treated with Myr and non-modulation of *clf*A and *clf*B genes. Also, the expression of *hla* was slightly up-regulated. Although *srt*A and *hla* expression was not significantly different in bacteria treated with Myr and Myr-gly, based on the docking calculations we obtained greater inhibition of enzymes (Hla and SrtA) by Myr and not by Myr-gly due to the increased molecular volume Myr-gly. Additionally, it is likely that there is a greater energy penalty for connecting the Myr-gly due to the presence of the very hydrophilic sugar, impairing the binding to enzymes.

Therefore, the inhibition of biofilm can be explained by the synergistic effect of Myr on iron depletion and modifications on cell-wall proteins involved in adhesion.

Antivirulence drugs represent a novel design therapeutic that interfere with virulence factors, which can help to overcome the spread of antibacterial resistance by potentially revitalizing the drug-development pipeline with new targets. Moreover, the classical antibacterials act on growing bacteria, being useless against the quiescent bacteria within biofilms. Not surprisingly, we demonstrate that Myr treatment led to reduction in *S. aureus* pathogenicity using the *in vivo* model of *G. mellonella*, evidencing that agents impairing virulence determinants are able to control infectious processes. In summary, the present study highlights the potential of Myr as a multi-target antivirulence agent, exhibiting antibiofilm, anti-hemolytic activities against the

important pathogen *S. aureus*. Additionally, Myr prevented biofilm formation even when immobilized as a thin coating and all effects observed *in vitro* culminated with a attenuated pathogenicity *in vivo*.

Methods

Reagents and surfaces. Myricetin (Myr), myricitrin (Myr-gly) and vancomycin hydrochloride were purchased from Sigma-Aldrich (USA). For *in vitro* assays, stock solutions were prepared in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, USA) while for *in vivo* studies, the substances were dissolved in sterile phosphate-buffered salt (PBS) buffer pH 7.0. Sterile 96-well polystyrene flat-bottom microtiter plates (Costar 3599) were purchased from Corning Inc. (USA) and hydrophobic modified polystyrene (Permanox™) slides were purchased from NalgeNunc International (USA). A 10µL Hamilton® Microliter™ syringe was used to inject inoculum aliquots into *G. mellonella*.

Bacterial growth kinetics. A kinetic study was performed to assess the effect of the compounds, at concentrations up to 200 µM, on *S. aureus* ATCC 6538 and *S. aureus* Newman growth. The OD_{600} was measured at 0, 1, 2, 4, 6, 8, 12 and 24h after incubation (37°C) in BHI broth[40]. Samples were replaced with sterile water as a control for bacterial growth and vancomycin was used as control for bactericide action. The results are expressed as mean ± standard deviation (SD).

Bacterial strains and culture conditions. *Staphylococcus aureus* Newman ATCC 25904, *S. aureus* ATCC 6538 and *S. aureus* ATCC 29213 were grown in Mueller Hinton (MH) agar (Oxoid Ltd., England). Bacterial suspension in sterile saline or Brain

Heart Infusion (BHI) broth (Oxoid Ltd., England), corresponding to optical density at 600 nm (OD_{600}) of 0.150 (3×10^8 CFU/mL), was used in the assays.

Initial adhesion and biofilm formation assays. Initial adhesion and biofilm formation were evaluated using the crystal violet assay in 96-well microtiter plates, as described by Trentin, et al. [41]. The incubation period at 37°C was 1.5, 3, 6 and 24h. Myr and myr-glyc were tested in concentrations ranging from 5 to 200 µM while untreated control received sterile water in order to correspond to 100% of adhesion or biofilm formation.

Scanning electron microscopy (SEM). *Saphylococcus aureus* ATCC 6538 and ATCC Newman biofilms were grown in 96-well microtiter plates (37°C during 24 h) with a piece of Permanox™ slide. The samples were prepared and examined according to Silva, et al. [42].

***S. aureus* clumping assay.** The clumping assay was performed as previously described by Weiss, et al. [13] with the following modifications. The strains ATCC 6538 and ATCC Newman were cultured in BHI broth during 24 h at 37 °C in the presence of compounds. The cultures were harvested by centrifugation and washed twice times with sterile saline. After washings, the pellets were resuspended in a fibrinogen solution consisting of 1 mg/mL of fibrinogen in PBS solution. The absorbance (OD_{600}) was measured for each sample 2 h after resuspension in the fibrinogen solution. In the presence of an active sortase protease, clumping factor protein, anchored in the cell wall by sortase, actively recognizes and binds to fibrinogen in solution, forming aggregation or ‘clumps’ which fall out of solution and are traced by a decreasing OD over time. The

percent change in absorbance was determined by dividing the absorbance at the time points by that obtained at the initial time point multiplied by 100.

Microbial surface hydrophobicity index. Bacterial surface hydrophobicity was determined using the microbial adhesion to hydrocarbon (MATH) test, according to Trentin, et al. [43]. *Staphylococcus aureus* ATCC 6538 and ATCC Newman were cultured in BHI broth during 24 h at 37 °C in the presence of compounds. The cultures were harvested by centrifugation and washed twice times with sterile saline solution. The suspensions were adjusted to an absorbance (Ai) of about 0.3 at 600 nm using a spectrophotometer. Toluene (200 µL) was added to 1 mL of each adjusted bacterial suspension and mixed. The new absorbances of aqueous phase (Af) were measured after phase separation. The hydrophobicity index (HPBI) was expressed as: (Ai−Af)/Ai × 100%.

Iron chelating assessment. To evaluate whether iron chelation could account for Myr activity against *S. aureus* biofilm formation, the ferrozine assay was performed using Fe^{2+} as source of iron. A standard curve was established to determine the Fe^{2+} concentration to be used in the ferrozine assay. Formation of the iron-ferrozine complex was measured at 562 nm to assess the amount of iron chelated by Myr and Myr-gly. For comparison of iron-chelating activity, 2,2-bipyridyl (Sigma-Aldrich Co., USA) was used as a standard iron chelator.

Biofilm formation assay under iron supplementation. *S. aureus* strains were cultured in BHI broth in the presence of Myr (50 µM) plus Fe^{2+} (50 or 100 µM) in 96-well microtiter plates at 37°C for 24 h. The amount of biofilm formation was determined by

crystal violet and the sample that was not treated with Myr or Fe^{2+} was set as 100% biofilm formation.

Green-coated surfaces: preparation and characterization. Permanox™ surface was coated as previously described by Trentin, et al. [22]. Precisely 200 μL of a 0.5 mg/mL and 2.0 mg/mL in 70% aqueous metanol (Merck, Germany) was spin-coated onto a 1 cm^2 fragment of Permanox™ during a cycle of 500 rpm (5s) and then accelerated to 5000 rpm (40 s) in the spin coater Laurell Model WS-650MZ-23NPP/LITE. After this first coating step, the specimens were heat-treated (30 min at 60°C) to allow for film annealing and to remove any excess solvent. The coating process was repeated twice and the second coating step was followed by 1h 30 min anneling at 60°C. Samples with coating film double were sterilized with UV light during 20 minutes. As controls, samples were spin-coated with 70% aqueous methanol solution but without Myr and other samples without coating were just heated to 60°C and UV-treated.

Permanox™ samples were characterized before and after coating with Myr using water contact angle (WCA). Contact angle measurements were carried out using the sessile drop technique and milliQ water. The drop was observed directly using an Optical Tensiometer Theta Lite (OneAttension, Biolin Scientific, Finland). The reported water contact angles are means of more than five measurements performed on different areas of each sample surface.

Green-coated surfaces: confocal microscopy. Slides of the non-coated and coated Permanox™ were placed in the wells of 24-well tissue culture plates wherein biofilms were grown (37°C during 24 h). After, the slides were gently washed with PBS and were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies,

USA). Images were obtained using an Olympus IX81 confocal microscope and UPLSAPO 60X W NA:1.20 objective and were overlaid using Image J software.

Staphyloxanthin assay: qualitative and quantitative analysis. The bright golden color of this virulence factor facilitates the anti-virulence screening by the simple observation of color change[7], however we also applied a quantitative carotenoid evaluation according[44], with minor modifications. Briefly, *S. aureus* ATCC 6538 cells were inoculated in BHI medium and incubated for 24 h at 37 ^{o}C with or without flavonoids. Bacterial cells harvested by centrifugations and washed twice with sterile saline and at this point, cell pellets were photographed to compare the staphyloxanthin production. For the extraction of carotenoid pigments, the cell pellets were resuspended in 0.2 mL of methanol by vortexing, and this mixture was heated at 55 ^{o}C for 30 min. Pigment extraction was separated from cell debris by centrifugation at 16,600g for 10 min. The procedure of pigment extraction was repeated 3 times in order to maximize staphyloxanthin extraction, and the optical densities of collected extractions were measured at 465 nm using a spectrophotometer. Each data point was averaged from at least three independent cultures.

Staphyloxanthin assay: hydrogen peroxide resistance evaluation. The resistance assay (survival test) with hydrogen peroxide (H_2O_2) was adapted from previous study of Liu, et al. [44]. *Staphylococcus aureus* ATCC 6538 were cultured in the presence and absence flavonols during 24 h and then harvested by centrifugation and washed twice with sterile saline. Bacterial suspensions prepared with saline solution in order to obtain a suspension of OD_{600} of 0.150 and incubated with H_2O_2 (final concentration of 1.5%) for 60 min at 37°C with shaking (150 rpm). The percentage of cells surviving the stress

was calculated by the counting of the number of colony-forming units (CFU)/mL in comparison with the non-treated control (bacteria exposed to water instead of flavonols and then challenge to survive after addition of H_2O_2). The results are the average of at least three independent cultures.

Hemolysis assay. Firstly, we carried out a simple assay to assess the possible injury caused by flavonoids in red blood human cells. Myr and Myr-gly were tested at 5, 50 and 200 µM, and as reference samples, we used water (for baseline values) and Triton X-100 (for 100% hemolysis). To avoid the interference of sample color, a blank sample of flavonoids and PBS (without erythrocytes) was developed. The assay was calculated as (Abs treatment - Abs blank/ Abs Triton- Abs Water) x 100.

Then, the lysis efficacies of human red blood cells also were measured using *S. aureus* supernants grown in the presence of flavonoids. Briefly, *S. aureus* ATCC 29213, a well-known α-hemolysin producer, was cultured in BHI medium with or without flavonoids during 24 h at 37°C and 150 rpm. *S. aureus* treated-supernatants were added to 3% human red blood cells suspension and were incubated at 37 °C for 1 h at 100 rpm. Supernatants were collected by centrifugation at 3000×g for 10 min and optical densities were measured at 543 nm.

All the blood donors were healthy researchers and students who signed specific form for consent to participate in the study. The Universidade Federal do Rio Grande do Sul Ethical Committee approved all documents, procedures and project under authorization number 1.202.565 (2015).

***In vivo* toxicity and survival assay in *Galleria mellonella* larvae.** The whole cycle of *G. mellonella* were maintained in our laboratory at 28°C. Insects were fed with artificial

diet, consisting of honey and several flours. Groups of ten larvae of the greater wax moth in the final instar larval stage weighing 220-260 mg were used in all assays. Larvae were injected using a 10 μL Hamilton syringe into the hemocoel in the last right proleg. For toxicity assay, larvae were treated with 10 and 50 mg/kg of Myr. Controls included a group of larvae that did not received any injection and a group of larvae inoculated with vehicle (PBS 2% DMSO).

For infection assay, in order to determine the appropriated concentration of each bacterium strain to be injected in larvae, we performed a curve with different bacterial inoculums, ranging from 1 $x10^6$ to 1.0 x 10^7 CFU/larvae. Then, larvae were infected with 10 uL of about bacterial suspension in saline (5 x 10^6 CFU/larvae). After 30 minutes of incubation at 37°C, larvae received 10 μL of flavonoid or vancomycin or vehicle in the last left proleg. Subsequently, all larvae were incubated at 37°C in sterile petri plates. The following control groups were included: untreated control (larvae not administered any injection), PBS 2% DMSO control (larvae inoculated with vehicle), and negative control (larvae inoculated with the *S. aureus* and treated with PBS) and positive control (larvae inoculated with the *S. aureus* and treated with vancomycin 10 and 50 mg/kg). To evaluated the possible synergistic effect between Myr and vancomycin, larvae were treated with Myr 50 mg/kg plus vancomycin 10 mg/kg after 30 minutes of infection.

For both toxicity and infection assays, larvae were assessed daily for survival up to 5 days post-treatment status and were evaluated according survival, considering dead when they displayed no movement in response to touch.

Computational details. Atomic coordinates for α-HL were taken from the crystallographic structure of the monomeric form solved at 2.8 Å from the sequence of

S. aureus (PDB ID 4YHD)[24]. The X-ray structure was obtained for the single mutant H35A. The mutation was reversed to the wild-type sequence prior the docking and molecular dynamics (MD) simulations. Missing residues (129-TGKIGGLIG-137) in the X-ray structure were homology modeled using the X-ray structure of the α-HL heptamer (PDB ID 3ANZ)[45] as target. Molecular docking calculations were also performed for Sortase A using the X-ray structure solved at 2.8 Å from the sequence of *S. aureus* (PDB ID 1T2W) (DOI 10.1074/JBC.M401374200). All the structural modeling was performed with the SWISS-MODEL software[46].

Molecular docking calculations were performed using the Autodock 4.2[47-49] and AutoGrid4[50] software combined with the AutoDock Tools[51]. Partial charges for the receptor atoms were assigned according to AMBER86 force field parameters[52] while ligand charges were calculated with the Gasteiger method[53]. Dihedral angles were treated as fully flexible for ligands. Grid resolution and center were adjusted to each one of the systems. Grid maps of 126 x 126 x 126 points, point spacing of 2.5 Å and centered at 31.156 13.82 -31.295 Å were used for α-HL prior to MD simulations. After the MD simulations, grid maps of 122 x 122 x 124 points, point spacing of 2.5 Å and centered at 68.492 44.987 68.103 Å were used. For Sortase A, grid maps with dimensions of 126 x 126 x 126 Å and point spacing of 1.4 Å were centered at 34.798 -14.795 3.594 Å. The Lamarckian genetic algorithm was used with the following parameters: 150 random individuals in an initial population, a maximum number of 2500000 energy evaluations, a maximum number of 27000 generations with mutation and crossover rates of 0.02 and 0.08, respectively. An optional elitism parameter equal to 1 was applied, determining the number of top individuals that will survive into the next generation. A maximum of 300 iterations per local search was allowed. The probability of performing a local search on an individual was 0.06 where the maximum

number of consecutive successes or failures before doubling or halving the search step was 4. A total of 100 LGA runs were performed. After the conformational search, docked conformations were sorted in order of increasing energy. The coordinates of the lowest energy conformation were clustered based a root-mean-squared-deviation of 2.0 Å[47-49]. A more detailed description of the methodology employed has been previously presented[54,55].

MD simulations were performed for the free and Myr-bound α-HL receptor. Optimized atomic coordinates and atomic parameters for Myr were obtained from the Automated Topology Builder Repository version 2.2[56]. The GROMOS force field parameter set 54A7 was used in the MD simulations[57]. The simulations were performed in explicit solvent using Single Point Charge (SPC) model[58] in a cubic box of 10.0 x 10.0 x 10.0 nm3. Periodic boundary conditions were applied in all directions. The system was neutralized with 3 Cl- counter ions described by the GROMOS parameter set 53A6[59,60]. The systems were first energy-minimized for 5000 steps. Under NPT conditions, a time step of 0.001 ps was applied during the equilibration and production phases. Center of mass motion was removed at every 5 steps. The temperature of 298 K was maintained by using the Berendsen thermostat[61], coupling separately the temperatures of the protein and the solvent via a time constant of 0.2 ps for each. The pressure was maintained by weakly coupling the particle coordinates and box dimension to an isotropic pressure bath at 1.0 bar. The relaxation time was 0.1 ps and a compressibility of 4.5 × 10−5 (kJ mol−1 nm−3)−1 as appropriate for water[61]. The generalized reaction field [19] was applied to treat long-range electrostatic interactions with a dielectric constant of 66[62]. A cutoff of 1.4 nm was used for both van de Waals and long-range interactions. The pair list for short-range non-bonded and long-range

electrostatic interactions was updated with a frequency of 10 fs for all simulations. Configurations of the trajectory were recorded every 0.5 ps.

All MD simulations and analyses were performed with GROMACS v.4.6.7[63]. Coordinates and trajectories were visualized with the software VMD version 1.9.1[64].

Statistical analysis. Biological assays were carried out at least in triplicate. Data were analyzed by the Student t-test in relation to the untreated samples and $p \leq 0.01$ was considered to be significant. Survival analysis and statistical significance were determined using the log-rank test and the Kaplan–Meier survival curves (Graphpad Prism 6.0).

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Author contributions: L.N.S., A.J.M. and D.S.T. conceived and designed the research; L.N.S. and D.S.T. performed all biological experiments with exception of qRT-PCR

analysis. M.S.B conducted the qRT-PCR experiments, under supervision of H.I. G.C.A.H. and T.A.S. carried out the *in silico* docking analysis. L.N.S., A.J.M, D.S.T., G.C.A.H. and T.A.S wrote the manuscript and the other authors revised the paper critically for important intellectual content.

Supplementary Information

Myricetin protects *Galleria mellonella* from *Staphylococcus aureus* infection via inhibition of the several virulence factors

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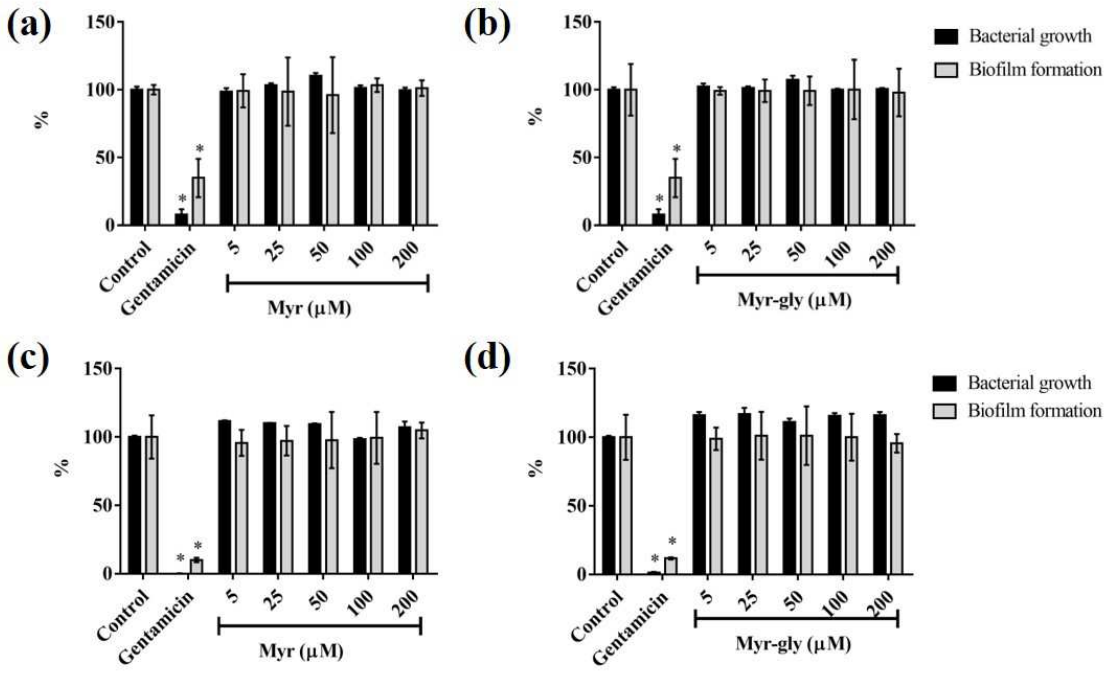
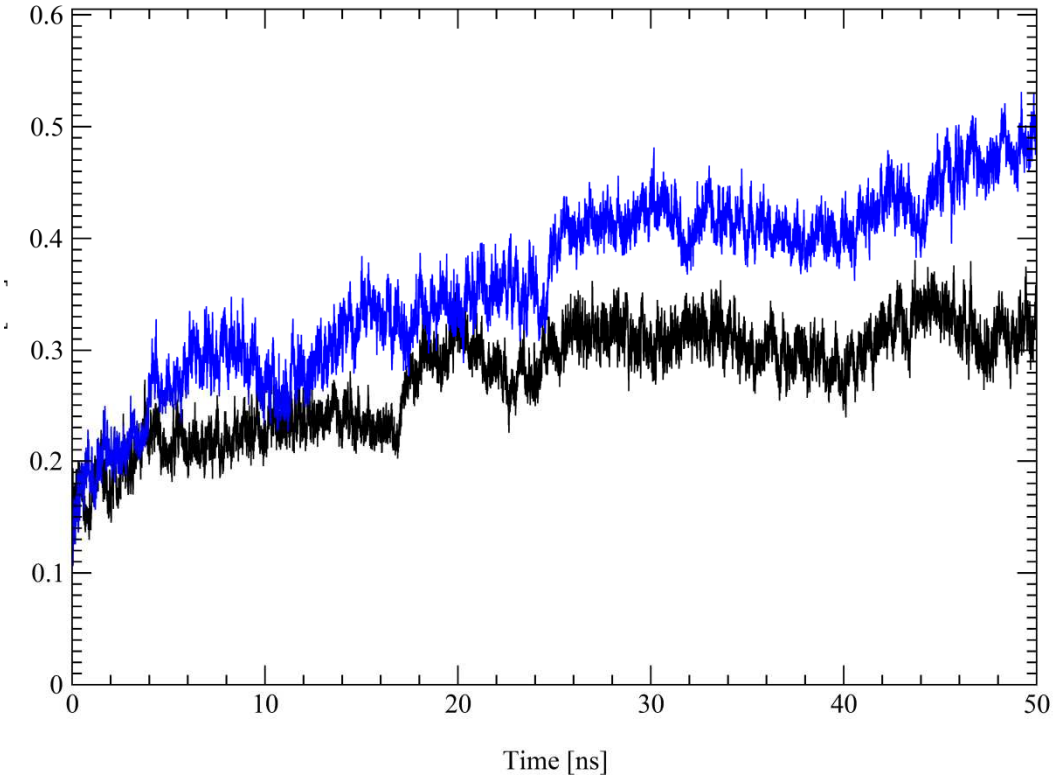
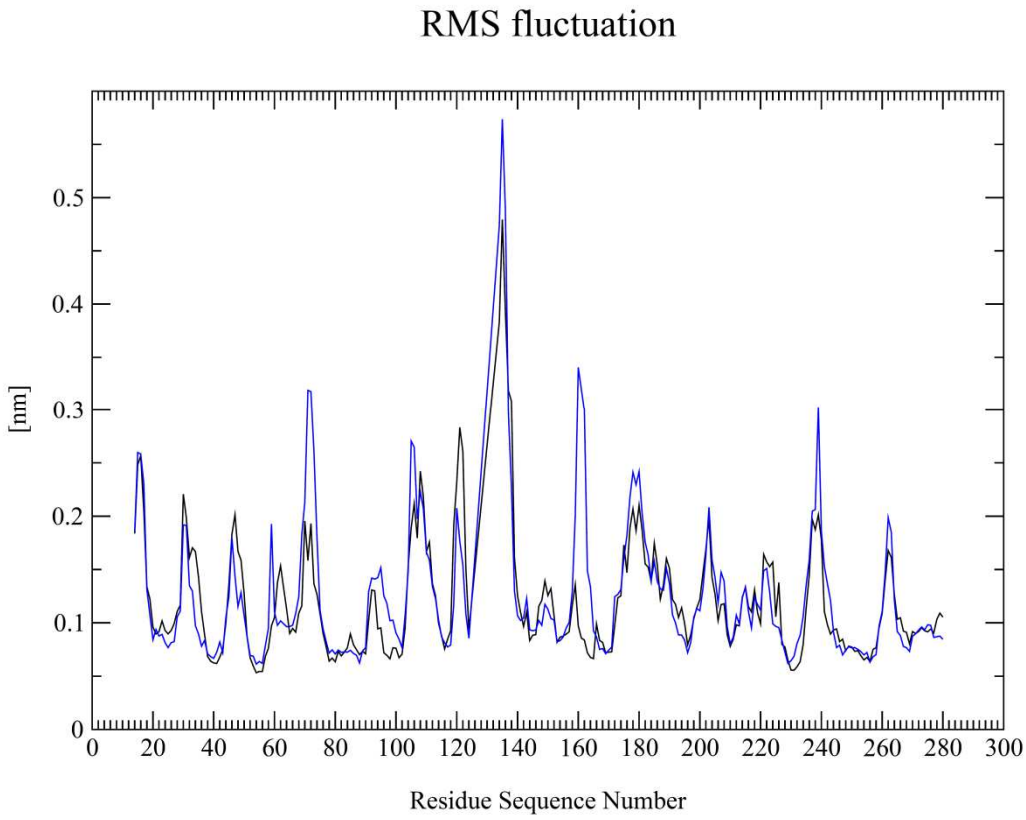


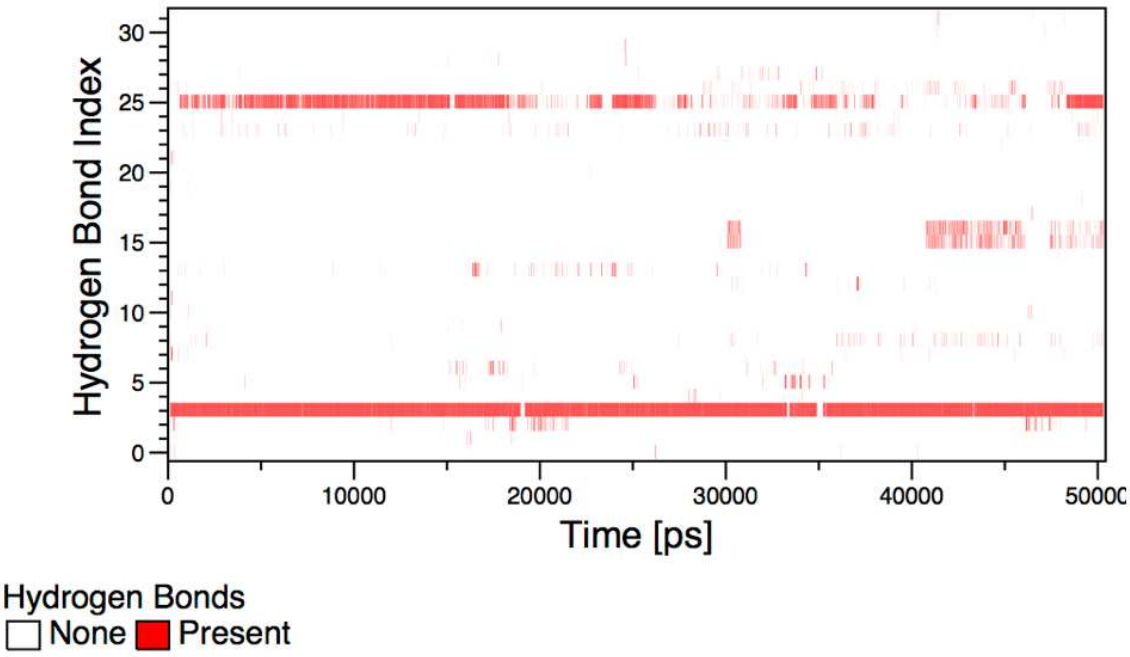
Supplementary Figure S1 Dose-response curve of Myr and Myr-gly tested against *K. pneumoniae* and *P. aeruginosa* biofilm formation and bacterial growth. Biofilms of *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 were quantified by the crystal violet staining method and bacterial growth was determined by measuring optical density at 600 nm. * represent statistically significant differences (*p*-value <0.01).



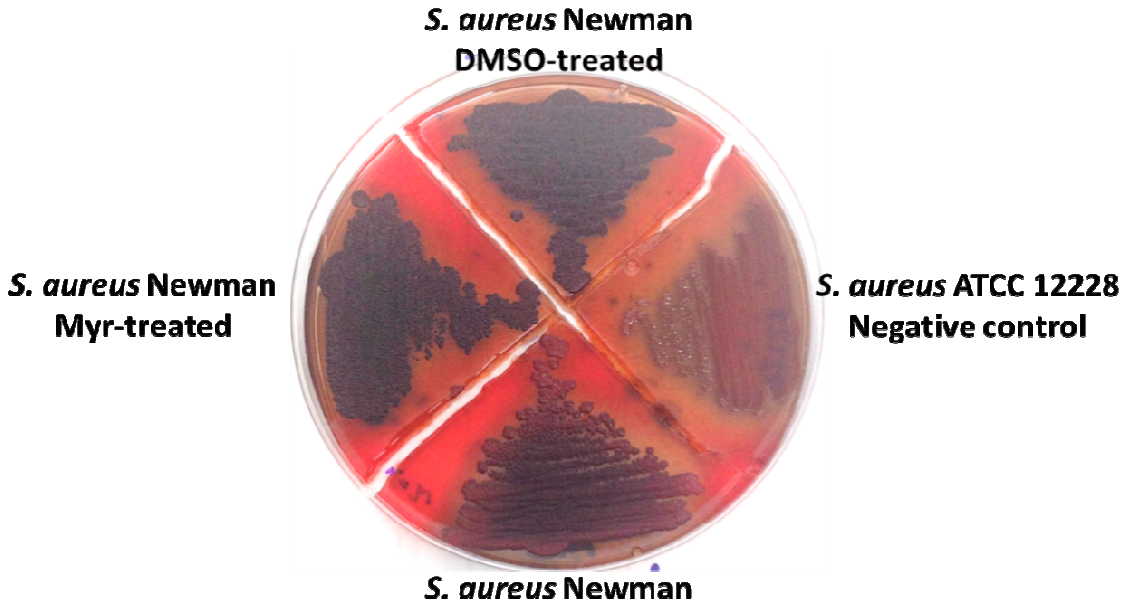
Supplementary Figure S2 Root-mean-square deviation (RMSD) of Cα atoms of free (black line) and Myr-bound (blue-line) α-HL from the X-ray structure (4YHD) as function of time.Rotational and translational fitting of pairs of structures was applied using Cα from residues 15-250 atoms.



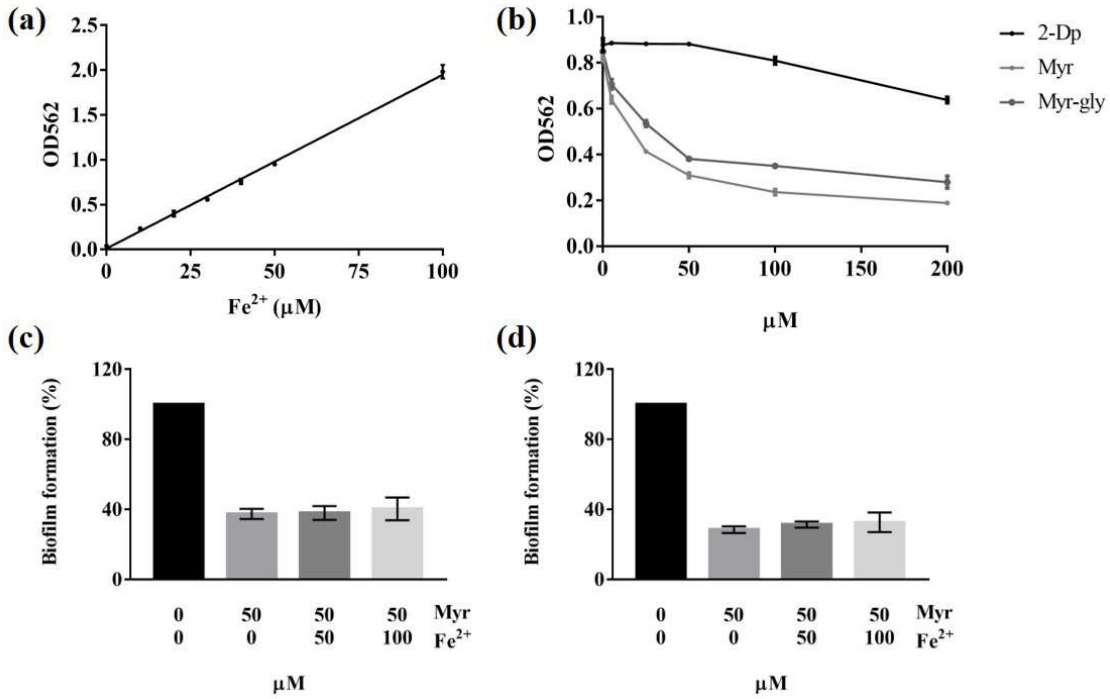
Supplementary Figure S3 Root-mean-square atom-positional fluctuations (RMSF) of Cα atoms of free (black line) and Myr-bound (blue-line) α-HL from the X-ray structure (4YHD) as function of residue sequence number, calculated for the final 2 ns of the two MD trajectories.



Supplementary Figure S4 Hydrogen bond occurrence map for interactions between Myr and α-HL obtained from explicit solvent MD simulations of the complex. The hydrogen bond index correspond to hydrogen bonds between hydroxyl groups in the ligand and i. carbonyl group of K50 (index 3), ii. hydroxyl group of Y118 (indexes 25-26) and iii. amino group of I5 (indexes 16-17).



Supplementary Figure S5 Slime production of *S. aureus* treated with Myr. Treated cells and control strain were plated on Congo Red Agar in order to detect a modulation on the polysaccharide intercellular adhesin (PIA) production.



Supplementary Figure S6 Iron-chelating assessment of Myr and Myr-gly and its influence on *S. aureus* biofilm formation. (a) The standard curve established to determine the Fe^{2+} concentration in a ferrozine complex; (b) Ferrozine-Fe^{2+} complex quantified in the presence of increasing concentrations of Myr, Myr-gly and positive-chelator 2,2-bipyridyl; (c-d) Biofilm formation by *S. aureus* Newman and ATCC 6538 strains, respectively, in the presence of Myr and Fe^{2+} supplementation.

SILVA LN, TRENTIN DS, SILVA DB, LOPES NP, SILVA MV, MACEDO AJ. **PEPTIDES FROM *Harpochilus neesianus* Mart. ex Nees IMPAIR *Staphylococcus epidermidis* BIOFILM FORMATION.**

Resultados preliminares

Peptides from *Harpochilus neesianus* Mart. ex Nees impair *Staphylococus epidermidis* biofilm formation

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Abstract

Most of bacterial infections are related to multicellular communities known as biofilms which demonstrate a 10 to 1000-fold increased resistance profile to conventional antibacterials. Due to the growing emergence of resistant strains to available antimicrobials, there is a great interest in the discovery of agents capable of controlling infections by attenuating bacterial virulence. In this work, two peptides isolated from *Harpochilus neesianus* Mart. ex Nees leaves were shown to be capable of interfering with the adherence and biofilm formation by *Staphylococcus epidermidis*, without antibacterial effect. These peptides are being identified in order to correlate their chemical structures with the biological activities evaluated by crystal violet and turbidimetric assays and by scanning electron microscopy. Herein we show that peptides might act as important agents by reducing bacterial virulence through inhibition of biofilm formation, contributing to development of antibiofilm prototypes.

Introduction

Biofilms can be defined as communities of microbial cells enclosed in self-produced matrix of extracellular polymeric substances required for irreversible cellular attachment to surfaces. Bacteria growing in biofilm form contribute to chronicity of persistent infections such as those associated with implanted medical devices (DONLAN, 2001). This lifestyle allows pathogens to escape from host immune defenses and resist to antibacterial treatments, which is correlated with their resilience in most medical settings (HOIBY *et al.*, 2010). Devices that are prone to biofilm formation include central venous catheters, contact lenses, endotracheal tubes, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, replacement joints and urinary catheters (DONLAN, 2001; DEL POZO e PATEL, 2007).

Staphylococcus epidermidis is a common colonizing harmless bacterium present in human skin. Nowadays, this bacterium is considered an important opportunistic pathogen. The emergence of *S. epidermidis* infections is closely linked to its capacity to adhere on indwelling medical device surfaces during device insertion, forming multilayered biofilm agglomerations (OTTO, 2009). Furthermore, *S. epidermidis* produces a series of protective surface polymers and exoenzymes (OTTO, 2014). The difficulty of effectively treating biofilm infections and the increasing resistance of strains to traditional treatments cause pressure to find novel antimicrobials with novel mechanisms of action.

In recent years, several new antibiofilm approaches have been proposed, including the application of novel natural, synthetic or bioengineered agents as alternatives to classical antibiotic treatment (CHEN *et al.*, 2013). New trends point peptides as a class of compounds that can be rapid-acting and potent, and possess an unusually broad spectrum of activity (HANCOCK e SAHL, 2006). Natural peptides are biochemically

similar wherein the majority are cationic with a net charge of +2 to +9 (due to the presence of several Arg or Lys residues), short with range size from 12 to 50 amino acids and around 50% hydrophobic amino acids, presenting diverse amino acid sequences and structures (HANCOCK e SAHL, 2006). The anti-biofilm peptides are a distinct group of the antimicrobial/host defense peptides with ability to prevent biofilm formation or to eradicate established biofilms, by different mechanisms of action. These include: (1) inhibition of microbial cell adhesion to the surface; (2) up-regulation of genes related to motility; (3) down-regulation of extracellular matrix synthesis; and (4) direct bacterial killing (DI LUCA *et al.*, 2015).

In previous screening studies performed with Caatinga (a semiarid biome inserted in Brazil territory) plants, various extracts displayed antibiofilm activity against *S. epidermidis* (TRENTIN D *et al.*, 2011; SILVA *et al.*, 2015; TRENTIN *et al.*, 2015). The aqueous extract obtained from the leaves of *Harpochilus neeesianus* Mart. ex Nees inhibited *S. epidermidis* biofilm formation on polystyrene, without interfering with bacterial growth and with limited cytotoxicity *in vitro*. Scanning electron microscopy (SEM) images of surfaces demonstrated the high capacity of this extract in preventing bacterial adhesion (SILVA *et al.*, 2015). Since there is absolutely no report in the literature about this plant genus and few studies about bioactive peptides from plants, the aim of this study is to elucidate the compounds responsible for the inhibition of biofilm development by *S. epidermidis*.

Materials and methods

Plant material and extracts

Plant samples were collected in Parque Nacional do Catimbau (PARNA do Catimbau), Pernambuco, Brazil, in 2013. Specimens were identified at the herbarium of the Instituto Agronômico de Pernambuco (IPA), under authorization of the responsible authority Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) using the license SISBIO 16.806. Aqueous extracts of *H. neesianus* leaves were obtained according to TRENTIN D *et al.* (2011) and the powder was stored at -20°C.

Bacterial strain and culture conditions

Staphylococcus epidermidis ATCC 35984 were grown in Mueller Hinton (MH) agar (Oxoid Ltd., England) overnight, at 37°C, and a bacterial suspension in 0.9% sterile saline, corresponding to 3 x 10^8 CFU/ml, was used in the assays.

Antibiofilm activity and bacterial growth assays

The antibiofilm activity assay was performed as established by TRENTIN D *et al.* (2011), employing the crystal violet technique. The bacterial growth was evaluated by the difference between initial (t = 0) and final (t = 24 h) absorbance values at 600 nm in 96-well microtiter plates (Costar 3599, Corning, Inc., USA). In the control sample (untreated), the extracts or fractions were replaced by water.

Proteinase K test

In order to figure out whether antibiofilm activity is the cause of peptides (or proteins) in the crude extract (Hnee), Hnee was treated with Proteinase K. The proteinase K 100 μg/mL(Sigma-Aldrich, St. Louis, MO) and PMSF (5mM) was added into 600 of Hnee

extract (1 mg/mL protein) . The treatment reaction was performed at 37°C overnight and then applied on antibiofilm test.

Peptides content

Protein content of samples was determined by the Coomassie dye binding method (BRADFORD, 1976).

Bioguided Peptides Purification

The liofilized aqueous extract of leaves of *H. neesianus* (Hnee) was extracted with methanol and the active insoluble fraction (Nmet) was used for further column chromatography. The fraction Nmet was suspended in 2 ml of milliQ water and applied onto a packed Sephadex G-50 Fine (GE Healthcare Life Sciences). Elution was performed by using milliQ water and 5mL-fractions were collected, liofilized and tested for their antibacterial and antibiofilm activities. The active fractions (Peps) were pooled according to detection by Coomassie, and the purification of the peptides was performed by RP-HPLC on a Waters Alliance apparatus with Millennium software (Millford, MA, USA). Peps (80 l) was injected into a semi-preparative C18 column (Symmetry 300; 5 m; Spherical 300 Å; 150 × 4.6 mm, Waters, Guyancourt, France) equilibrated in 0% solvent C (0.045% TFA in H2O) and 50% solvent D (80% methanol, 20% H2O, 0.05% TFA). The elution was performed with a linear gradient from 30% to 100% solvent D in 30 min. The flow rate was adjusted to 0.8 ml min-1. The eluted peaks were detected by spectrophotometry measuring the absorbance between 220 and 270 nm with a photo diode array detector (PDA 996; Waters) and collected manually. Each fraction was sequentially filtered and screened for antibiofilm activity by using cristal violet assay.

MALDI ToF mass spectrometry analysis

High-resolution mass spectrometry (MS) analyses were performed using an UltrafleXtreme MALDI-TOF/TOF equipment (Bruker Daltonics, Bremen, Germany). The ions were generated by irradiation with a nitrogen laser (337 nm) and accelerated at 20 kV. For MS analyses, the experimental conditions were: pulsed ion extraction of 100 ns, laser frequency of 1000 Hz, reflectron mode, positive ion mode, and 600 laser shots were averaged to record a mass spectrum. In addition, the selected ions were accelerated to 19 kV in the LIFT cell for MS/MS analyses. The matrix of choice was DHB (2,5-dihydroxybenzoic acid) at 20 mg/mL (in 30% acetonitrile [ACN] and 70% H2O with 0.1% trifluoroacetic acid). All samples were suspended in ACN:H2O (3:7) and mixed with DHB containing 0.1 M solution of NaCl. These mixtures (1 µL) were spotted onto a ground stainless steel MALDI target.

Statistical analysis

Experiments were carried out in triplicate and data are presented as percentage mean ± standard deviation. Differences between groups were evaluated by Student's t-test (p value ≤ 0.05).

Results and discussion

Plants are constantly exposed to attack by a large range of pathogens. Under stress conditions, plants produce a large number of secondary metabolities, including antimicrobial peptides (AMPs) that act as the first line of defense made by innate defense. This report presents the purification and partial characterization of two active peptides derived from *H. neesianus* leaves which impair *S. epidermidis* biofilm formation. *H. neesianus* Nmet fraction was first characterized by MALDI-TOF analyses which indicated the presence of a mixture of polyphenolics with low molecular weight (Figure

1A) and compounds with molecular weight ranging from 4-10 KDa (Figure 1B). In an attempt to gain information about the active compounds, we tested the effect of the treatment of the *H. neesianus* Nmet fraction with proteolytic enzyme on antibiofilm activity. The fraction was sensitive to heating and proteinase K treatments, reinforcing thus the hypothesis of the proteinaceous nature of the active compounds (Figure 2).

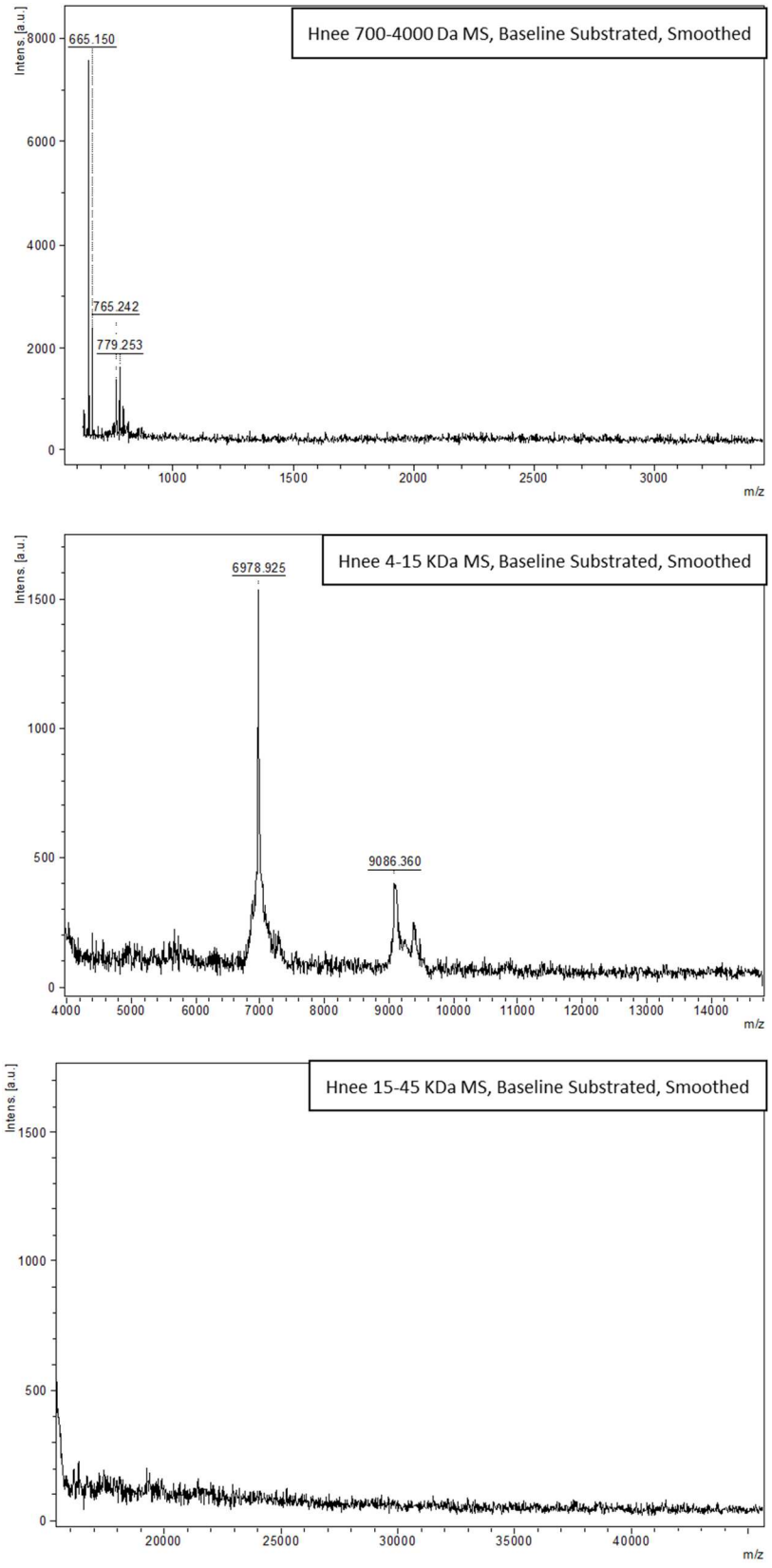

Figure 1. MALDI-MS spectra of *Harpochilus neesianus* leaves extract. The analysis was performed in different molecular weight ranges: A. From 700 - 4000 Da; B From 4 - 15 KDa and C. from 15-45 KDa.

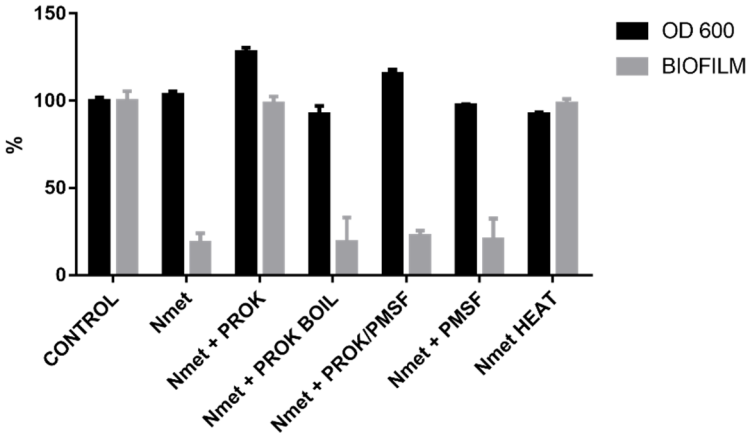

Figure 2. Effect of treatment of Nmet fraction (400 µg/ml) with proteolytic enzyme followed by *S. epidermidis* biofilm formation and bacterial growth. (PROK – proteinase K; BOIL – boiled; PMSF - phenylmethylsulfonyl fluoride; HEAT – heated).

Further we applied Nmet fraction onto a size exclusion chromatography column packed with G-50 in order to separate by molecular size the fractions with higher antibiofilm activity, consequently separating the low molecular weight compounds from proteinaceous compounds. Using this approach, we obtained a proteinaceious-enriched fraction (Peps) free from low molecular weight polyphenols (Fig. 3). Peps fraction was able to inhibit *S. epidermidis* at very low concentrations (Fig. 3, insert), without inhibiting bacterial growth (data no show).

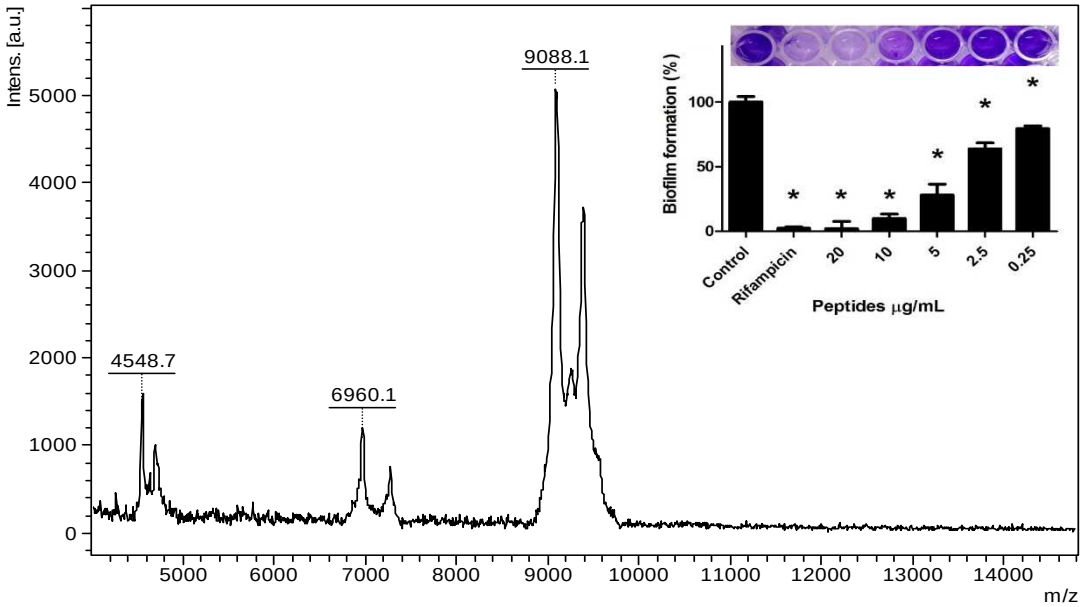

Figure 3. MALDI-MS spectra of Peps and its activity on biofilm formation of *S. epidermidis*.

The semi-preparative and preparative liquid cromatography (LC) are well described methods to purify small molecules due to the advantage of high speed, high sensitivity and good reproducibility to separate fractions. Then, we used semi-preparative LC as strategy to isolate the peptides. As shown in the Fig. 4A and B, the Peps fraction was separated in thirteen peaks, in which peak numbers 4, 8, 10 and 12 presented slightly higher absorption at 270 nm than the other nine peaks. All peaks were individually collected and tested against *S. epidermidis* biofilm formation. Among all peaks, only peaks number 1 and 3 showed antibiofilm activity (Figure 4C), preventing at least 90% *S. epidermidis* biofilm formation. These peptides are being identified in order to relate its chemical structure with the biological activity.

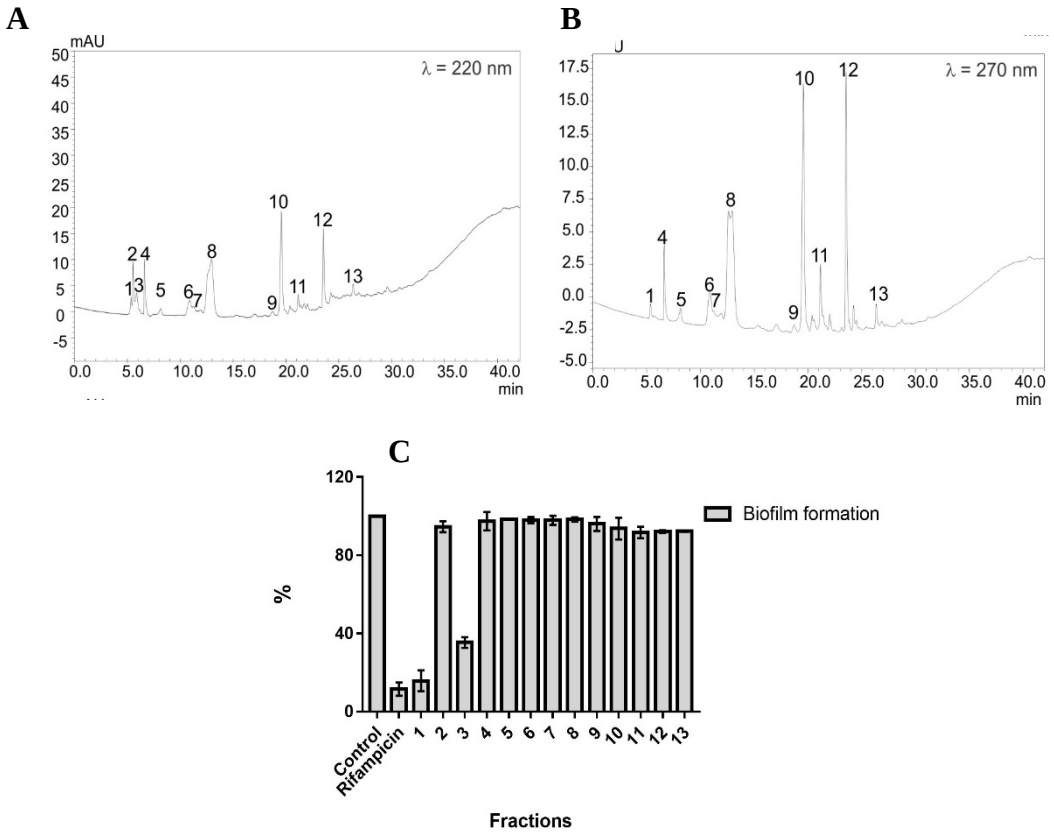

Fig. 4. Separation of Peps fraction by RP-HPLC. (A)Absorption at 220 nm; (B) absorption at 270 nm and (C) activity on biofilm formation of *S. epidermidis.*

The AMPs have diverse structures and functions, being extensively studied against planktonic bacteria but a lower number of works is dedicate to investigate their action against biofilm development. Since biofilm resistance mechanisms to antibacterials is mainly atributted to the slow growth rate and metabolic activity, their use to prevent biofilm formation could be potentially an attractive therapeutic strategy. Also, the peptide structures are relatively easy to modify and to immobilize on surfaces. In this sense, it was demonstrated that the AMP nisin hinders *S. aureus* biofilm formation without inhibiting the growth when immobilized in multi-walled carbon nanotubes (QI *et al.*, 2011). Another example is the cathelicidin-2 derived peptide, which prevents *S. epidermidis* biofilm formation at a concentration four times below the minimal inhibitory concentration (MIC) and impairs development of mature biofilms (MOLHOEK *et al.*, 2011).

In summary, this is the first report of antibiofilm peptides from *H. neesianus* species against the human pathogen, *S. epidermidis*.

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4.0 DISCUSSÃO GERAL

A presente dissertação teve como tema a busca por agentes atenuadores da virulência, abordando um conceito recente que é a terapia antivirulência, o qual pode oferecer oportunidades promissoras para controlar a patogênese bacteriana. Esta nova estratégia consiste em inibir mecanismos específicos que promovem a infecção, sejam eles essenciais para a sua persistência bacteriana e/ou que acarretem desenvolvimento de danos teciduais ao hospedeiro. Neste sentindo, alguns fatores de virulência considerados mais promissores foram revisados e indicados como potenciais alvos para fármacos e novas intervenções farmaco-terapêuticas. Há uma necessidade urgente de novos e melhores agentes anti-infecciosos e, de fato, a taxa atual de introdução de novos agentes antimicrobianos pode não ser suficientes para lidar com a emergência de bactérias resistentes aos agentes já disponíveis (HOGBERG *et al.*, 2010).

Neste contexto, os produtos naturais desempenham um papel dominante na descoberta de protótipos para o desenvolvimento de novos fármacos. Historicamente, os produtos naturais provenientes de microrganismos, plantas e animais foram a fonte de praticamente todos os agentes farmacológicos e, mais recentemente, têm continuado a entrar em ensaios clínicos ou serviram de protótipo para o desenvolvimento de fármacos, com destaque para a atividade anticâncer e antimicrobiana (NEWMAN e CRAGG, 2012; HARVEY *et al.*, 2015). Além disso, metabólitos secundários de plantas mostram uma diversidade estrutural única, que muitas vezes não pode ser facilmente sintetizada *in vitro*, mas que complementa compostos e bibliotecas sintetizadas quimicamente em programas de descoberta de fármacos.

Como apontado na revisão presente no capítulo 1 desta dissertação, a qual abordou 371 estruturas químicas provenientes de plantas com ação sobre fatores de virulência bacterianos, os fenólicos destacaram-se como a classe qúimica de produtos derivados de

plantas com o maior número de compostos bioativos descritos para diferentes fatores de virulência. Estes compostos possuem como alvo diversos componentes bacterianos, afetando diferentes funções da célula. Uma das propriedades de destaque dos compostos fenólicos é a capacidade de formar complexos com proteínas, particularmente através de ligações de hidrogênio. Esta característica está intimamente relacionada à capacidade que os fenólicos possuem de inativar adesinas microbianas, enzimas, toxinas, proteínas de transporte, bem como muitas outras proteínas importantes para a célula bacteriana. Além disso, fenólicos são conhecidos por serem agentes quelantes de metais, tais como o ferro, o qual é um íon essencial para o crescimento de diversas bactérias de tal modo que muitas espécies produzem compostos cuja função é captar ferro do ambiente ou mesmo das proteínas humanas de transporte de ferro. Não menos importante, outros mecanismos envolvidos no controle de micro-organismos também já foram descritos para os compostos fenólicos, como: inibição da síntese de ácidos nucleicos, inibição da função da membrana citoplasmática e inibição do metabolismo energético (CUSHNIE e LAMB, 2005; DAGLIA, 2012).

Duas abordagens foram seguidas para o rastreio de fitocompostos com o propósito de investigar possíveis atividades antivirulência: i) triagem através de uma classe selecionada de compostos como alcalóides, flavonóides, terpenoídes, etc., ii) triagem bioguiada de compostos partindo de um extrato aquoso previamente identificado pelo nosso grupo de pesquisa como bioativo.

A partir de uma biblioteca de compostos disponível no Laboratório de Biofilme e Diversidade Microbiana, escolheu-se rastrear o efeito dos flavonóides miricetina e de seu glicosídeo (miricitrina) contra diferentes fatores de virulência utilizando uma bactéria reconhecidamente produtora de um arsenal destes fatores, *S. aureus*. Neste estudo, investigou-se as vias de ação destes flavonoides através do emprego de ensaios

fenotípicos, ensaios genotípicos e simulações de modelagem molecular, levando em consideração a relação estrutura-atividade dos compostos. A importância deste estudo é a comprovação de que a miricetina pode: (i) reduzir a formação de biofilme, (ii) bloquear os danos causados pela hemolisina às células humanas, e (iii) inibir a produção do pigmento estafiloxantina, culminando com a redução da patogenicidade *in vivo*. Além disso, este estudo proporciona uma visão sobre o mecanismo molecular dos efeitos inibitórios antivirulência da miricetina em *S. aureus* através da modulação das propriedades da superfície, homeostase de ferro e da ligação competitiva a proteínas importantes na patogênese estafilococal. Publicações recentes também demonstraram que a miricetina é capaz de atenuar a atividade hemolítica da hemolisina de *Listeria monocytogenes* através da ligação direta na proteína (WANG *et al.*, 2015) e também inibir a formação de biofilme de *E. coli* e *S. aureus* (ARITA-MORIOKA *et al.*, 2015), suportando a ideia de que a atividade desta molécula pode não se restringir a espécies de *S. aureus* . Coletivamente, com base nos resultados deste estudo, é razoável inferir que a miricetina pode ser útil para o tratamento de infecções por *S. aureus*, atuando sobre múltiplos alvos de virulência, podendo, potencialmente, conferir efeito sinérgico quando combinada com antibacterianos.

Baseado na segunda estratégia utilizada nesta dissertação, deu-se continuidade ao trabalho de SILVA *et al.* (2015) que indicou o potencial de diversos extratos de plantas da Caatinga contra o patógeno *S . epidermidis*. Como o extrato de *H. neesianus* apresentou elevada atividade antibiofilme e baixa citotoxicidade contra células mamíferas (linhagem Vero), prosseguiu-se o fracionamento bioguidado deste extrato. Uma vez que não existe nenhum relato na literatura sobre os possíveis compostos presentes nesta espécie nem tampouco neste gênero e como a purificação de compostos é uma "ciência" dependente da amostra, da extração inicial e da complexidade dos constituintes químicos, foram

usadas diversas estratégias para o isolamento e a identificação dos compostos. Após a realização de ensaios biológicos posteriores ao tratamento da amostra ativa com protease e com calor, juntamente com as análises por MALDI-TOF, conclui-se que os compostos ativos eram de origem proteica. Primeiramente, os compostos foram separados por tamanho, utilizando a resina de Sephadex G-50 e, logo depois isolados através de uma fase estacionária hidrofóbica (RP-HPLC), sempre acompanhados de atividade biológica. RP-HPLC é uma técnica reconhecida por ser capaz de separar polipeptídeos de sequências próximas, não apenas de tamanho pequeno, mas mesmo para proteínas maiores (AGUILAR, 2004). Assim que as sequências estiverem definidas, será possível sugerir homologias e potenciais vias de ação responsáveis pela inibição da adesão de *S. epidermidis*. É importante destacar que esta investigação é o primeiro estudo fitoquímico da planta endêmica da Caatinga *H. neesianus*.

A terapia antivirulência, capaz de desarmar patógenos em vez de agir sobre sua viabilidade, está emergindo como uma nova estratégia para o controle de doenças infecciosas. Esta estratégia têm atraído o interesse da indústia, haja vista o sucesso no caso dos anticorpos ou compostos neutralizadores de toxinas, onde pelo menos seis candidatos já estão em ensaios clínicos (CEGELSKI *et al.*, 2008). Os sucessos iniciais desses agentes antitoxinas fornecem evidência empírica e apoiam a investigação de novos compostos antivirulência. Portanto, os compostos antivirulência citados neste trabalho podem levar, futuramente, ao desenvolvimento agentes antivirulência que poderiam ser utilizados em monoterapia ou como adjuvantes para a terapia convencional com antibacterianos ou ainda para aplicações em biomateriais.

5.0 CONCLUSÕES E PERSPECTIVA

Em resumo, os dados obtidos nesta dissertação:

- Mostram a importâncias dos produtos naturais derivados de plantas como agentes atenuadores da virulência bacteriana e como potenciais agentes sinérgicos aos antimicrobianos, com especial atenção à classe química dos flavonóides.

- Relatam a atividade do flavonóide miricetina contra diferentes fatores de virulência de *S. aureus* independemente de efeitos no crescimento, sendo ainda ativo contra as bactérias quiescentes dentro de biofilmes.

- Demonstram que agentes antibiofilme, como a miricetina, possuem potencial de recobrimento de superfícies afim de gerar materiais antiaderentes para bactérias.

- Evidenciam que agentes que atenuam fatores de virulência, como a miricetina, são capazes de controlar processos infecciosos, como demostrado pela redução da patogenicidade de *S. aureus* utilizando o modelo *in vivo* de *G. mellonella*.

- Descrevem pela primeira vez a presença de peptídeos com atividade antibiofilme das folhas de *Harpochilus neesianus.*

Como perspectivas deste trabalho, temos:

- Investigar a capacidade da miricetina e vancomicina em sinergismo no tratamento da infecção por *S. aureus* no modelo de *G. mellonella* (capítulo 2).

- Concluir a parte de elucidação estrutural dos peptídeos de *Harpochilus neesianus* (capítulo 3).

- Estudar as vias de ação pelas quais os peptídeos inibem a adesão e formação de biofilme de *S. epidermidis* (capítulo 3).

- Desenvolver superfícies funcionalizadas de silicone com os peptídeos imobilizados, uma vez que este é um dos biomateriais frequentemente utilizados na fabricação de cateteres e outros dispositivos médicos.

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7.0 Anexo

Decision Letter (cr2016001847. R1)
From: jamisonoffice@ chemrev.acs.org
To: alexandre.macedo@ufrgs.br
Subject: Decision on Manuscript ID cr2016001847. R1
Body: 16Jun2016

Dr. Alexandre Macedo
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Journal: Chemical Reviews
Manuscript ID: cr2016001847. R1
Title: "Plant Natural Products Targeting Bacterial Virulence Factors"
Author(s): Silva, Laura; Zimmer, Karine; Macedo, Alexandre; Trentin, Danielle

Dear Dr. Macedo:
We are pleased to inform you that your manuscript has been accepted for publication and sent to the Production Department. This is clearly an outstanding manuscript and we are pleased to have the opportunity to publish it. Publication has been scheduled for an upcoming regular issue of Chemical Reviews.

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Sincerely,
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- **FORMAÇÃO:**

2014-2016 Mestrado em Biologia Celular e Molecular

Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Título: Fitocompostos capazes de inibir a adesão e outros fatores de virulência bacterianos

Orientador: Alexandre José Macedo.

Coorientador: Danielle da Silva Trentin.

Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.

2008 – 2013 Graduação em Ciências Biológicas.

Universidade Federal do Rio Grande do Sul, UFRGS, Brasil com período sanduíche em Faculdade de Engenharia da Universidade do Porto (Orientador: Filipe Mergulhão).

Título: Triagem etno-dirigida de plantas medicinais da caatinga brasileira contra biofilmes de bactérias patogênicas.

Orientador: Alexandre José Macedo.

- **ESTÁGIOS:**

Universidade do Porto, Portugal

2013 - 2013

Vínculo: Estágio, Enquadramento Funcional: Bolsista de Graduação Sanduíche - CNPQ, Carga horária: 35

Estágio no Laboratório de Engenharia de Processos Ambiente e Energia (LEPAE) na Faculdade de Engenharia da Universidade do Porto sob a orientação do professor Dr. Filipe Mergulhão

Laboratório Aqualab

2013 - 2013

Vínculo: Estágio, Enquadramento Funcional: Estágio Obrigatório, Carga horária: 20

Estágio no Laboratório Acqualab (Laboratório de Análises Médicas e Ambientais), sob a orientação da bióloga Ana Paula Costantin.

Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2011 - 2013

Vínculo: Estágio, Enquadramento Funcional: Bolsista de Iniciação Científica CNPQ, Carga horária: 20, Regime: Dedicação exclusiva.

Estágio no Laboratório de Biofilmes e Diversidade Microbiana da Universidade Federal do Rio Grande do Sul sob a orientação do professor Dr. Alexandre Macedo

2009 - 2010

Vínculo: Estágio, Enquadramento Funcional: Bolsista de Iniciação Científica CNPQ, Carga horária: 20, Regime: Dedicação exclusiva.

Estágio no Laboratório de Proteínas Tóxicas da Universidade Federal do Rio Grande do Sul, sob Orientação da Professora Dra. Célia Carlini

- **PRÊMIOS E DISTINÇÕES**

- **EXPERIÊNCIA PROFISSIONAL OU DIDÁTICA ANTERIOR**

- **ARTIGOS COMPLETOS PUBLICADOS**

(*in press*) **SILVA, LN**; ZIMMER, K. R.; MACEDO, A. J.; TRENTIN, D. S. Plant Natural Products Targeting Bacterial Virulence Factors. **Chemical Reviews**, 2016.

(*in press*) NETO, TDN; LEITE, AJM ANA; FELIPE, KF; ALMEIDA, CG; **SILVA, LN**; ROQUE, AA; BARBOSA, EM; MACEDO, AJ; ALMEIDA, MV; GIORDANI, RB *Activity of pyrrolizidine alkaloids against biofilm formation and *Trichomonas vaginalis*. **Biomedicine & Pharmacotherapy**, 2016.

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- **RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS**

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SILVA, L. N.; TRENTIN, D. S. ; ZIMMER, K. R. ; TRETER, J. ; BRANDELLI, C. L. C. ; FRASSON, A. P. ; TASCA, T. ; SILVA, A. G. ; SILVA, M. V. ; MACEDO, A. J. . Anti-infective effects of Brazilian Caatinga plants against pathogenic bacterial biofilm formation. In: XXII Congreso Latinoamericano de Microbiología, 2014, Cartagena. XXII Congreso Latinoamericano de Microbiología. Cartagena: Legis S.A., 2014. v. 5.

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- **ORGANIZAÇÃO DE EVENTOS**

SILVA, LN; TRENTIN, D. S. ; MACEDO, A. J. . III Workshop Adesão Microbiana e Superfícies. 2015.

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