UNIVERSIDADE ESTADUAL DE LONDRINA

VIVIANE FERREIRA CARDOZO

AVALIAÇÃO DA ATIVIDADE ANTIBACTERIANA DA FRAÇÃO SEMI-PURIFICADA F3 PRODUZIDA POR *PSEUDOMONAS AERUGINOSA* SOBRE AMOSTRAS DE *STAPHYLOCOCCUS AUREUS* METICILINA-RESISTENTE (MRSA)

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Dissertação apresentada ao Programa de Pós-graduação em Microbiologia da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Microbiologia.

Orientador: Prof. Dr. Gerson Nakazato

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Londrina, 27 de março de 2013.

Por plantarem em mim a semente do saber e permitirem que mais esse sonho se realizasse, dedico este trabalho aos meus queridos pais.

“Somos do tamanho dos nossos sonhos”

Fernando Pessoa

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CARDOZO, V.F. **Avaliação da atividade antibacteriana da fração semi-purificada F3 produzida por *Pseudomonas aeruginosa* sobre amostras de *Staphylococcus aureus* meticilina-resistente (MRSA).** 2013. 50 f. Dissertação de Mestrado em Microbiologia, Universidade Estadual de Londrina - UEL, Paraná.

RESUMO

O surgimento de bactérias multirresistentes é um grave problema na saúde pública mundial. *Staphylococcus aureus*, incluindo *S. aureus* meticilina-resistente (MRSA), é um dos mais importantes patógenos humanos associado às infecções hospitalares e comunitárias. O objetivo deste trabalho foi avaliar a atividade antibacteriana da fração F3 (acetato de etila) obtida de *Pseudomonas aeruginosa* contra cepas de MRSA. Trinta cepas de MRSA isoladas no Hospital Universitário de Londrina e três cepas padrão de MRSA foram utilizadas neste estudo. As frações semi-purificadas contendo os compostos extracelulares de *P. aeruginosa* foram obtidas por cromatografia líquida a vácuo. A avaliação da atividade antibacteriana foi realizada através da técnica de disco-difusão em ágar, determinação da concentração inibitória mínima, curva de crescimento-viabilidade e microscopia eletrônica de varredura. A fração F3 (acetato de etila) e sua derivada F3d (diclorometano-acetato de etila) demonstraram atividade antibacteriana frente às cepas de MRSA. A fenazina-1-carboxamida foi identificada e purificada a partir da fração F3d, demonstrando uma discreta atividade antibacteriana contra MRSA, porém quando combinada com nanopartículas de prata produzidas por *Fusarium oxysporum* apresentou efeito sinérgico. Um composto organohalogenado também foi purificado a partir desta fração mostrando forte efeito antibacteriano. Por meio da microscopia eletrônica de varredura, alterações morfológicas na parede celular das cepas de MRSA foram observadas na presença da fração F3d. Estes resultados sugerem que compostos produzidos por *P. aeruginosa* podem se tornar uma boa alternativa no controle e tratamento de infecções causadas por MRSA.

Palavras-chave: Atividade antibacteriana Meticilina-resistente. *Pseudomonas aeruginosa. Staphylococcus aureus.*

CARDOZO, V.F. **EVALUATION OF ANTIBACTERIAL ACTIVITY OF THE F3 SEMI-PURIFIED FRACTION *Pseudomonas aeruginosa*-DERIVED AGAINST METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA) STRAINS.** 2013. 50p. Dissertação de Mestrado em Microbiologia, Universidade Estadual de Londrina - UEL, Paraná.

ABSTRACT

The emergence of multidrug-resistant bacteria is a great problem in world public health. *Staphylococcus aureus,* including methicillin-resistant *S. aureus* (MRSA) strains, is one of the most important human pathogens associated with hospital and community-acquired infections. The aim of this work was to evaluate the antibacterial activity of F3 fraction (ethyl acetate) from *Pseudomonas aeruginosa*-derived against MRSA strains. Thirty MRSA strains isolated from Hospital Universitário de Londrina, and three standard MRSA strains were used in this study. The semi-purified fractions containing extracellular compounds were obtained by vacuum liquid chromatography. Evaluation of antibacterial activity was performed by agar disc-diffusion technique, determination of the minimal inhibitory concentration, curve of growth-viability and scanning electron microscopy. The F3 (ethyl acetate) fraction and F3d-derived (dichloromethane-ethyl acetate) demonstrated antibacterial activity against the MRSA strains. Phenazine-1-carboxamide was identified and purified from the F3d fraction and demonstrated slight antibacterial activity against MRSA, but when combined with silver nanoparticles produced by *Fusarium oxysporum* showed synergic effect. Organohalogen compound was also purified from this fraction showing high antibacterial effect. Morphological alterations on cell wall of the MRSA strains with F3d fraction were showed using scanning electron microscopy. These results suggest that *P. aeruginosa*-produced compounds may be a good alternative control and treatment for MRSA infections.

Keywords: Antibacterial activity. Methicillin-resistant, *Pseudomonas aeruginosa*, *Staphylococcus aureus*.

LISTA DE SIGLAS E ABREVIATURAS

6-APA	6-Aminopenicillanic Acid
APUA	Alliance for the Prudent Use of Antibiotics
CA-MRSA	Community-associated Methicillin-resistant *Staphylococcus aureus*
CDC	Center for Disease Control and Prevention
ECDC	Europe Centre for Disease Prevention and Control
ESBL	Extended-spectrum Beta-lactamase
FDA	Food and Drug Administration
HCA-MRSA	Health-care Associated Methicillin-resistant *Staphylococcus aureus*
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
KPC	*Klebsiela, Enterobacter e Citrobacter* Produtores de Carbapenemases
MIC	Minimum Inhibitory Concentration
MR	Multirresistentes aos antimicrobianos
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
OMS	Organização Mundial de Saúde
PBP	Penicillin-binding Protein
PVL	Panton-Valentine Leukocidin
SCC*mec*	*Staphylococcal Cassette Chromosome mec*
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant *Enterococcus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*

SUMÁRIO

1 INTRODUÇÃO

A intensa pressão de seleção decorrente do uso de antimicrobianos de amplo espectro para o controle das infecções induz à evolução e disseminação de micro-organismos altamente resistentes aos mesmos, os MR. As infecções nosocomiais são reconhecidas como um importante problema, pelas consequências graves no tratamento dos pacientes e pelos custos acrescidos na assistência a estes pacientes. A proporção de infecções associadas aos serviços de saúde causadas por bactérias patogênicas MR tem aumentado significativamente nas últimas décadas, e o controle nos pacientes infectados ainda é um desafio tendo em vista os antimicrobianos disponíveis no mercado.

MRSA é um dos principais agentes causadores de infecções em ambiente hospitalar e ambulatorial; tais como bacteremia, pneumonia, endocardite, artrite séptica, osteomielite e formação de abscessos profundos. As cepas de MRSA podem se disseminar rapidamente, sendo necessário implementar efetivos programas epidêmicos e de controle. Além disso, *S. aureus* é uma das causas mais importante de infecções adquiridas na comunidade que podem resultar em alta morbidade e mortalidade.

Durante anos, a vancomicina tem sido usada como antibiótico de escolha no tratamento de infecções por MRSA, sendo recomendada como tal por diretrizes clínicas. No entanto, o surgimento de VRSA e VISA tem dificultado a terapia antibacteriana.

Com o crescente aumento da resistência bacteriana aos antimicrobianos, torna-se imprescindível a busca por novas drogas, como também o desenvolvimento de novos derivados de compostos já existentes que possam ser usados com maior eficácia. A utilização de vegetais é uma possibilidade promissora pois estes contêm uma grande diversidade química de bioativos, e devido à exploração desses compostos deu-se o desenvolvimento de centenas de drogas farmacêuticas. Novos compostos bioativos produzidos por bactérias também representam uma alternativa no controle e tratamento de infecções bacterianas, principalmente as MR. As substâncias produzidas por bactérias na competição por um nicho podem ser metabólitos secundários com ação antibiótica. Esses metabólitos matam ou inibem o crescimento de outras espécies microbianas, mesmo em pequenas quantidades.

A descoberta de novos antimicrobianos é importante no tratamento e prevenção de infecções bacterianas incluindo as MR, representando uma nova alternativa terapêutica.

2 OBJETIVO

2.1 OBJETIVO GERAL

Avaliar a atividade antibacteriana da fração F3 obtida de uma cepa de *Pseudomonas aeruginosa* sobre cepas de MRSA.

2.2 OBJETIVOS ESPECÍFICOS

- Verificar a sensibilidade das cepas de MRSA para a fração F3.

- Avaliar a dinâmica do efeito da fração F3 sobre MRSA.

- Verificar a existência de sinergismo entre um composto identificado da fração F3 e a prata biológica sobre MRSA.

- Verificar as alterações morfológicas provocadas pela fração F3 em MRSA, por meio da microscopia eletrônica.

3 REVISÃO BIBLIOGRÁFICA

3.1 *STAPHYLOCOCCUS AUREUS*

O gênero *Staphylococcus*, pertencente à família Micrococcaceae, é composto por 38 espécies, sendo que 17 destas podem ser isoladas de amostras biológicas humanas. São cocos Gram-positivos, medindo cerca de 0,5 -1,5 μm de diâmetro, os quais podem apresentar-se isolados, aos pares, em cadeias curtas, ou grupos em forma de cachos irregulares. Os membros dessa espécie são imóveis, não esporulados, anaeróbios facultativos; porém, desenvolvem-se melhor em atmosfera aeróbia. Suas colônias, em meio sólido, são usualmente lisas e algumas convexas com a borda contínua. Algumas colônias podem apresentar pigmentação amarela, ou amarelo-alaranjado, que se torna mais pronunciada após incubação em temperatura ambiente, enquanto outras colônias podem ser esbranquiçadas ou acinzentadas. Além disso, algumas cepas de *S. aureus* cultivadas em ágar sangue podem produzir uma zona difusa de β-hemólise ao redor da colônia, evidenciada após incubação prolongada (BANNERMAN et al., 1995; KLOOS, 1997).

Estafilococos são produtores de catalase, sendo essa prova importante para diferenciá-los dos estreptococos (catalase negativos). Um dos fatores de patogenicidade que diferencia *S. aureus* das demais espécies estafilocócicas é a capacidade de coagular o plasma, por reação direta com o fibrinogênio. Essa reação produz fibrina, que recobre as células bacterianas, permitindo sua rápida aglutinação e resistência aos processos de opsonização e fagocitose. Tal propriedade é conferida tanto pela presença da coagulase livre como pela coagulase ligada, ou fator de agregação na superfície da parede celular (KLOOS, 1997).

Fermentação do manitol, endonuclease termoestável e teste da desoxirribonuclease são provas confirmatórias adicionais utilizadas para a caracterização do patógeno. São micro-organismos que se multiplicam sob condições de alta pressão osmótica e pouca umidade, o que explica, parcialmente, sua sobrevivência nas secreções nasais e na pele (BANNERMAN, 1995).

O gênero *Staphylococcus* é amplamente encontrado na natureza, sendo parte da microbiota de pele e mucosas de animais. Algumas espécies de *Staphylococcus* são frequentemente caracterizados como agentes etiológicos de infecções oportunistas em humanos e outros animais. *S. aureus, S. epidermidis, S. saprophyticus* e *S. haemolyticus* são as espécies mais importantes causadoras de infecções nosocomiais e comunitárias humanas. Entre seus representantes, *S. aureus* é a espécie mais virulenta e o patógeno mais importante, mas a incidência de infecções causadas por estafilococos coagulase-negativos apresentou um aumento em todo o mundo (SAKAI, 2004).

No indivíduo sadio, *S. aureus* é usualmente um comensal das fossas nasais, pele e até do intestino. Por isso, as infecções frequentemente resultam da introdução dessas cepas em locais previamente estéreis, após um trauma, abrasões de pele e mucosas ou durante procedimentos cirúrgicos (CROSSLEY et al., 1997).

S. aureus é bem evidenciado como um importante patógeno humano e está normalmente envolvido em infecções de pele e tecidos moles tais como furúnculos, celulite, foliculite, impetigo, carbúnculos, síndrome da pele escaldada e abscessos. Além disso, ele pode causar algumas infecções graves incluindo pneumonia, bacteremia, endocardite aguda, meningite, osteomielite e síndrome do choque tóxico (SIANGLUM et al., 2011).

Este micro-organismo é a principal causa de infecções bacterianas Gram-positivas, e responsável por um amplo espectro de doenças, variando de infecções mais simples até as mais complexas. Embora as infecções por *S. aureus* sejam historicamente tratáveis com antibióticos comuns, o surgimento de micro-organismos MR tornou-se uma grande preocupação para a saúde pública (TSERING, 2011).

3.2 MRSA

As infecções nosocomiais são uma das mais comuns complicações de hospitalização e levam ao aumento da morbidade e mortalidade. Estas infecções prolongam a hospitalização e tratamento, e estão associadas a custos adicionais. A infecção causada por micro-organismos MR pode também dificultar o tratamento.

Deve-se dar ênfase à prevenção de infecções hospitalares e contenção dos micro-organismos MR, ambos para melhorar a saúde do paciente e otimizar o uso de recursos financeiros cada vez mais limitados (GEFFERS; GASTMEIER, 2011).

As infecções causadas por cepas MR quando comparadas com as suscetíveis da mesma espécie, estão sempre associadas com um longo tempo de internação, um alto custo de tratamento dos pacientes e altas taxas de mortalidade. O insucesso da antibioticoterapia está relacionado a inúmeros fatores, tais como severidade da infecção, o diagnóstico e tratamento tardio, e a baixa efetividade dos antimicrobianos utilizados no tratamento. A presença desses micro-organismos MR em hospitais, onde é elevado o número de pessoas debilitadas, representa um dos maiores problemas de saúde pública, por causarem infecções nosocomiais (BURD et al., 2003).

As organizações internacionais dedicadas à promoção da saúde pública (OMS, CDC, APUA e ECDC) reconhecem a grande importância de promover medidas sanitárias para preservar a eficácia dos antimicrobianos, devido ao crescente desenvolvimento de resistência bacteriana à quase todas as classes de antimicrobianos conhecidos (ASENCIO; CARRANZA; HUERTAS, 2012).

Atualmente, MRSA, *Escherichia coli* sorotipo O157:H7, *Mycobacterium tuberculosis* e *Pseudomonas aeruginosa* são considerados alguns dos mais preocupantes patógenos para a população (HAFIDH et al., 2011).

O CDC define os MR predominantemente como bactérias que são resistentes a uma ou mais classes de agentes antimicrobianos, dentro deste critério incluímos cepas de MRSA, VRE e as produtoras de beta-lactamases e carbapenemases. Programas de prevenção a infecções utilizam várias medidas para controlar e reduzir infecções resistentes aos antibióticos no ambiente hospitalar, incluindo isolamento e precauções de contato, vigilância ativa e restrição ao uso de antibióticos (POGORZELSKA, 2012).

O aumento contínuo de infecções causadas por MR continua a preocupar a saúde mundial. MRSA é atualmente o patógeno MR mais comumente identificado em muitas partes do mundo incluindo Europa, Américas, África do Norte, Oriente Médio e Ásia (IPPOLITO et al., 2010).

Antes da introdução dos antimicrobianos na prática clínica, a letalidade da bacteremia por *S. aureus* ultrapassava 80%, e mais de 70% dos pacientes desenvolviam infecções metastáticas. No início da década de 1940, com a introdução da penicilina, o prognóstico desses pacientes melhorou bastante (LOWY, 2003). Porém a resistência à penicilina foi reconhecida e aumentou inicialmente em cepas hospitalares e, depois, na comunidade. Contudo, no final da década de 1960, as taxas de resistência tanto hospitalares como comunitárias chegavam a 90% e 70%, respectivamente, em algumas regiões da Europa (OLIVEIRA; TOMASZ; DE LENCASTRE, 2002).

Em 1959, o isolamento do 6-APA tornou possível a produção de penicilinas semi-sintéticas. Modificações na cadeia desse precursor da penicilina resultaram em proteção do anel beta-lactâmico contra a ação hidrolítica das beta-lactamases. Os primeiros agentes antimicrobianos disponíveis para uso clínico foram a oxacilina e a meticilina, que solucionaram temporariamente o problema causado pela resistência de *S. aureus* à penicilina. Porém, o uso desses agentes resultou no surgimento de cepas resistentes em 1961. Desde então as taxas de resistência de *S.aureus* à oxacilina aumentaram vertiginosamente (BELKUM; VERBRUGH, 2001; CHAMBERS, 2001; LOWY, 2003).

No início, cepas de MRSA estavam restritas a centros médicos de referência e hospitais terciários, mas logo se alastraram para serviços e centros de saúde menores. Estudos de vigilância feitos em várias partes do mundo mostraram uma prevalência variável de MRSA, dependendo do país e, principalmente, do hospital ou setor do hospital estudado. Em alguns locais foram relatadas taxas acima de 80% (FARR, 2004; OLIVEIRA; TOMASZ; DE LENCASTRE, 2002). A porcentagem de bacteremia por MRSA chega a 20% nos hospitais americanos e 31% nos hospitais espanhóis (YAMADA et al., 2011).

No entanto, cepas de MRSA não podem mais ser consideradas um patógeno relacionado exclusivamente às infecções de serviços de saúde. A partir da década de 1990, começaram os relatos de infecções por cepas de CA-MRSA em pacientes sem fatores de risco identificáveis para aquisição de cepas de MRSA, ou seja, não tinham contato frequente, direto ou indireto, com serviço de saúde que pudesse explicar a infecção por cepas de HCA-MRSA (CHAMBERS, 2001). Cepas de CA-MRSA já foram descritos em várias regiões do mundo, entre elas o Brasil (RIBEIRO et al., 2005). Além de infectarem indivíduos sem fatores de risco aparentes, essas cepas têm perfil de resistência e virulência peculiares. Quanto à resistência, são menos resistentes a outras classes de antimicrobianos não beta-lactâmicos do que HCA-MRSA (DAUM, 2007; KAPLAN et al., 2005; NAIMI, et al., 2003). Em relação à virulência, nessas cepas existe alta prevalência dos genes que codificam a produção da PVL, uma exotoxina associada a infecções de pele e partes moles graves, bem como a pneumonia necrotizante (DAUM, 2007; GILLET et al., 2002; LABANDEIRA-REY et al., 2007; SAID-SALIM et al., 2005) .

Para HCA-MRSA, nas últimas décadas, uma das poucas opções têm sido os glicopeptídeos, principalmente a vancomicina. Porém, em 1996, foi identificado no Japão o primeiro isolado de *S. aureus* com suscetibilidade reduzida à vancomicina (HIRAMATSU et al., 1997). Desde então, diversos relatos de isolados de VISA ocorreram no mundo, inclusive no Brasil (MARLOWE et al., 2001; OLIVEIRA et al., 2001).

Vancomicina, um glicopeptídeo introduzido a mais de 50 anos atrás, é considerado a principal escolha no tratamento de infecções por MRSA. (PARK et al., 2012). Este glicopeptídeo apresenta nefrotoxicidade tornando-se necessário monitorar o paciente; pode ser ototóxico e deve ser administrado com cuidado em recém-nascidos prematuros e lactentes jovens devendo-se controlar a concentração sérica de vancomicina (YAMAMOTO et al., 2010).

O surgimento de VISA ameaçou o posto da vancomicina como o antibiótico de primeira linha para estas infecções (PARK et al., 2012).

Em junho de 2002, o primeiro VRSA foi identificado em Michigan (EUA) (CHANG et al., 2003). Apenas dois meses depois, um segundo VRSA foi isolado na Pensilvânia (EUA) (CDC, 2002) e uma terceira cepa de VRSA foi descrita em Nova Iorque (EUA) após mais de um ano e meio (CDC, 2004). O quarto, quinto e sexto isolados de VRSA também foram isolados em Michigan, este último em janeiro de 2006. VRSA foi isolado não só nos EUA, mas também na Índia e Irã. VRSA é altamente associado com USA100 (Nova York / clone do Japão) e USA800 (clone pediátrico) nos EUA. VISA também foi encontrado no clone dos EUA CA-MRSA, USA300 (YAMAMOTO et al., 2010).

O isolamento de cepas resistentes à vancomicina, β-lactâmicos, macrolídeos, tetraciclina, aminoglicosídeos e quinolonas mostra que é necessário o surgimento de novas drogas para fortalecer o arsenal antibacteriano. Ao longo dos últimos 30 anos, apenas duas novas classes de antibióticos para o tratamento de infecções por estafilococos, foram introduzidas: as oxazolidinonas representadas pela linezolida e o lipopeptídeo daptomicina. Contudo, resistência a estes antibióticos também têm sido relatadas (CHUNG; CHUNG; NAVARATNAM, 2013).

As oxazolidinonas são uma importante classe de compostos antimicrobianos sintéticos. Linezolida, o único agente comercializado da classe, foi aprovado em 2000 para o uso no tratamento de infecções de pele. Com um espectro de atividade predominantemente contra bactérias Gram-positivas, linezolida é frequentemente usado no hospital e na comunidade para o tratamento de infecções causadas por estafilococos e enterococos, incluindo os que exibem susceptibilidade reduzida à meticilina e vancomicina. A resistência clínica à linezolida está associada a tratamentos prolongados e, estes, podem causar supressão de medula óssea e trombocitopenia; e em casos não reversíveis, neuropatia periférica, lesão do nervo óptico e acidose lática (SANTORO; BUSH; ABBANAT, 2010).

Daptomicina foi aprovada pela FDA, nos EUA, em 2003, para o tratamento de infecções complicadas da pele e, posteriormente, para o tratamento de bacteremia por *S. aureus*. Esta droga pode ser miotóxica e não pode ser usada em infecções respiratórias (KAUR et al., 2012).

3.3 MECANISMOS DE RESISTÊNCIA DE MRSA

A resistência à oxacilina em *S. aureus* é determinada, na grande maioria das vezes, pela presença de um gene localizado no cromossomo, o gene *eca* (LOWY, 2003). Este gene é responsável pela síntese da PBP2a ou PBP2', que substitui as outras proteínas ligadoras de penicilina na membrana e têm baixa afinidade não só para a oxacilina como para os outros antimicrobianos beta-lactâmicos (SCHITO, 2006). O gene *eca* faz parte de uma ilha genômica de resistência chamada SCC*mec*, podendo essas ilhas conter também outros genes de resistência a antimicrobianos (LOWY, 2003). Enquanto cepas de HCA-MRSA carregam SCC*mec* dos tipos I a III, cepas de CA-MRSA estão mais associados aos tipos IV, V e VI (COOMBS et al., 2006; ITO et al., 2004). Os tipos IV, V e VI são elementos genéticos menores e com mais mobilidade que os outros. Esses tipos carregam menos genes determinantes de resistência do que os tipos I, II e III. Por isso, cepas de CA-MRSA caracteristicamente tendem a ser menos multirresistentes que as cepas de HCA-MRSA, mantendo em geral sensibilidade à clindamicina, por exemplo (DAUM, 2007; FRIDKIN et al., 2005; NAIMI et al., 2003).

Atualmente são conhecidos onze tipos de SCC*mec* (I a XI), sendo oito tipos (I a VIII) descritos em literatura, os demais que ainda não foram descritos estão listados no website SCC*mec* pelo IWG-SCC (eca://www.sccmec.org/Pages/Scc_TypesEN.html) (SHORE et al., 2011).

A resistência fenotípica à oxacilina é extremamente variável e depende da expressão do gene *eca*. Essa variabilidade é reconhecida como heterorresistência fenotípica, e se caracteriza pelo fato de que de toda população bacteriana heterogeneamente resistente, carrega o gene *eca*, marcador genotípico da resistência, porém nem todas expressam fenotipicamente sua resistência da mesma forma (MARANAN et al., 1997) .

A expressão da resistência à oxacilina em *S. aureus* é regulada por genes homólogos aos reguladores do gene *bla*Z, determinante da resistência à penicilina. Esses genes (*mec*I e *mec*R1) regulam a resposta do *eca* aos beta-lactâmicos de maneira similar à regulação do *bla*Z pelos genes *bla*R1 e *bla*I ante a exposição à penicilina (CHAMBERS, 1997; LOWY, 2003).

Outros mecanismos (mais raros) de resistência à oxacilina que podem ocorrer incluem a superprodução de beta-lactamases e a produção de PBPs habituais (não a PBP2a), porém com graus variados de afinidade pelos beta-lactâmicos (HACKBARTH et al., 1995; TOMASZ et al., 1989).

3.4 ALTERNATIVAS TERAPÊUTICAS

O surgimento da resistência de MRSA aos antimicrobianos convencionais tem gerado esforços para o desenvolvimento de novos agentes antimicrobianos, que sejam efetivos contra essa bactéria MR. Produtos naturais provenientes de plantas têm fornecido muitos compostos à indústria farmacêutica, sendo que 40% dos fármacos modernos são derivados de fontes naturais.

O extrato bruto metanólico de *Brassica oleracea L.* (couve roxa) foi investigado para possível atividade antimicrobiana. A atividade anti-MRSA do extrato de couve roxa e o seu mecanismo de ação parecem ser novos e diferentes de outros antibióticos já conhecidos (HAFIDH et al., 2011).

Outro exemplo, os óleos de copaíba obtidos das espécies *Copaifera martii, C. officinalis* e *C. reticulata* foram eficazes contra bactérias Gram-positivas (*S. aureus* incluindo MRSA, *S. epidermidis, Bacillus subtilis* e *Enterococcus faecalis*), com MICs variando de 31,3-62,5 µg/mL (SANTOS et al., 2008).

Alguns agentes antibacterianos naturais como compostos de *Hylomecon hylomeconoides*, também apresentaram atividade antibacteriana contra MRSA, com MICs variando de 1,95 a 250 µg/mL (CHOI et al., 2010).

Oito novos produtos naturais antimicrobianos denominados chrysophaentinas A-H, pertencentes a uma nova classe estrutural, foram isolados a partir da alga *Chrysophaeum taylori.* Chrysophaentina A, o mais potente destes antibióticos, inibiu o crescimento de bactérias Gram-positivas clinicamente relevantes, incluindo MRSA (PLAZA. et al., 2010).

Estudos mostraram que ácidos motualévico A-E obtidos da esponja marinha Siliquariaspongia apresentam propriedades antibacterianas. Os ensaios antimicrobianos revelaram que ácido motualévico A inibiu o crescimento de *S. aureus* e MRSA em baixas concentrações (CHERUKU et al., 2010).

A obtenção e utilização de moléculas com ação antimicrobiana produzidas por micro-organismos é uma alternativa de grande potencial para o controle de bactérias MR, como MRSA. Essas substâncias eliminadas por micro-organismos, principalmente bactérias, quando em competição por nicho, são produtos do metabolismo secundário produzidas durante a fase estacionária do crescimento microbiano e não são diretamente essenciais à sobrevivência dos micro-organismos que as produzem. No entanto, os micro-organismos produtores de antibióticos são favorecidos em relação aos não produtores. Uma das formas de serem mais competitivas é através da produção de compostos extracelulares que inibem o crescimento de outras populações microbianas favorecendo assim a manutenção da sua população (ANDRADE, 2004). Esses tipos de substâncias químicas, matam ou inibem o crescimento de outras espécies microbianas, mesmo em pequenas quantidades (OLIVEIRA, 2011). O processo de isolamento, seleção e produção dos micro-organismos antagonistas pelo homem, permite que eles sejam utilizados no controle de micro-organismos patogênicos que não respondem ao tratamento com antimicrobianos convencionais.

Thiomarinols [1-4], produzidos por bactérias marinhas, formam uma nova família de compostos naturais com potente atividade antimicrobiana. Estes compostos são produzidos por bactérias pertencentes gênero *Pseudoalteromonas* e são híbridos de duas espécies ativas de forma independente: a mistura de ácido pseudomônico, mupirocina, que é utilizado clinicamente contra MRSA; e o núcleo de pyrrothina holomycina (FUKUDA et al., 2011).

Novos alcalóides 1,4-dihydroxy-5-phenyl-2-pyridinona (1–4) foram isolados de um extrato do meio de cultura de *Septoria pistaciarum,* um fungo patógeno de plantas. O composto 2 foi moderadamente ativo contra MRSA e MSSA (KUMARIHAMY et al., 2010).

O ambiente marinho é uma fonte importante de novos metabólitos, onde encontramos um número significativo de micro-organismos produtores de antibióticos. A bactéria marinha, *Alteromonas rava* SANK 73390, mostrou-se produtora de vários metabólitos secundários; thiomarinols A-G que têm amplo espectro contra bactérias Gram-positivas e Gram-negativas.

Dois metabólitos secundários MC21 e MC21-A-B foram isolados a partir da bactéria marinha *Pseudoalteromonas phenolica* O-BC30, e demonstraram atividade contra 10 isolados clínicos de MRSA, com MICs variando de 1-4 µg/mL. Significativamente o nível de atividade destes compostos foi comparável ao da vancomicina (MICs variando de 0,25-2 mg/mL) (RAHMAN et al., 2010).

O cultivo da cepa de actinomiceto CNQ-418, recuperado a partir de uma amostra do oceano da Califórnia, forneceu marinopyrroles A-F. Estes possuem marcante atividade antibacteriana contra MRSA (HUGHES et al., 2010).

Extratos de uma *Pseudoalteromonas sp.* marinha coletados em Kaneohe Bay, apresentaram atividade antimicrobiana significativa contra MRSA (FEHÉR et al., 2010).

A maioria dos antimicrobianos utilizados na clínica hoje, é derivada de metabolitos secundários de actinomicetos. Este grupo de bactérias produziu um grande número de antibióticos habitualmente utilizados na clínica como, gentamicina, rifampicina e vancomicina. Compostos produzidos pelo actinomiceto marinho *Streptomyces sp.* CNQ-525, demonstraram rápida e potente atividade bactericida contra cepas de MRSA que apresentam o perfil de resistência encontrado atualmente (HASTE et al., 2011).

Fungos filamentosos são bem conhecidos pela produção de substâncias com atividade antimicrobiana, algumas das quais foram a base para o desenvolvimento de novos agentes antimicrobianos clinicamente importantes. Fungos filamentosos, prosperando em um ambiente extremamente contaminado com altos níveis de antibióticos de amplo espectro e, consequentemente, inibidos por bactérias resistentes a antibióticos, são capazes de produzir metabólitos secundários com atividade bactericida.

Alguns fungos isolados do sedimento de um rio indiano altamente contaminado com antibióticos produziram substâncias com atividade contra várias bactérias clinicamente importante como MRSA, *Escherichia coli* produtoras de ESBL, VRE e *Candida albicans* (SVAHN et al*.,* 2012).

Fenazinas são metabólitos produzidos por *Pseudomonas* spp., *Streptomyces* e alguns outros gêneros, no habitat terrestre e marinho, que apresentam atividade antimicrobiana. Em um estudo observou-se a atividade antimicrobiana de duas fenazinas produzidas por *P. aeruginosa*, a piocianina e seu precursor biossintético 5-metil-phenazinium-1-carboxilato frente à *Candida albicans* (MORALES et al., 2013)

A utilização de compostos extracelulares bacterianos com atividade antibiótica foi relatada por OLIVEIRA e colaboradores (2011) no controle do Cancro cítrico causado pela *Xanthomonas citri* pv. *citri* (Xcc). Os resultados obtidos estimularam novos estudos, que visam explorar o uso desses compostos com atividade antibiótica no controle de micro-organismos MR como MRSA, VRE e KPC, uma vez que as formas de controle atuais não são satisfatórias.

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5 ARTIGO

Este artigo foi submetido à revista "Annals of Clinical Microbiology and Antimicrobials".

Antibacterial activity of extracellular compounds produced by a *Pseudomonas* strain against methicillin-resistant *Staphylococcus aureus* (MRSA) strains

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Abstract

Background: The emergence of multidrug-resistant bacteria is a world health problem. *Staphylococcus aureus,* including methicillin-resistant *S. aureus* (MRSA) strains, is one of the most important human pathogens associated with hospital and community-acquired infections. The aim of this work was to evaluate the antibacterial activity of a *Pseudomonas aeruginosa*-derived compound against MRSA strains.
Methods: Thirty clinical MRSA strains were isolated, and three standard MRSA strains were evaluated. The extracellular compounds were purified by vacuum liquid chromatography. Evaluation of antibacterial activity was performed by agar diffusion technique, determination of the minimal inhibitory concentration, curve of growth and viability and scanning electron microscopy. Interaction of an extracellular compound with silver nanoparticle was studied to evaluate antibacterial effect.
Results: The F3 (ethyl acetate) and F3d (dichloromethane- ethyl acetate) fractions demonstrated antibacterial activity against the MRSA strains. Phenazine-1-carboxamide was identified and purified from the F3d fraction and demonstrated slight antibacterial activity against MRSA, and synergic effect when combined with silver nanoparticles produced by *Fusarium oxysporum*. Organohalogen compound was purified from this fraction showing high antibacterial effect. Using scanning electron microscopy, we show that the F3d fraction caused morphological changes to the cell wall of the MRSA strains.

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Conclusion: These results suggest that *P. aeruginosa*-produced compounds such as phenazines have inhibitory effects against MRSA and may be a good alternative treatment to control infections caused by MRSA.
Keywords: Antibacterial activity, Methicillin-resistant, *Pseudomonas aeruginosa*, *Staphylococcus aureus*.

Running-title: Antibacterial from *Pseudomonas* against MRSA

INTRODUCTION

The emergence of multidrug-resistant bacteria is a world health problem [1,2]. *Staphylococcus aureus* is one of the most important human pathogens associated with hospital and community-acquired infections. Over the last few decades, the number and proportion of methicillin-resistant *S. aureus* (MRSA) infections in different countries has increased due to the rise of epidemics in humans[3,4,5] and other animals, such as dogs, cats, cattle, pigs and exotic species [6,7]. In Brazil, according to data obtained from the first five years of the SENTRY Antimicrobial Surveillance Program, MRSA strains were among the most prevalent pathogens and contributed to 56% of the nosocomial and community infections [8]. One of the major global clones is the MRSA Brazilian epidemic clone (BEC), a hospital-acquired MRSA strain. Isolates of this strain are typically resistant to multiple antimicrobials [9].

The expense incurred to control MRSA may be considerable; however, several economic evaluations have indicated that MRSA control programs are cost-effective in terms of reducing the costs of MRSA infections. In a study comparing two neonatal ICUs, the cost of instituting control measures in a stepwise, delayed approach was US$ 49-69 million (€ 38-52 million), while the cost of introducing effective and immediate measures was US$ 1.3 million (€ 1 million) [10]. Another study calculated that the total cost per case of bacteremia that was caused by an antibiotic-resistant strain, including MRSA (50% of the cases), was US$ 88,445 [11].

The health risks associated with MRSA infections, including its potential to produce invasive infections, particularly in vulnerable patients, and its resistance to multiple antibiotics, warrant the implementation of monitoring programs to control its dissemination. There is a considerable epidemiological interest in tracking strains to gain a more complete picture of the distribution of strains in the population and the dynamics of clonal spread [12]. For years, vancomycin has been used as the drug of choice to treat MRSA infections and was recommended by clinical guidelines; however, the emergence of the vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) has made antibacterial therapy difficult. Therefore, new chemotherapeutic compounds to treat and control infections by these microorganisms have been broadly studied and developed [13].

Recently, some natural antibacterial agents, such *Quercus dilatata*, *Hylomecon hylomeconoides*, *Eleutherine Americana*, *Chelidonium majus* Linn. (*Papaveraceae*) and *Tabebuia avellanedae* compounds, have been tested against MRSA [14, 15, 16, 17, 18].

The ability of antibacterial compounds obtained from other bacteria to inhibit methicillin-sensitive *S. aureus* (MSSA) and MRSA has also been tested [19, 20, 21]. Other bacterial compounds known to have antibacterial activity have not been tested against MRSA. We have tested an extracellular compound derived from *Pseudomonas aeruginosa* that has previously been shown to have antibacterial effects against *Xanthomonas citri* pv. Citri, which causes citrus cancer lesions [22].

The aim of this work was to evaluate the antibacterial activity of a compound from *P. aeruginosa* against MRSA strains.

MATERIALS AND METHODS

Bacteria strains

Thirty MRSA strains from bacteria collection of the hospital of Londrina State University, and isolated in 2011, Londrina-PR, Brazil. The MRSA strains were isolated from blood, urine, trachea and secretion cultures. Three standard MRSA strains were also used in this work. The strains MRSA N315 [23], BEC9393[24] and rib1[25] were provided by Dr. Elsa Masae Mamizuka (Universidade de São Paulo, São Paulo-SP, Brazil), Dr. Agnes Marie Sá Figueiredo (Universidade Federal do Rio de Janeiro, Rio de Janeiro-RJ, Brazil), and Dr. Wanderley Dias da Silveira (Universidade Estadual de Campinas, Campinas-SP, Brazil), respectively. All strains were stored at - 80ºC in stocks containing glycerol (2.5 M).

Extracellular compounds from *Pseudomonas aeruginosa*

The extracellular compounds were provided by Dr. Galdino Andrade from the Laboratory of Microbial Ecology (Londrina State University, Londrina-PR, Brazil). The method of production has been patented (Patent, 2008#PI0803350-1; http://www.inpi.gov.br). These antibacterial compounds were obtained from the *P. aeruginosa* LV strain that was isolated from an old citrus canker lesion on the leaves of orange (*Citrus sinensis* cv. Valence) plants and collected in Astorga, Brazil [26]. The production and purification of these compounds by vacuum liquid chromatography (VLC) were performed as described by Oliveira and collaborators (2011) [22]. The culture supernatants were treated with dichloromethane 1:1 (v:v). The dichloromethane phase (DP) was fractionated using the mobile phase (v/v): hexane (100:F1); dichloromethane (100; F2); ethyl acetate (100; F3); methanol (100; F4); methanol-water (1:1; F5); and water (100; F6). Fractionation was performed again using the following phase (v/v): hexane (100; F3a); hexane-dichloromethane (1:1; F3b); dichloromethane (100; F3c); dichloromethane- ethyl acetate (1:1; F3d); ethyl acetate (100; F3e); ethyl acetate-methanol (1:1; F3f); methanol (100; F3g); methanol-water (1:1; F3h); and water (100; F3i). In this study, the F3 and F3d fractions were used to evaluate the antibiosis effect. All reagents were purchased from Sigma-Aldrich, USA.

Silver nanoparticles from *Fusarium oxysporum*

The silver nanoparticles were obtained following the method of Durán and collaborators (2005) [27]. After the growth of *F. oxysporum* culture, 10 g of the biomass was added in 100 ml of distilled water. After incubation of 72 h at 28°C, the solution components were separated by filtration, and AgNO3 at concentration of 10^{-3}M was added and the system was kept for several hours and then the absorbance at 420 nm that corresponds to the plasmon resonance value was determined. The silver nanoparticles were characterized by Transmission Electron Microscopy (TEM) (Carl Zeiss CEM-902, 80 KeV).

Cytotoxicity assay

The LLC-MK_2 cell line was cultured in a 96-well culture plate at a density of 2.5 x 10^5 cells/well and incubated for 24 h. When the cells were confluent, the non-adherent cells were removed by washing with sterile 0.01 M phosphate buffered saline (PBS). The medium containing different concentrations of F3d (1 to 2000 μg/ml) was added to each well containing the cells, and the plates were incubated for 72 h. For the controls, the cells were cultured in the growth medium alone or in the presence of 1% dimethyl sulfoxide (DMSO). Cell viability was determined by the dimethylthiazol diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) method, according to the manufacturer’s recommendation. The concentration of the compounds needed to inhibit the viability of cells by 50% (IC_{50}) was determined by regression analysis. The 50% cytotoxic concentration ($CC_{50/72h}$) and the selectivity index (SI) were calculated using the equation: $SI = CC_{50}/IC_{50}$.

Evaluation of the antibiosis effect by the agar diffusion technique

The experiment was carried out with three replicates of two fractions obtained from the dichloromethane phase at two concentrations (100 and 500 μg/ml). The negative control was the dichloromethane phase alone (compound solvent). The antibiotic effect of the fraction on the MRSA strains was evaluated on Mueller Hinton agar plates (Difco, USA). MRSA suspensions of 10^8 colony-forming units (CFU)/ml were grown to log phase, and the diffusion disks were treated with the antibiotic compounds. The plates were incubated at 35°C for 24 h, and the size of the inhibition halos diameter was evaluated (mm). The experiment was repeated three times, and the antibiosis effect was determined by measuring the size of inhibited halos formed around the wells.

Determination of the minimal inhibitory concentration (MIC)

The minimal inhibitory concentrations (MICs) were determined by micro-dilution assays in 96-well plates, as suggested by the CLSI (2011) [28]. In brief, single colonies of bacterial cultures grown in Mueller-Hinton agar (Sigma-Aldrich, USA) media were diluted in saline solution and adjusted to 0.5 on the MacFarland scale, which corresponds to 1.5×10^8 CFU/ml. Then, the bacterial suspensions were diluted in Mueller-Hinton broth (Difco, USA) and plated in 96-well plates at a density of 5.0×10^5 CFU/well. Finally, different concentrations of the analyzed antibiotics and compounds were added to each well to determine the MIC values. As negative control, DMSO (Sigma-Aldrich, USA) alone was added in equal concentrations as the ones used to solubilize the antibiotic compounds. The plates were incubated at 37ºC for 18 h, and then the optical density values at 600 nm were determined using a Bio-Rad Microplate Reader model 3550.

Curve of growth and viability

To quantify the effect of compounds on the bacterial growth, a time-response growth curve was obtained in the presence of these antimicrobials. In brief, a single colony forming unit (CFU) of each MRSA strain was diluted in Mueller-Hinton broth and grown for 18 hours at 37ºC with constant stirring at 200 rpm. Then, each culture was adjusted to 0.5 index in MacFarland scale and inoculated at a cell density of 10^6 CFU/ml in 2 ml of Mueller-Hinton broth. For each strain culture was divided in two new cultures of 1 ml each. One culture received the antimicrobial compound and other received only the solvent (control). The bacterial cultures were then incubated at 37°C with constant stirring (200 rpm). In different times, an aliquot of the broth was collected, serial diluted in saline solution, plated on Mueller-Hinton agar media and grown for 18 h at 37°C in order to determine the total CFU of each culture.

Drug interaction studies

To evaluate the antibacterial effects and interactions of phenazine-1-carboxamide combined with silver nanoparticle produced by *Fusarium oxyporum* against MRSA, assays of microdilution in double-antimicrobial gradient were used. Briefly, the MIC values for phenazine and silver nanoparticle used alone were determined, and several concentrations of phenazine were combined with different concentrations of the silver nanoparticle. The MIC of the combination, which is the lowest concentration of phenazine that, when combined with the lowest concentration of silver nanoparticle, were determined. To evaluate the interaction between both antimicrobials, the fractionated inhibitory concentration (FIC) index was used as described by Chin and collaborators (1998) [29]:

$$FIC = MIC(Pc)/MIC(Pa) + MIC(Sc)/MIC(Sa)$$

Where MIC(Pc) is the MIC of phenazine used combined with the silver nanoparticle, MIC(Pa) is the MIC of free phenazine used alone, MIC(Sc) is the MIC of the silver nanoparticle used combined with phenazine and MIC(Aa) is the MIC of the silver nanoparticle used alone. FIC indexes were interpreted as follows: FIC $\leq$ 0.5 = synergic interaction; 0.5 < FIC $\leq$ 1.0 = additive interaction; 1.0 < FIC $\leq$ 4.0 = no interaction; FIC > 4.0 = antagonist interaction.

Scanning Electron Microscopy (SEM)

For scanning electron microscopy (SEM), suspensions of the MRSA strains (10^{10} CFU/ml) with and without the antibacterial compound (at about MIC) were spotted onto polylysine-coated glass slides. Each slide was then fixed by immersion in 1 ml of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 12 h and then post-fixed in 1% OsO_4 for 2 h. The fixed samples were dehydrated in an ethanol gradient (70, 80, 90 and 100˚GL) and then were critical point dried in CO_2 (BALTEC CPD 030 Critical Point Dryer). Finally, the slides were taped onto stubs, coated with gold (BALTEC SDC 050 Sputter Coater) and observed under a FEI Quanta 200 SEM. All reagents were purchased from Sigma-Aldrich, USA.

RESULTS

Extracellular compound

Phenazine-1-carboxamide and organohalogen were identified from the F3d fraction. These compounds were extracted, purified, and evaluated for antibiosis effects. The organohalogen specific structure does not identified yet.

Cytotoxicity assay

It was not possible to determine the 50% cytotoxic concentration of the F3d fraction on LLC-MK_2 cells because even with the highest concentration tested (2000 μg/ml), 84% of the cells were viable, according to the MTT assay.

Diffusion disc-mediated antibiotic treatments against MRSA strains

As an initial screen to evaluate the antimicrobial activity again MRSA, we measured the diameters of the zone of inhibition generated by the F3 fraction against the MRSA standard and clinical isolates (Table 1). There was no difference in the size of the zones for the MRSA standards; however, there was some variation in the zones for the clinical isolates. Discs treated with only dichloromethane (solvent) were also tested and showed no inhibition zones. Discs were impregnated with 25 µg of organohalogen from F3d fraction showed high zones of inhibition for MRSA strain N315 (Table 1). The strains MRSA N315, BEC9393 and rib1 showed resistance for erythromycin, gentamicin, penicillin and ampicillin antimicrobials (data not shown).

Minimal Inhibitory Concentration (MIC)

The MICs of the F3 fraction for MRSA strains (N315, BEC9393 and Rib1) were in the range of 125 μg/ml. The MIC for the more purified F3d fraction was equal to the F3 fraction. The MIC for phenazine-1-carboxamide was 250 μg/ml for the MRSA strain N315 (Table 1). These compounds do not have breakpoints because they are new antibiotics (Table 1). The MRSA strain N315 was further selected for curve of growth and viability tests and scanning electron microscopy.

Curve of growth and viability

At 125 μg/ml, the F3 fraction significantly reduced the number of CFUs over the incubation time (data not shown). A similar effect occurred with the F3d fraction (200 μg/ml) (Figure 1) and with phenazine-1-carboxamide (250 μg/ml) (Figure 2). The number of CFUs after 7 h of incubation with the F3d fraction (200 μg/ml) and phenazine-1-carboxamide (250 μg/ml) was about 10,000-fold lower than the control (without treatment) (Figures 1 and 2). After 24 h of incubation with the F3d fraction, all the bacteria were eliminated (Figure 1).

Drug interaction studies

Phenazine-1-carboxamide was combined with silver nanoparticle for evaluation synergic effect against MRSA N315 strain. The MICs for phenazine and nanoparticle alone were 250 μg/ml and 125 μM respectively. The MICs for these compounds combined were 7.81μg/ml and 31.25 μM, showing the FIC at 0.281 (synergic interaction).

Scanning Electron Microscopy (SEM)

The SEM analysis showed that at a low concentration of F3d, morphological changes in the bacteria could be observed within a few hours (Figures 3C and 3D). No morphological changes were observed after 30 min of incubation with F3d, but the number of cells was reduced (Figure 3B). After 2 h, we observed the cell wall sinking into the bacterial body (Figure 3C). Some cells were deformed after 4 h (Figure 3D). In contrast, the untreated cells (treated only with solvent) appeared intact, and the cell wall was not deformed (Figure 3A). This assay was performed with 100 µg/ml of the F3d fraction.

DISCUSSION

The continuous selection of bacteria that are resistant against a wide range of antibiotics necessitates the discovery of novel unconventional sources of antibiotics. Methicillin-resistant *S. aureus* (MRSA), *Escherichia coli* O157:H7, *Mycobacterium tuberculosis* and *P. aeruginosa* have been considered some of the most virulent microorganisms for the human population.

Notably, MRSA and VISA strains have a thickened cell wall that is believed to deplete the vancomycin available to kill the bacterium; this mechanism of resistance would significantly impact the near future prospects of the current anti-MRSA therapies. The methanol crude extract of *Brassica oleracea* L. (red cabbage) was investigated for possible antimicrobial activity. The anti-MRSA activity of the red cabbage extract and its underlying mechanism of action appear to be novel and different from other known antibiotics. Accordingly, the discovery of natural, effective, and cheap drugs against this resistant bacterium may be a breakthrough solution for this worldwide problem [30].

The pentacyclic triterpenoids α-amyrin, betulinic acid and betulinaldehyde and other related triterpenes, such as imberbic acid, oleanolic acid (oleanic acid), ursolic acid, ulsolic acid, rotundic acid and zeylasteral, have been reported to possess antimicrobial activity [31]. Preliminary studies have shown that the pentacyclic triterpenoids have weak antibacterial activity against the reference strains of methicillin-resistant (ATCC 43300) [31]. All three triterpenoids exhibited a bacteriostatic effect against the reference strains of *S. aureus* at the concentrations tested. Synergism against the two reference strains was reproducibly observed between the three compounds and cell wall inhibitors of the β-lactam and glycopeptide classes. The best synergistic combination was betulinic acid and methicillin [31].

The bactericidal effect of the ethanolic extracts of the stem bark of cinnamon (*Cinnamomum zeylanicum*; CIN), the flower bud and stalk of clove (*Syzygium aromaticum*, CLV) and the seed of cumin (*Cuminum cyminum*, CMN) were tested on MRSA. In decreasing order, the antibacterial activities for the spices were *C. zeylanicum* > *S. aromaticum* > *C. cyminum*. All three spices were excellent bactericidal agents and are potential anti-MRSA agents [32].

Thiomarinols, produced by marine bacteria belonging to the genus *Pseudoalteromonas*, are hybrids of two independently active species: the pseudomonic acid mixture, mupirocin, which is used clinically against MRSA, and the pyrrothine core of holomycin. Thiomarinols are a novel family of natural compounds with potent antimicrobial activity. Understanding how complex antibiotics are synthesized by their producer bacteria to create new families of bioactive compounds [33].

Recently, the violacein produced by *Chromobacterium violaceum* has an inhibitory effect against *S. aureus* isolated from bovine mastitis and displays synergism with penicillin [20].

The F3 compound studied in this work is effective against *Xanthomonas citri* pv. Citri, which causes citrus canker lesions [22]. The F3 fraction was initially tested against other bacteria such as *Staphylococcus* spp., *Enterococcus* spp., *Klebsiella pneumoniae*, *E. coli*, *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium and Enteritidis. The initial results demonstrated that the compound was effective against *S. aureus*, *S. epidermidis*, *Enterococcus faecium* and *K. pneumoniae* (data not shown). Among these bacteria, *Staphylococcus* spp. had the largest inhibition zones in the diffusion disc test.

Because many hospital infections involve MRSA, we evaluated the effect of the F3 fraction standard and clinical strains on MRSA.

The zones of inhibition in the diffusion disc test and the MICs for the F3 fraction were similar among the standard and clinical MRSA and MSSA strains. The MICs for MRSA were higher than for *X. citri*; however, these results are very significant and important for therapies used to treat diseases caused by these strains. For the experiments discussed, the F3d fraction was purified from the F3 fraction.

When we evaluated the F3d effect, we found that within a few hours (2 to 5 h), the number of CFUs decreased significantly, indicating that this compound acts rapidly on these strains. In our *in vitro* tests, the F3d fraction had a bactericidal effect at 200 μg/ml.

By electron microscopy, we observed cellular morphological alterations within a few hours of incubation with lower concentrations of the F3d fraction. The alterations were similar to the effects on *X. citri* [22]. In addition to reducing of the number of CFUs, we observed deformation and sinking of the bacterial cell wall (Figures 3C and 3D).

The results of the cytotoxicity assay demonstrated that the F3d compound does not have cytopathic effects and is not cytotoxic to LLC-MK_2 cells, suggesting low toxicity to the host.

Phenazines are natural products found in *Pseudomonas* spp., *Streptomyces* and a few other genera from soil or marine habitats. Phenazines are large family of colorful, nitrogen-containing tricyclic molecules with antibiotic, antitumor, and antiparasitic activities [34]. Phenazines isolated from *Pseudomonas* species (e.g. *aeruginosa*, *aureofaciens*, *fluorescens* and *cepacia*) are mostly simple hydroxyl- and carboxyl-substituted structures. Pyocyanin (5-N-methyl-1-hydroxyphenazine), phenazine-1-carboxylic (PCA) and phenazine-1-carboxamide (Figure 4) are among the phenazines produced by Pseudomonads, mainly rhizosphere isolates [35]. In our study, we identified phenazine-1-carboxamide (Figure 4) in the F3d fraction. This substance was effective against *S. aureus*, including the MRSA strain N315. The MIC (250 μg/ml) of phenazine for these strains was greater than for the F3 and F3d fraction (125 μg/ml) (Table 1). In other words, the phenazine was less effective than the F3 and F3d compounds. The growth and viability curve with phenazine was similar to F3 and F3d treatment (Figure 2). These results demonstrate that phenazine-1-carboxamide from the F3d fraction has a slight inhibitory effect on *S. aureus*, including MRSA. The higher MIC of phenazine suggests that the F3d fraction contains other inhibitory substances or synergistic compounds. Physiologically, phenazine physiological inhibits and controls nucleic acid and protein synthesis [34]. Therefore, the modes of action for phenazines may include interactions with DNA (intercalation or groove binding), topoisomerases, anti-oxidants or charge-transferring molecules [34].

Some studies have demonstrated that phenazine efficiently inhibits the growth of bacterial and fungi [36, 37]. There are no studies showing that phenazine-1-carboxamide has an antibacterial effect on MRSA. Another study has reported that phenazine has antimicrobial activity on major rice pathogens, such as *Rhizoctonia solani* and *Xanthomonas oryzae* pv. Oryzae [38]. Ecological investigations of the action and crucial role of phenazines have focused in suppressing fungal pathogens of plants such as *Fusarium oxysporum* and *Gaeumannomyces graminis* [39].

The combination of phenazine-1-carboxamide and silver nanoparticle produced by *F. oxysporum* showed synergic effect decreasing up to 32 times the MIC value of phenazine. Studies involving synergism have been very important for antibacterial therapy mainly for multiresistant bacteria. Some natural products have shown anti-staphylococcal activities weaker than others antibiotics, but synergic interactions may use different mechanism of action or pathways to demonstrate their antimicrobial effects, as resulting in the lowered MICs [40]. The combination of current antibiotics with plant-derived antibacterial agents has showed synergic effect against MRSA [41].

An organohalogen compound also was identified from F3d fraction and showed high inhibitory activity against MRSA strain N315. The specific structure has not been identified, but future studies on this organohalogen will conducted. Like penicillin, morphine, vincristine, aspirin and other natural products, several natural organohalogens have important medicinal value [42, 43]. The 2,4-dibromophenol-6-chloro compound isolated from a marine filamentous bacterium, *Pseudoalteromonas luteoviolacea*, shows antibacterial activity against MRSA [44].

The ecological interactions that occur between different bacteria, such as between the *Pseudomonas* genus and other microorganisms in the environment, is extremely important for understanding the antimicrobial effects of some natural compounds. Therefore, we wanted to study the medicinal activities of natural compounds on hospital infections.

There are few effective antimicrobials against multiresistant bacteria including MRSA strains. These antimicrobials are often associated with high costs and serious side effects for the patients. Many different natural antimicrobials have been studied as an alternative to control these infections. Our study suggests that the use of a secondary metabolite from bacteria such as *P. aeruginosa* could be effective again MRSA strains that cause diseases in humans and other animals. This compound may be a good alternative to treat and control of infections caused by MRSA.

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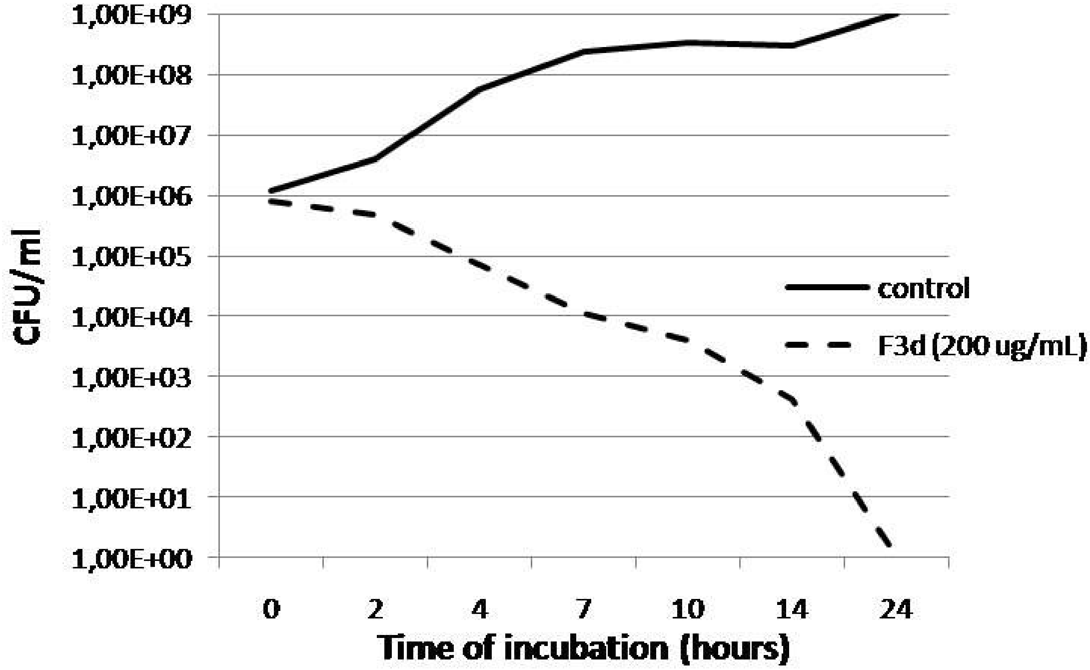
Table 1 - The MIC and diameters of the zones of inhibition (mm) generated by the F3 fraction diffusion discs against standard MRSA strains grown on agar.

MRSA strains	F3 disk (500 μg) zone (mm)	F3 disk (100 μg) zone (mm)	Organohalogen (25 μg) zone (mm)	MIC F3 (μg/ml)	MIC F3d (μg/ml)	MIC phenazine (μg/ml)
N315	22	12	28	125	125	250
BEC9393	23	12	NT	125	125	NT
Rib1	22	12	NT	125	NT	NT
MRSA clinical	16 - 27	08 - 15	NT	NT	NT	NT

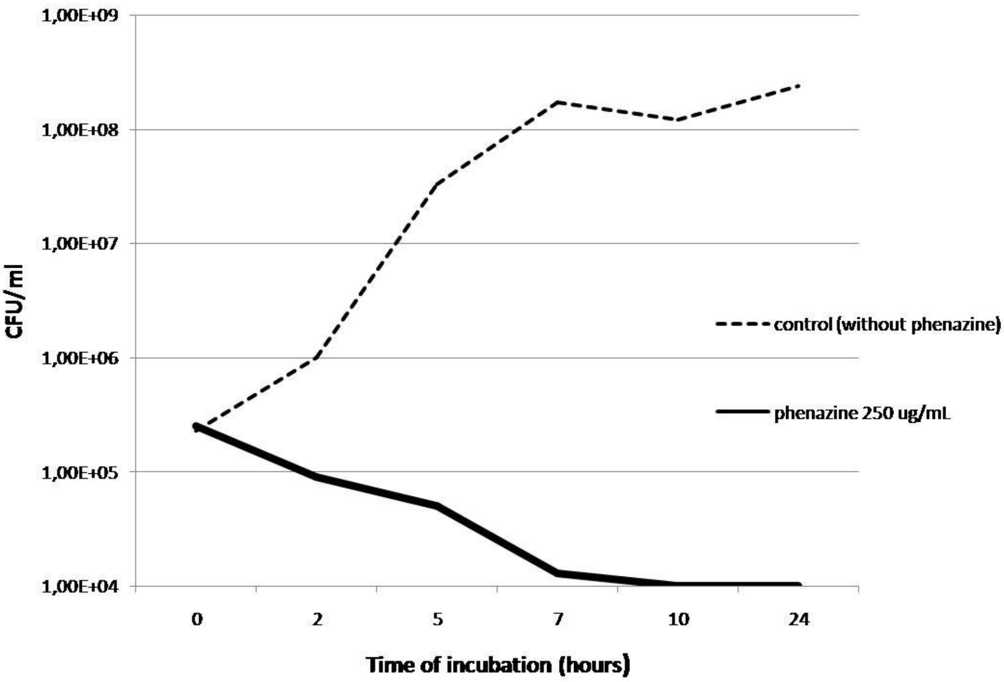
NT – not tested
MIC – Minimal Inhibitory Concentration
MRSA – Methicillin-resistant *Staphylococcus aureus*
MSSA - Methicillin-sensitive *Staphylococcus aureus*

Figure 1- Time-kill curves of *Staphylococcus aureus* N315 strain exposed to F3d fraction



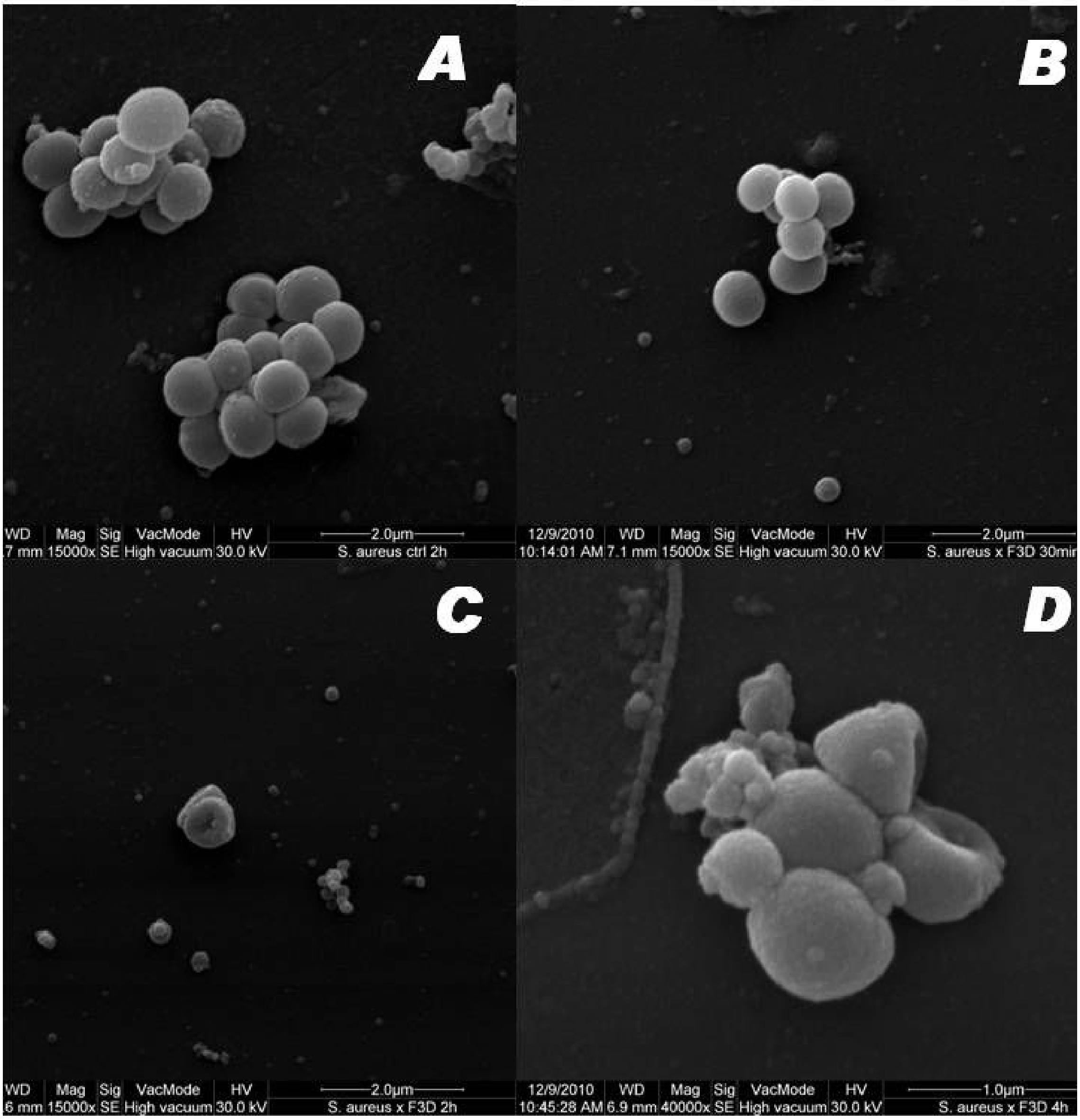
Notes: straight line: without antibiotic treatment; dash line: F3d treatment (200 μg/ml).

Figure 2- Time-kill curves of *Staphylococcus aureus* N315 strain exposed to phenazine-1-carboxamide.



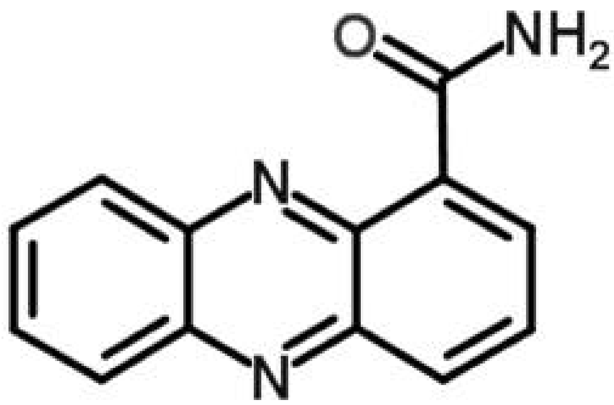
Notes: dash line: control; straight line: phenazine treatment (125 μg/ml); large line: phenazine treatment (250 μg/ml).

Figure 3- Scanning electron microscopy images of the antibacterial effect of the F3d fraction (200 µg/ml) against the MRSA N315 strain at different times.



A: negative control (2 h without antibiotic); B: F3d (30 min); C: F3d (2 h); D: F3d (4 h). When the bacteria were incubated with the F3d fraction for 2 and 4 h, morphological alterations were observed. No morphological cellular alterations were observed with 30 min incubation.

Figure 4- Chemical structure of phenazine-1-carboxamide.



ANEXOS

ANEXO A

Certificado de revisão da língua inglesa

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7 CONCLUSÃO

Metabólitos obtidos de bactérias como *Pseudomonas aeruginosa* apresentam atividade antibacteriana e efeito sinérgico sobre as cepas de *S. aureus,* incluindo cepas meticilina-resistente. O trabalho mostrou que estes compostos extracelulares bacterianos podem ser uma das alternativas para o controle de infecções bacterianas, principalmente para as MR.