UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Avaliação da atividade antibiofilme de *Capsicum baccatum* var. *pendulum* (Solanaceae)

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Avaliação da atividade antibiofilme de *Capsicum baccatum* var. *pendulum* (Solanaceae)

Dissertação apresentada por **Rafael Gomes Von Borowski** para a obtenção do GRAU DE MESTRE em Ciências Farmacêuticas

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“Recordar é reconstruir um novo amanhã.

A partir daí, refeito de antigos conceitos.

Feliz diante de novos paradigmas que nos nortearão.

Assim são as nossas escolhas e perspectivas de vida.

E assumindo que o mundo é seu, preparas-te para altos voos.

Leveza, o lúdico e o prazeroso deverão fazer parte de seu labor.”

Agradeço as grandes professoras-amigas que mais do que dedicar essas palavras em meu nome, fizeram espelhar o sentido da minha busca profissional.

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Resumo

Muitas espécies de pimentas vermelhas do gênero *Capsicum* são utilizadas em práticas medicinais tradicionais. Essas plantas são empregadas em algumas preparações para tratar uma variedade de doenças, incluindo infecções. Algumas bactérias produzem biofilme como um importante fator de virulência, pois a estrutura do biofilme intermedia a adesão bacteriana a superfícies, como em dispositivos implantados, sondas e cateteres além de promover proteção física contra os antibióticos ou as respostas do sistema imunológico. Dessa maneira, este estudo investigou a capacidade do extrato e de produtos isolados das sementes de *Capsicum baccatum* como agentes antibiofilme. Este estudo demonstra, pela primeira vez, que um extrato de *C. baccatum* apresentou importante atividade antibiofilme contra *Staphylococcus epidermidis* e *Pseudomonas aeruginosa*. A fração ativa foi obtida através de ensaios bioguiados e analisada por HPLC-DAD-MS, MALDI-TOF MS e MALDI-MS/MS, identificando-a como peptídeos da proteína 2S sulfur-rich seed storage protein 2-like. Estes peptídeos (2mg/ml) foram potentes no controle da formação de biofilme de *S. epidermidis* (>96%) em solução e adsorvidos em lâminas de Permanox® recobertas. De modo interessante, não inibiram o crescimento bacteriano, indicando que a inibição do biofilme é independente da morte celular bacteriana. Ainda, esses peptídeos foram capazes de preservar eritrócitos, bem como a integridade de linfócitos humanos após 24 e 48 horas de exposição, demonstrando que o fracionamento do extrato de *C. baccatum* potencializou a sua atividade antibiofilme e reduziu significativamente a sua citotoxicidade. Nossos resultados corroboram com a pesquisa de novas estratégias não antibióticas para combater microrganismos com reduzida possibilidade para o desenvolvimento de resistência.

Palavras-chave

Antibiofilme, peptídeos, 2S sulfur-rich seed storage protein 2-like, *Capsicum*, citotoxicidade.

Abstract

"Anti-biofilm evaluation of *Capsicum baccatum* var. *pendulum* (Solanaceae)"

Many species of *Capsicum* red peppers are used in traditional medicinal practices. These plants are utilized in a number of preparations to treat a variety of illnesses including infections. Some bacteria produce biofilm as an important virulence factor, due to this its structure mediates the adhesion to surfaces as implanted devices, probes, catheters and also promotes physical protection against the antibiotics or the immune system response. Accordingly, this study investigated the ability of the extract and isolated products from seeds of *Capsicum baccatum* as anti-biofilm agent. This study demonstrates by the first time that an extract from *C. baccatum* presented relevant anti-biofilm activity against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa.* The active fraction was obtained by bio-guided assays and analyzed by HPLC-DAD-MS, MALDI-TOF MS and MALDI-MS/MS, identifying it as peptides from 2S sulfur-rich seed storage protein 2-like. It strongly controlled (2mg/ml) the *S. epidermidis* biofilm formation (>96%) when the compound was in solution and adsorbed on Permanox™ slides. Interestingly, it did not inhibit the growth of this bacterium, indicating the inhibition of biofilm is independent of bacterial cell death. Moreover, this peptides preserved human erythrocytes and lymphocytes integrity after 24-48 h of exposure, suggesting the fractionation potentiated the anti-biofilm activity of the *C. baccatum* crude extract while absolutely reduced its cytotoxicity. Our results corroborate to the search of new non-antibiotic strategies to combat microorganisms with a reduced pressure for resistance development.

Keywords

Anti-biofilm, peptides, 2S sulfur-rich seed storage protein 2-like, *Capsicum*, cytotoxicity.

Sumário

Introdução

Os vegetais participam da vida humana desde os primórdios, como fonte de alimentos, como defesas e ataque, como meio de restauração da saúde entre outras tantas (Simões, 2010), estabelecendo considerável relevância científica frente à fragilidade do homem. Neste contexto, as pimentas, em especial as do gênero *Capsicum* (Solanaceae) nativas do continente Americano, são importantes exemplares de vegetais popularmente utilizados como alimento e agente medicinal. De acordo com a literatura, os frutos, folhas e sementes de *Capsicum* spp., isoladas ou combinadas, tem sido utilizadas em práticas medicinais tradicionais e incorporadas a algumas preparações para tratar uma variedade de doenças, incluindo feridas infectadas, queimaduras, problemas respiratórios, picadas e dores de ouvido (Cichewicz e Thorpe, 1996). Seus frutos são fontes de compostos antioxidantes como vitaminas, carotenoides, capsaicinoides e compostos fenólicos podendo variar seus níveis fitoquímicos nos diferentes genótipos bem como nas diferentes partes da planta (Kappel *et al.*, 2008; Zimmer *et al.*, 2012a). As pimentas desse gênero são conhecidas popularmente como tili, pimenta-quente, páprica e pimenta-vermelha. Em especial, a espécie *Capsicum baccatum* (Figura 1) é popularmente conhecida como pimenta dedo-de-moça e cambuci, com diferentes colorações e formas, sendo produzidas algumas dezenas de variedades dessas pimentas no Brasil. O seu cultivo ocorre praticamente em todas as regiões do país e é um dos melhores exemplos da agricultura familiar, sendo o Rio Grande do Sul um dos cinco Estados de maior produção, atrás apenas de Minas Gerais, Goiás, São Paulo e Ceará (Embrapa, 2002).

Os registros mais antigos do consumo de pimentas datam de aproximadamente 9 mil anos, resultado de explorações arqueológicas em Tehuacán, México. Outros sítios arqueológicos pré-históricos (2500 a.C.) são conhecidos no Peru, nas localidades de Ancon e Huaca Prieta. O cultivo de pimentas era uma característica de tribos indígenas brasileiras quando do descobrimento do Brasil. Com a imensa variabilidade de pimentas nativas,

certamente pode-se supor que diversas tribos cultivavam e colhiam pimentas; e o plantio de pimenta por tribos indígenas continua até hoje, como entre os índios mundurucus, da bacia do rio Tapajós (Embrapa, 2002).

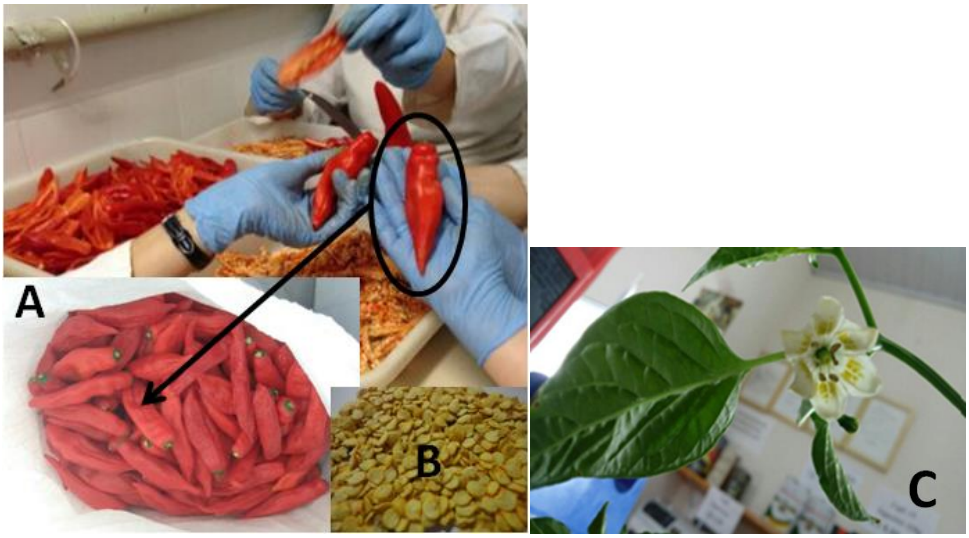

Figura 1. Fotos da pimenta dedo-de-moça (*C. baccatum var. pendulum*), arquivo do autor; A) Frutos com 7 x 1,5 cm (comprimento x largura), coloração vermelha (maduros); B) Sementes de coloração amarelo claro e C) Flor com corola branca, um par de manchas amareladas ou esverdeadas na base de cada lobo das pétalas e anteras geralmente amareladas (uma a duas flores por nó).

O gênero *Capsicum* apresenta propriedades anti-inflamatórias, antioxidante, anti-hipertensiva, hipoglicemiante e hipocolesterolêmica *in vitro* e *in vivo*, embora o potencial farmacológico da espécie *C. baccatum* seja pouco explorado. Neste sentido o trabalho de ZIMMER et al. (2012a), demonstrou atividades antioxidante (frente ao radical DPPH) e anti-inflamatória (modelo de pleurisia em camundongos) dos extratos etanólico e butanólico de frutos de *C. baccatum var. pendulum* (200mg/kg, v.o.). Alguns peptídeos e proteínas de defesa são comuns na composição química das sementes de *Capsicum* spp., assim como peptídeos antimicrobianos (AMPs) entre outros (Lee *et al.*, 2004; Dias *et al.*, 2013). Dessa forma, alguns trabalhos reportam que frações proteicas derivadas de plantas podem exibir propriedades antiadesivas (Lengsfeld *et al.*, 2004; Bensch *et al.*, 2011).

Porém, relatos sobre a composição química da espécie são muito escassos, fazendo-se necessário a ampliação de estudos nessa área (Zimmer *et al.*, 2012a).

No trabalho de KAPPEL et al. (2008) foi testada a atividade antimicrobiana de diferentes partes dos frutos de *C. baccatum var. pendulum*, incluído as sementes, contra *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Enterococcus faecium*, *Candida glabrata*, *Candida albicans*, *Candida krusei*, *Candida dubliniensis*, *Candida parapsilosis*, *Cryptococcus neoformans* e outras duas cepas de isolados clínicos, *C. albicans* e *C. parapsilosis*, pelo método de difusão em disco. Neste estudo não foi demonstrada nenhuma atividade antimicrobiana para os extratos de *C. baccatum* nas concentrações testadas (2000, 1000 e 200 µg de extrato por disco). Esses resultados são consoantes com os *screenings* iniciais realizados pelo nosso grupo de pesquisa que encontrou significante atividade antibiofilme, com ausência de atividade antimicrobiana, contra cepas padronizadas de *P. aeruginosa* e *Staphylococcus epidermidis* para algumas frações do extrato das sementes.

Embora esses resultados apontem para a ausência de atividade antibacteriana, poucos estudos correlacionam os compostos derivados de pimentas com a atividade antiadesiva, entre eles, um que estudou extratos dos frutos de *Capcisum annum* contra *Campylobacter jejuni* (Bensch *et al.*, 2011) e outro, com extratos das folhas ou com produtos isolados de *Piper nigrum* contra *Enterococcus faecalis* e *Staphylococcus aureus* (Geethashri *et al.*, 2014; Lee *et al.*, 2014), porém, nenhum para *C. baccatum*.

A avaliação da atividade sobre biofilme justifica-se, pois atualmente, as infecções associadas a bactérias formadoras de biofilme estão envolvidas em 80% das infecções humanas tais como endocardites, infecções em pacientes com fibrose cística, osteomelites, rino-sinusites crônicas, colites, vaginites, uretrites, conjuntivites, otites, periodontites, caracterizando-se como importante problema de saúde pública. Considerando que as bactérias nessa forma séssil são de 10 a 1000 vezes mais resistentes do que no seu estado planctônico, o biofilme é um

importante fator de virulência e um dos principais desafios na clínica médica (Hall-Stoodley *et al.*, 2012; Madeo e Frieri, 2013). Bactérias resistentes a antibioticoterapia têm preocupado a comunidade médica sendo a causa primária de morte em unidades de tratamento intensivo em todo o mundo (Cegelski *et al.*, 2008). *Pseudomonas aeruginosa* (bacilos Gram-negativos) e *S. epidermidis* (cocos Gram-positivos) são importantes exemplos de bactérias multirresistentes, formadoras de biofilme e causadoras de uma ampla gama de infecções oportunistas em humanos. Estes microorganismos estão associados às infecções nosocomiais mais prevalentes em neonatos e imunocomprometidos (*S. epidermidis* mais prevalente que *S. aureus*), as quais frequentemente são originadas a partir de biomateriais contaminados com estes microorganismos, tornando-se importantes patógenos oportunistas. A infecção por essas bactérias resulta em danos graves ao tecido alvo podendo levar o indivíduo à morte, necessitando urgentemente de novos métodos de prevenção e tratamento (Kerr e Snelling, 2009; Da Silva *et al.*, 2012; Taylor *et al.*, 2014).

O conceito de biofilme o define como uma matriz complexa que envolve comunidades de microorganismos aderidas irreversivelmente a uma superfície biótica / abiótica cujos níveis de estrutura, desenvolvimento, organização, metabolismo e regulação são afetados por diversos fatores. Essa matriz é composta principalmente por exopolissacarídeos, proteínas e DNA, dando origem á uma substância polimérica extracelular (EPS) separada por canais de água que atuam como um sistema circulatório primário, os quais transportam nutrientes e removem metabólitos ou comunicam-se através de moléculas mensageiras. A composição dessa matriz ou das demais moléculas varia em função de um controle genético de formação e diferenciação. Ainda, as micro colônias (grupos com mais de 50 células) são o início do biofilme tridimensional maduro e em função de suas estruturas com distintas propriedades, podem adsorver ou reagir com agentes externos (adesão a biomateriais, por exemplo), além de proteger fisicamente contra a penetração do agente antimicrobiano e expressar fatores de proteção específicos,

o que explica a resistência antibiótica, bem como ao sistema imune (Davies, 2003; Romling e Balsalobre, 2012; Haussler e Fuqua, 2013).

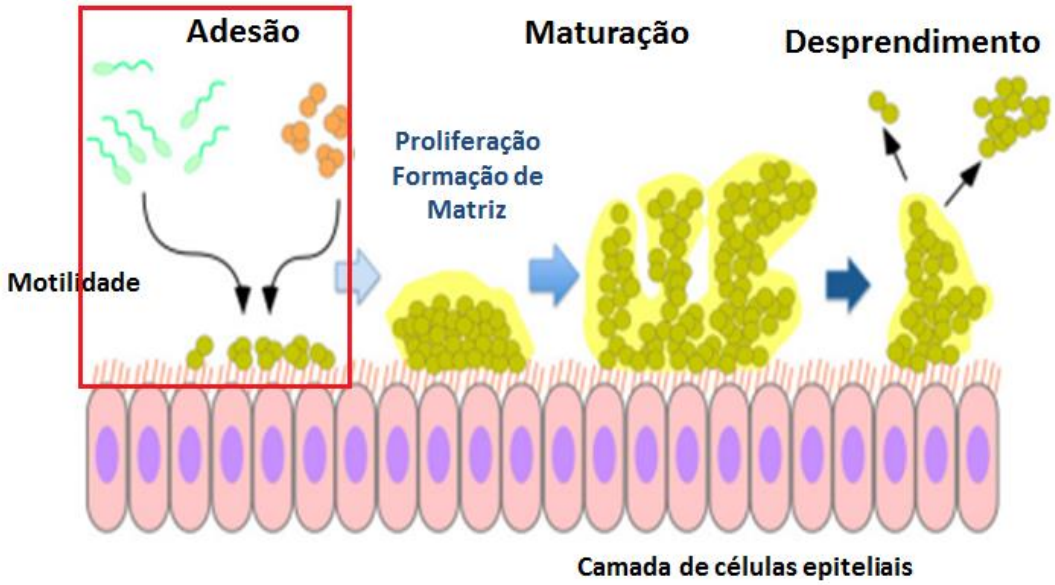

Figura 2. Fases do desenvolvimento do biofilme: **Adesão** é o início do processo de fixação e colonização, por exemplo, em superfícies poliméricas (hidrofobicidade da superfície, auto lisinas e ácidos tecóicos) ou em matrizes proteicas do hospedeiro; **Maturação** é onde ocorre a proliferação celular e a formação substancial de matriz (fatores adesivos e outras proteínas, inclusive de fatores de desprendimento) e **Desprendimento** é onde há o rompimento do biofilme já estabelecido e o desprendimento celular, podendo dar início a um novo processo de infecção (fatores de desprendimento, proteases e nucleases). Destacado em vermelho a fase onde atua o extrato/produto isolado de *C. baccatum* (Adaptado de Otto, 2014).

As fases do desenvolvimento do biofilme (adesão, maturação e desprendimento) abrangem o ilustrado e descrito na Figura 2. O processo de adesão mantém as bactérias juntas/agregadas e estabelece o início da infecção, a maturação do biofilme compreende a proliferação celular além da formação substancial de matriz e o processo de desprendimento, além de formar canais entre a estrutura do biofilme (comunicação e nutrição celular nas camadas mais profundas do biofilme), contempla o rompimento do mesmo, possibilitando a

liberação destas bactérias. Ainda, a importância desta fase de desprendimento do biofilme reside na disseminação da infecção, podendo gerar uma infecção aguda não associada ao biofilme, como a sepse (Laverty *et al.*, 2013; Otto, 2014).

Problemas associados à formação de biofilme em biomaterias tais como próteses ortopédicas, cateteres e sondas, que são particularmente susceptíveis a colonização microbiana e favorecem o desenvolvimento de infecções, são fontes importantes de transmissão de patógenos resistentes em hospitais e clínicas. Estas infecções vêm crescendo globalmente e impactando significativamente no desfecho final dos casos, bem como nos custos econômicos para os sistemas de saúde. Nesse sentido, a pesquisa em biomateriais ou em substâncias capazes de impedir a infecção dos mesmos, deve basear-se no profundo conhecimento entre a interação dessas bactérias e o biomaterial. O desenvolvimento de superfícies contendo agentes antimicrobianos e/ou antibiofilmes para recobrir biomaterias são exemplos de novas estratégias para a prevenção e o tratamento (Arciola *et al.*, 2012; Lim *et al.*, 2013).

Assim sendo, muitos antibióticos derivados de produtos naturais estão sendo testados, embora nos últimos 20 anos a indústria farmacêutica tem comercializado apenas variações das mesmas moléculas convencionais, com mecanismos de ação que não atuam sobre a produção de biofilmes bacterianos. Este é um importante ponto a ser analisado, pois o surgimento de novos alvos terapêuticos é um elemento fundamental no desenvolvimento de novos fármacos. Novas estratégias como a combinação de agentes antibiofilmes com a antibioticoterapia convencional são necessárias para combater essas bactérias (Cegelski *et al.*, 2008; Beloin *et al.*, 2014).

Desta forma, este trabalho se propôs a avaliar a atividade antibiofilme do extrato e de produtos isolados de *C. baccatum var. pendulum* (Solanaceae) visando explorar novos compostos que dificultem o rápido desenvolvimento de resistência, como terapia antivirulência. Ainda, visou a melhor caracterização química dessa

espécie vegetal, para a qual até o momento, não foram encontrados na literatura estudos sobre a investigação da atividade antibiofilme.

CAPÍTULO 1. ARTIGO: "RED PEPPER (*Capsicum baccatum*) IS SOURCE OF ANTI-BIOFILM COMPOUNDS AGAINST CLINICAL RELEVANCE BACTERIA *Pseudomonas aeruginosa* AND *Staphylococcus epidermidis"*

Este artigo será submetido para publicação na revista *Journal of Ethnopharmacology*.

Red pepper (*Capsicum baccatum*) is source of anti-biofilm compounds against clinical relevance bacteria *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*

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1. Abstract

Ethnopharmacological relevance: Many species of *Capsicum* red peppers are used in traditional medicinal practices. These plants are utilized in a number of preparations to treat a variety of illnesses including infections. These ethnopharmacological data can contribute significantly in the search of new natural bioactive compounds against bacterial biofilms. The aim of this study was to investigate the anti-biofilm activity of *Capsicum baccatum* extracts against clinically relevant bacteria.

Materials and methods: Antimicrobial and anti-biofilm activities were evaluated by the OD_{600} absorbance and crystal violet method, respectively. *Staphylococcus epidermidis* ATCC35984 and *Pseudomonas aeruginosa* PA14 were used as bacterial models for biofilm formation. Scanning electron microscopy (SEM) was performed to evaluate the results obtained by crystal violet assay. Color and precipitation reactions were applied for phytochemical evaluation of bioactive extract.

Results: The residual aqueous extract from seeds (RAqS) was the most promising extract, able to inhibit in 80% and 60% *S. epidermidis* and *P. aeruginosa* biofilm, respectively. SEM analysis showed the presence of only small clusters or individual cells on the surface of treated samples with RAqS. Interestingly, RaqS did not inhibit the growth of these bacteria, indicating that the inhibition of biofilm is independent of bacterial cell death or growth inhibition. The phytochemical evaluation of RaqS indicated the presence of total polyphenols (quantified in 70.29 mg/g), amino acids / proteins, saponins, triterpenes and tannins (quantified in 15 mg/g of tannic acid equivalents for hydrolysable tannins and 174.61 mg/g of catechin equivalents for condensed tannins).

Conclusion: This study demonstrates by the first time that an extract from *Capsicum baccatum* presented promising anti-biofilm activity against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, two clinically significant pathogens.

Keywords: pepper, *Capsicum*, anti-biofilm, antibacterial, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*

2. Introduction

The genus *Capsicum*, belonging to Solanaceae family, is native to the tropical and humid zones of America and includes many species of red peppers (Govindarajan, 1986; Menichini *et al.*, 2009). These plants are valuable for their nutritional and medicinal constituents, being used for a variety of therapeutic applications worldwide (Osuna-Garcia *et al.*, 1998; Kappel *et al.*, 2008). The red pepper *Capsicum baccatum* var. *pendulum* is widely consumed in South America, especially Brazil, but there are few reports in the literature about its chemical composition and biological properties. Recently, we showed that *C. baccatum* extracts have antioxidant and anti-inflammatory activities (Zimmer *et al.*, 2012a) and pharmacological safety after long-term use in mice (Zimmer *et al.*, 2012b).

Some studies have investigated the antimicrobial activity of *Capsicum* spp. (Cichewicz e Thorpe, 1996; Dorantes *et al.*, 2000; Iorizzi *et al.*, 2002; Careaga *et al.*, 2003; Diz *et al.*, 2006; Bensch *et al.*, 2011; Peralva Santos *et al.*, 2012) including one that screened the antimicrobial activity of fruits and seeds extracts from *C. baccatum* using Gram-positive and Gram-negative bacteria and fungi, and all tested extracts revealed no activity against bacteria strains (Kappel *et al.*, 2008). Accordingly to literature, fruits, leaves, roots or seeds of *Capsicum* genus, alone or in combination, have been used in traditional medicinal practices and incorporated into a number of medicinal preparations to treat a variety of illnesses, including infected wounds, fresh burns, respiratory problems, bowel complaints, earaches, and sores (Cichewicz e Thorpe, 1996).

Microbial biofilms are ubiquitous in natural, clinical and industrial environments. Increased resistance to antibiotics and host immune defenses is a feature usually associated to bacteria living in biofilms (Parsek e Singh, 2003). Biofilms are communities of microorganisms attached to a biotic and/or abiotic surface and encapsulated by a polymeric extracellular matrix of microbial origin (Karatan e Watnick, 2009). Biofilms are involved in a multitude of different infections and contribute significantly to the therapeutic failures (Bryers, 2008).

Pseudomonas aeruginosa and *Staphylococcus epidermidis* are bacterial species well established as models for biofilms formation and development, being causal agents of several chronic human infections.

Considering the importance of investigating antiadhesive agents as an alternative strategy to prevent bacterial infection, we evaluated the potential of *C. baccatum* extracts to inhibit biofilm formation by clinically relevant bacterial models.

3. Materials and methods

3.1 Chemicals

All reagents and the HPLC reference standards (quercetin, rutin, capsaicin, catechin and tannic acid) were purchased from Sigma Chemical Company® (St. Louis, MO, USA). All solvents were purchased from Vetec® 20 AG (Rio de Janeiro, Brazil) and all reagents were of analytical or HPLC grade.

3.2 Plant material and extraction

Capsicum baccatum var. *pendulum* (Willd.) Eshbaugh (Solanaceae) fruits were obtained from a cultivated area in Turuçu, Rio Grande do Sul (RS), Brazil. A voucher specimen (number P278) was identified and deposited at the Herbarium of Brazilian Government Research Institute EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária, Pelotas, RS, Brazil). The seeds were separated from fresh fruit and booths. Fruit and seeds were left to dry in a circulating air oven (40°C) and then triturated to powder. The extracts were prepared according to Zimmer et al. (2012) with modifications. Briefly, to obtain the ethanolic extracts, fruit and seeds were extracted with 70% ethanol (plant:solvent, 1:10, w/v) under reflux for 4 h. The fruits and seeds of *C. baccatum* were also submitted to successive extractions in a Soxhlet apparatus using dichloromethane, n-butanol and water until complete exhaustion. The organic solvents were filtered, evaporated under reduced pressure to dryness, and the aqueous extract was lyophilized, to obtain the respective residues from fruit and seeds named as follows: CE (crude ethanol extract), DCM (dichloromethane extract), BUT (butanol extract) and RAq (residual aqueous extract).

3.3 Phytochemical characterization

3.3.1 Qualitative identification of secondary metabolites

The extracts were evaluated through several classic phytochemical characterization reactions to detect secondary metabolites as alkaloids (Mayer, Bertrand and Dragendorff's Reactions), amino acids/proteins (Ninhydrin Reaction), anthraquinones (Bornträger's Reaction), coumarins (pH alteration under ultraviolet light observation), flavonoids (Cyaniding/Shinoda and Salkoski's Reactions), polyphenols (Ferric chloride Reaction), saponins (Foam test, Hemolysis assay and Liebermann-Burchard's Reaction), tannins (Gelatin test and Hydrolysis followed by thin layer chromatography - TLC) and triterpenes (Liebermann-Burchard and Salkoski's Reactions) (Zaia *et al.*, 1998; Lôbo *et al.*, 2010; Simões, 2010; Andrade Rezende *et al.*, 2011).

3.3.2 Thin Layer Chromatography

The extracts were applied on plates of TLC, 1 cm from the edge, and the chromatographic analysis was performed on silica gel 60 F_{254} plates (Merck, 5 × 6 cm, 0.5 mm thickness), using the follow systems as eluents: ethyl acetate-methanol-water-acetic acid (8:1:0.5:0.5); butanol-acetic acid-water (5:1.5:5.5; 4:2:2; 4:1:2; 1:2:4); methanol (100%); methanol-chloridric acid-water (7:1:2); chloroform-methanol-water-acetic acid (2:2:4:2); chloroform-ethanol-water (8:4:0.5; 10:10:1.25; 4:4:0.5; 5:10:1.25; 2:4:0.5); hexane-ethyl ether (2.5:0.25; 2:0.4); hexane-ethyl ether-acetic acid (9.7:0.3:0.1); ethyl acetate-formic acid-water (8.8:0.6:0.6); and ethyl acetate-methanol-ammonia hydroxide (5:5:1). A paper chromatography was also performed using chloridric acid-water (5:5) as eluent. After elution, TLC plates were dried in air and observed under UV light (254 nm and 365 nm). The plates were sprayed with solutions of NP (2-aminoethyldiphenylborinate 1% in methanol) followed PEG-400 (polyethylene glycol 5% in ethanol) for detection of flavonoids, sulfuric anisaldehyde for steroids and terpenes, and iodine vapor as an universal reagent (Stahl e Bolliger, 1965; Macek, 1972; Bruneton, 1991; Wagner e Bladt, 2009; Simões, 2010).

3.3.3 Quantitative analysis

3.3.3.1 Total phenolic contents, quercetin, rutin and capsaicin

The extracts from *C. baccatum* were characterized measuring total phenolic contents by Folin-Ciocalteau method and through the search of quercetin, rutin and capsaicin by high

performance liquid chromatography (HPLC) analysis with an Agilent Instrument (series 1200), according to the previous studies of Zimmer *et al*. (2012). .

3.3.3.2 Tannins content

The measurement of proanthocyanidins/condensed tannins content from active *C. baccatum* extract was performed according Vanillin reaction described by Prince *et al.* (1978). Briefly, to 1.0 ml of *C. baccatum* extract (2.0, 4.0 and 20.0 mg/mL) was added 5.0 mL of 1% methanolic vanillin solution and HCl 8% (in methanol). The samples were placed in a water bath at 30°C for 20 minutes, and the absorbance was determined at 500 nm. The blank sample received, at same conditions, only HCl 4% solution. The same procedure was performed for the construction of calibration curve, using catechin as standard in the concentration range of 0.1 to 0.5 mg/mL (Price *et al.*, 1978; Queiroz *et al.*, 2002).

The hydrolysable tannins content was estimated using the potassium iodate method, as follow: in a test-tube 1 mL of *C. baccatum* active extract (20 mg/mL) was added to 5 mL of 2.5% potassium iodate solution under stirring. The mixture was kept in water bath at 25°C for 35 minutes, and the absorbance was measured at 550 nm. The same procedure was performed for the construction of calibration curve, using tannic acid as standard in a concentration range of 0.1 to 1.5 mg/mL (Hagerman *et al.*, 1997; Willis e Allen, 1998; Mueller-Harvey, 2001).

3.4 Sample preparation for microbiological assays

The CE, DCM and BUT extracts from *C. baccatum* fruits and seeds were prepared in DMSO 1%, and the RAq extracts were dissolved directly in Milli-Q water. The extracts were filtered through a 0.2 µm pore membrane. All the extracts were tested at the concentration (into the well) of 4 mg/mL, with the exception of RAq extract from seeds that was also assayed in the concentrations of 0.25, 0.5, 1, and 2 mg/mL.

3.5 Bacterial strains and growth conditions

Pseudomonas aeruginosa PA14 and *Staphylococcus epidermidis* ATCC 35984 were grown in Luria-Bertani broth (peptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L), overnight, 100 mL into an Erlenmeyer (250 mL) on a rotary shaker (Incubator Shaker

Series Excella E25, New Brunswick Scientific), 150 rpm, at 37°C and adjusted to an optical density at 600 nm (OD_{600}), equivalent to 10^8 CFU/mL.

3.6 Anti-biofilm and antibacterial assays

The biofilm formation was evaluated employing standard sterile 96-well polystyrene flat bottom microtiter plates and crystal violet method (Costar 3599 Corning, Inc. NY, USA) according the protocol developed by Zimmer *et al.* (2013). In the biofilm formation inhibition assay 50 µL of LB broth, 100 µL of extract and 100 µL of bacterial suspension (final equivalent to 10^7 CFU/mL in the well) were added into each well. The control sample (untreated) received water Milli-Q or 1% DMSO instead of extract. The microtiter plates were incubated statically at 37°C for 24 h. After this period, the contents of the wells were removed and washed with sterile saline 0.9% three times. Layer biofilm formed on the microtiter plates was fixed at 60°C for 1 h. Biofilm was stained with 200 µL of crystal violet 0.4% for 15 min, the dye excess was removed with running water and adherent dye was solubilized with DMSO 99.5%. The absorbance was measured at 570 nm on Spectramax M2e Multimode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Bacterial growth was evaluated by the difference between the absorbance at 600 nm measured at the end and at the beginning of the incubation time in polystyrene 96-well microtiter plates. The control sample (untreated, considered 100% of bacterial growth) received water Milli-Q or 1% DMSO instead of extract. Gentamicin sulfate (6.5 µg/mL) was used as control for growth inhibition against *P. aeruginosa,* while rifampicin (6.5 µg/mL) was used as control against *S. epidermidis*.

3.7 Scanning electron microscopy (SEM)

Only the most active extract (RAqS – Residual aqueous from seeds) was selected to be visualized by SEM. Briefly, *S. epidermidis* and *P. aeruginosa* biofilms were cultured as described at item 3.6, holding a piece of Permanox™ slide (Nalge Nunc International, USA). The treated-sample received LB broth and extract, and the untreated sample received LB broth and Milli-Q water (24 h at 37°C). The samples were washed with sterile 0.9% NaCl to remove planktonic cells. The biofilm was fixed with 2.5% glutaraldehyde (in 100 mM cacodylate buffer pH 7.2), washed with 100 mM cacodylate

buffer (pH 7.2), dehydrated with increasing concentrations of acetone, dried with CO_2 as critical point (Bal-Tec 030 apparatus/CPD 030 Balzers, Liechtenstein) and placed on a mounting base. The samples were coated with gold and examined under a scanning electron microscope (model JEOL Instrument JSM-6060) at 10 kV.

3.8 Statistical analysis

Data were expressed as mean values ± standard deviation from three independent experiments. Differences between groups (treatments and control) were compared using unpaired two-tailed Student's t-Test (GraphPad Prism® version 5.00). A p value ≤ 0.05 was considered statistically significant.

4. Results

In the present study, we report the evaluation of anti-biofilm and antibacterial activities of different extracts from fruits and seeds of the red pepper *C. baccatum var. pendulum* against *P. aeruginosa* PA14 and *S. epidermidis* ATCC 35984. Notably, only the residual aqueous extract from seeds (RAqS), 10% extraction yield, significantly inhibited *S. epidermidis* and *P. aeruginosa* biofilm formation (Figure 1A and B) without inhibiting planktonic growth of these bacteria. The dose-response curve demonstrated that RaqS, in a concentration of 2 mg/mL and 4 mg/mL, inhibited in 60% and 80% *S. epidermidis* ATCC35984 biofilm, respectively, without bacterial growth inhibition. In other concentrations tested (0.25 to 1 mg/mL) RAqS did not prevent biofilm of *S. epidermidis* ATCC35984 (Figure 1B).

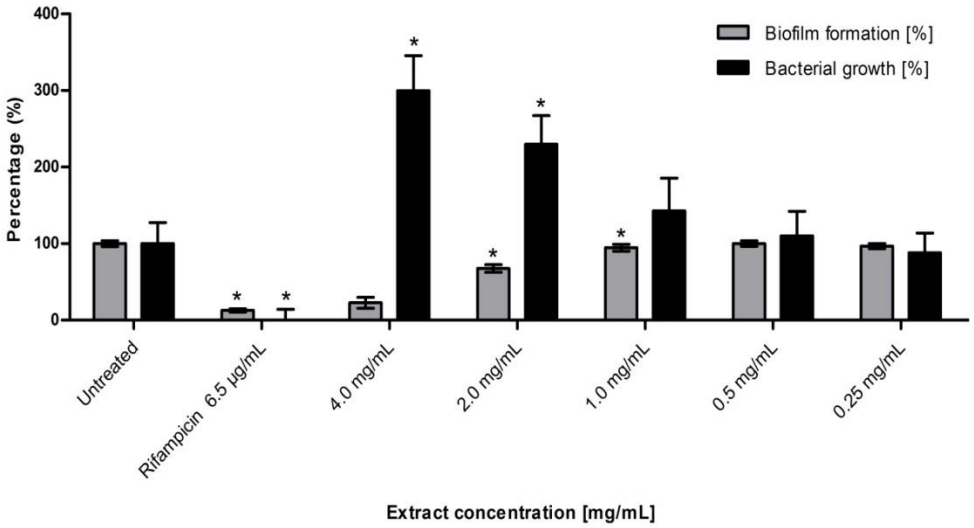

Figure 1A. Effects of RAqS on biofilm formation and bacterial growth by *S. epidermidis* ATCC 35984, after 24 h of treatment in 96-well plates. Biofilm formation and growth were quantified for optical density at 570 and 600 nm, respectively. Rifampicin was used as positive control. The results are presented as percentage and the untreated group is the 100% reference. *Represents statistical difference in growth and biofilm formation compared to the untreated sample.

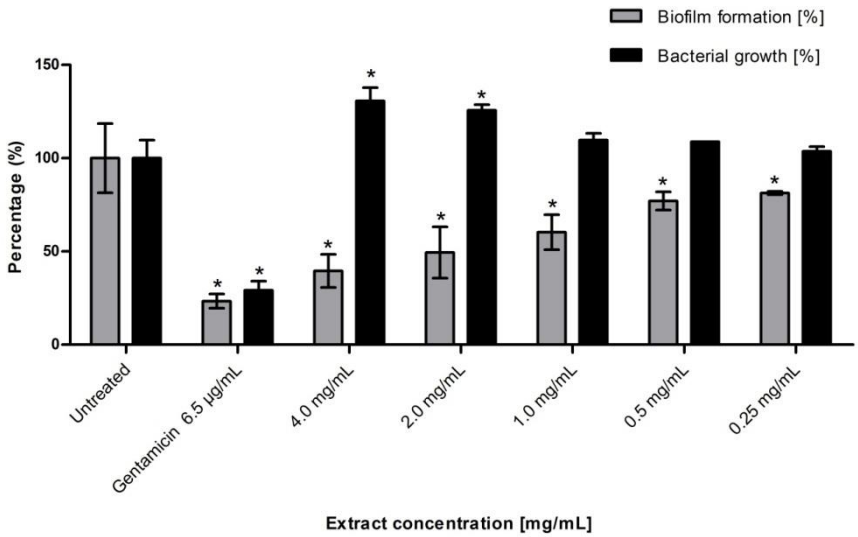

Figure 1B. Effects of RAqS on biofilm formation and bacterial growth by *P. aeruginosa* PA14, after 24 h of treatment in 96-well plates. Biofilm formation and growth were quantified for optical density at 570 and 600 nm, respectively. Gentamicin was used as positive control. The results are presented as percentage and the untreated group is the 100% reference. *Represents statistical difference in growth and biofilm formation compared to the untreated sample.

RaqS also inhibited in 50% and 60% *P. aeruginosa* biofilm at a concentration of 1 and 2 mg/mL, and 4 mg/mL, respectively. In concentrations of 0.5 and 0.25 mg/mL the biofilm of *P. aeruginosa* was inhibited in approximately 20% (Figure 1B). In any concentration tested RAqS prevented planktonic cell growth. The others extracts (CE, DCM and BUT) evaluated, in a concentration of 4 mg/mL, were not active against the bacteria tested (data not shown).

In order to visualize qualitatively the prevention of biofilm by RAqS, SEM images were performed. The images of SEM confirmed the results obtained by crystal violet method. In the untreated samples, a dense and compact biofilm can be visualized with extracellular matrix involving bacterial cells (Figure 2A, B, C for *S. epidermidis*; and 2G, H, I for *P. aeruginosa*). In contrast, only small clusters or individual cells can be seen on the surface of treated samples with RAqS. Furthermore, there is no presence of matrix in treated samples (Figure 2D, E, F for *S. epidermidis*; and 2J, K, L for *P. aeruginosa*).

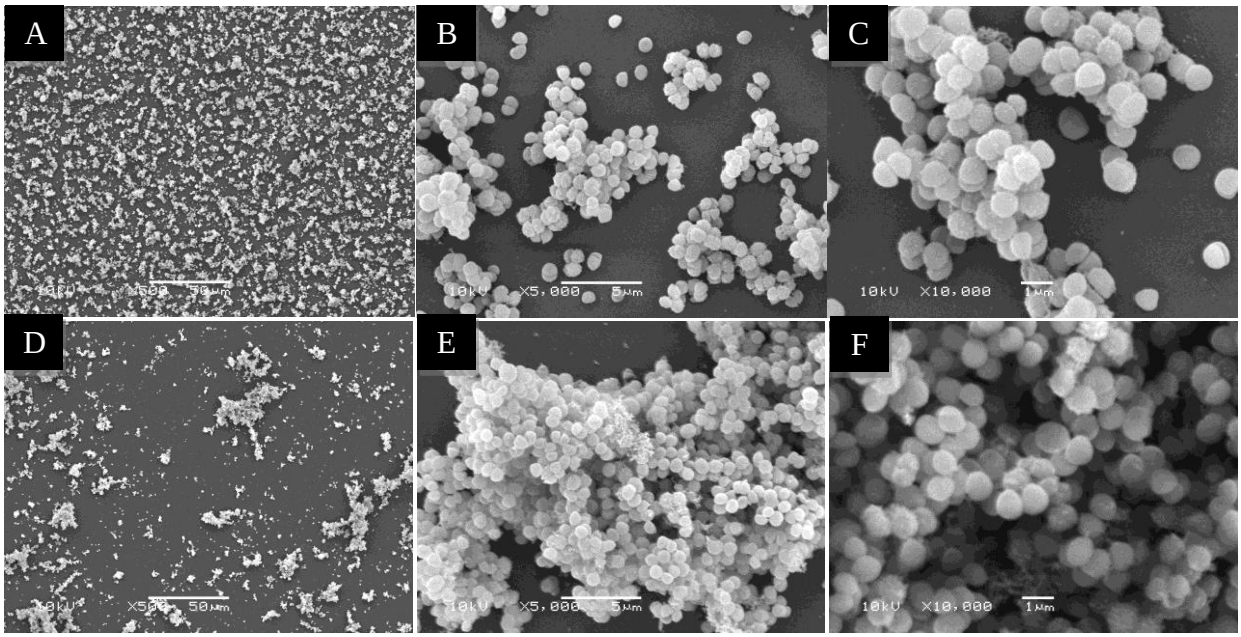

Figure 2. Scanning electron microscopy (SEM) images of biofilm by *S. epidermidis* ATCC 35984, upon Permanox™ slides. A, B, C – Untreated biofilm; D, E, F - RAqS-treated biofilm (4 mg/mL). Scale bars: 500, 5,000 and 10,000 x magnification.

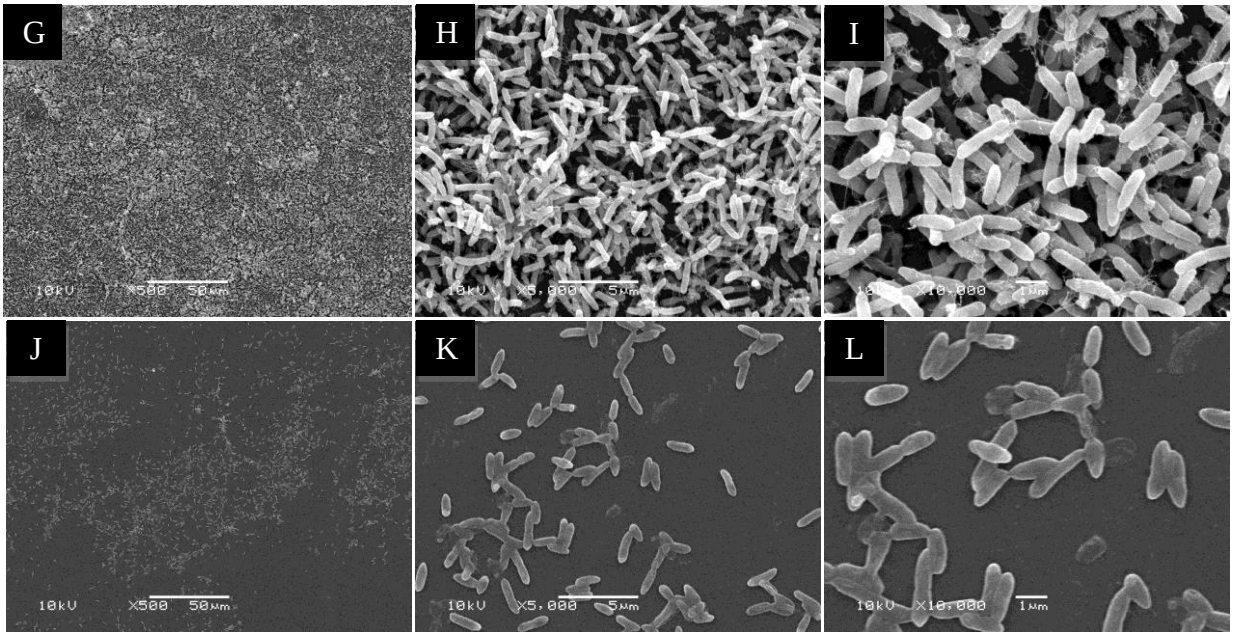

Figure 3. Scanning electron microscopy (SEM) images of biofilm by *P. aeruginosa* PA14, upon Permanox™ slides. G, H, I – Untreated biofilm; J, K, L - RAqS-treated biofilm (4 mg/mL). Scale bars: 500, 5,000 and 10,000 x magnification.

The phytochemical analyses of RAqS are summarized in the Table 1. The results suggested the presence of amino acids/proteins, polyphenols, saponins, tannins, triterpenes and the absence of alkaloids, anthraquinones, coumarins, and flavonoids. The separation of the components by TLC has not been successfully performed since the compounds were retained at the application point of the plates in different eluents phases used. The identification with the color reagents sprayed at the TLCs was not possible because the colors showed were not clear or specific and could fail the interpretation. This fact may have occurred due probably to high polarity of compounds present in RAqS.

Table 1. Phytochemical analyses of RAqS

PHYTOCHEMICAL CLASS	TESTS		
ALKALOIDS	Mayer Reagent -	Bertrand Reagent -	Dragendorff Reagent -
AMINO ACIDS / PROTEINS	Ninhydrin Reaction +		
ANTHRAQUINONES	Borntrager Reagent -		
COUMARINS	Ultra violet 360 nm / pH -		
FLAVONOIDS	Cyanidin / Shinoda +	Salkoski Reagent -	
POLYPHENOLS	Ferric Chloride Reagent +		
SAPONINS	Foam Test +	Hemolysis Test +	
TANNINS	Gelatin Test +		
TRITERPENES	Liebermann-Burchard Reagent (+) Triterpenes nucleus (-) Steroids nucleus	Salkoski Reagent (+) Glycosides	

The quantitative analyses of RAqS revealed the presence of total phenolic compounds in the order of 70.29 mg of gallic acid equivalents/g of extract. In the quantification of the tannins content the hydrolysable tannins were found 15.4 mg of tannic acid equivalents/g of RAqS, and for the condensed tannins were 174.61 mg of catechin equivalents/g of RAqS.

5. Discussion

This is the first report that demonstrated the anti-biofilm potential of the extracts obtained from red pepper *C. bacattum* against clinically relevant bacteria. The anti-biofilm activity was evidenced to RAqS through the crystal violet method, decreasing in 80% and 60% *S. epidermidis* and *P. aeruginosa* biofilm formation, respectively, in a concentration of 4.0 mg/mL. These findings were confirmed by images of SEM.

Interestingly, the results showed that inhibition of the bacterial biofilm is independent of cell death or growth inhibition. On the contrary, at concentrations where there was

inhibition of *S. epidermidis* and *P. aeruginosa* biofilms, the growth of these bacteria was stimulated. The stimulation of bacterial growth may be due to stress caused by the treatment with the extract since a virulence factor is being inhibited.

Biofilms often are the cause of difficulty in eradicating *S. epidermidis* and *P. aeruginosa* infections. *Staphylococcus epidermidis* is not usually pathogenic, however patients with compromised immune system are susceptible to its infection (Chusri *et al.*, 2012). *Staphylococcus epidermidis* is also a major concern for people with catheters, probes or other surgical implants because it is known to form biofilms on these devices, being this ability the major virulence factor of *S. epidermidis* (Otto, 2008). *Pseudomonas aeruginosa* is an important opportunistic pathogen causing infections especially in immune compromised patients and cystic fibrosis (CF) sufferers (Mulcahy *et al.*, 2008).

It is increasingly evident that alternative strategies are needed to combat drug-resistant organisms. Thus, rather than focusing on therapies that target bacterial growth, such as conventional antibiotics, an alternative approach is to search for new mechanisms of action, targeting virulence factors, including biofilm formation (Rasko e Sperandio, 2010). The use of traditional plants for treatment of diseases is a common trend and usually practiced in remote regions with inadequate infrastructure for health care (Sarkar *et al.*, 2014). In some stress situations, the response to pathogen attack or part of the normal development make the plants produce many bioactive compounds of interest for pharmacological uses. In addition, there are various natural products (like garlic, curcumin, ginger, clove and cinnamon) which have been found to interfere with the quorum sensing (QS) signaling in biofilm formation (Bjarnsholt *et al.*, 2005; Niu *et al.*, 2006).

In a classical phytochemical analysis of RAqS, we identified the presence of amino acids/proteins, polyphenols, saponins, tannins and triterpenes and the absence of alkaloids, anthraquinones, coumarins and flavonoids. Despite the Cyaniding/Shinoda reaction exhibited positive reaction, a light pink color was obtained, which is compatible with derivatives from anthocyanin and not for flavonoids. Considering the amino acids / proteins presence, some studies with plants derivatives have describing important antimicrobial and antifungal activities (Lee *et al.*, 2004; Ribeiro *et al.*, 2007; Fernando *et al.*, 2011; Dias *et al.*, 2013). Polyphenols have received some attention recently, regarding their antimicrobial and anti-biofilm effect upon microorganisms in biofilms, including a

small number of studies involving *S. epidermidis* (Ferrazzano *et al.*, 2009; Sampaio *et al.*, 2009; Prabhakar *et al.*, 2010; Schito *et al.*, 2011; Trentin *et al.*, 2011). However, antimicrobial activity of terpenes (excluding volatile oils) and steroids against bacteria and fungi has been widely described (Sparg *et al.*, 2004; Zhang *et al.*, 2008; Popova *et al.*, 2009).

There are just a few studies correlating some peppers compounds with antiadhesive activity. At this one, *Capsicum annum* (cayenne) fruits extract (hydroethanol) was tested against *Campylobacter jejuni* (IC50: 0.29 mg/mL) and provides evidence for additional beneficial effects of this herbal in gastrointestinal disorders. The main contains in this extract were capsaicinoids, such as capsaicin and dihydrocapsaicin, as well as, carotenoids and acyclic diterpene glycosides (Bensch *et al.*, 2011). One study with *Piper nigrum* (black pepper) showed that aqueous extract from the leaves disintegrated *Enterococcus faecalis* and *Staphylococcus aureus* biofilms significantly at 30 and 15 mg/mL, respectively (Geethashri *et al.*, 2014). Other study with *Piper nigrum* (black pepper) screened the essential oil as anti-biofilm, and showed a markedly inhibition of *S. aureus* biofilm formation. Furthermore, the essential oils of black pepper at below 0.005 % almost abolished the hemolytic activity of *S. aureus*. Transcriptional analyses showed that black pepper oil down-regulated the expressions of the α-toxin gene (*hla*), the nuclease genes, and the regulatory genes. In addition, it attenuated *S. aureus* virulence in the nematode *Caenorhabditis elegans* (Lee *et al.*, 2014). Moreover, one study with the isolated alkaloid piperine (found in black pepper), at sub-inhibitory concentrations (0.5 to 10 mg/mL) decreased bacterial swarming and swimming motilities, decreasing the expression of the flagellar gene (*fliC*) and motility genes (*motA* and *motB*), while increasing biofilm formation (increasing the expression of adhesin genes (*fimA, papA, uvrY*), despite of increase the penetration of ciprofloxacin and azithromycin into *Escherichia coli* biofilms aim to enhance the ability of these antibiotics to disperse pre-established biofilms (Dusane *et al.*, 2014).

Thus, considering that one of the crucial steps in biofilm development is the initial interaction of bacteria to abiotic or biotic surfaces that can ultimately lead to colonization and infection by pathogenic bacteria (Beloin *et al.*, 2014), our results demonstrate the anti-biofilm activity of RAqS, showing that *C. baccatum* is a potential source of innovative bioactive compounds which can interfere with the biofilm development of *S.*

epidermidis and *P. aeruginosa.* Additionally, the extracts from *C. bacattum* seem to be safe for use due to the low or the absence of toxicity *in vivo* (Zimmer *et al.*, 2012b), encouraging us to follow in the investigation of RAqS composition and their related bioactivity.

6. Conclusions

The study evidences the ability of *C. baccatum* to prevent bacterial biofilm formation and shows its potential as a promising source of compounds for the development of novel anti-virulence strategies to combat bacteria-mediated diseases.

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CAPÍTULO 2. ARTIGO: "2S SULFUR-RICH SEED STORAGE PROTEIN 2-LIKE FROM RED PEPPER *Capsicum baccatum* var. *pendulum* (SOLANACEAE), ARE POTENTIAL ANTI-BIOFILM AGENT AS ANTIVIRULENCE THERAPY AGAINST CLINICAL RELEVANCE BACTERIA"

Este artigo será submetido para publicação na revista *Apllied Microbiology and Biotechnology*.

2S sulfur-rich seed storage protein 2-like from Red pepper *Capsicum baccatum* var. *pendulum* (Solanaceae), is potential anti-biofilm agent as antivirulence therapy against clinical relevance bacteria

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Abstract

The biofilm structure mediates the adhesion to surfaces as implanted devices, probes, catheters and also promotes physical protection against the antibiotics or the immune system response, been an important virulence factor present in 80% of human infections. In this context, plant-derived compounds are widely accepted due to the perception of their safety and the long history of use in folk medicine, reporting prevention and treatment of infections. Accordingly, this study investigated the ability of isolated products from seeds of *Capsicum baccatum* as anti-biofilm agent against the biofilm procer *Staphylococcus epidermidis*. The active fraction was analyzed by HPLC-DAD-MS and MALDI-TOF MS, subsequently the 2S sulfur-rich seed storage protein 2-like was isolated and their digested peptides were analyzed by MALDI-MS/MS. It strongly controlled (2mg/ml) the biofilm formation (>96%) when the compound was in solution and adsorbed on Permanox™ slides. Interestingly, it did not inhibit the growth of this bacterium, indicating the inhibition of biofilm is independent of bacterial cell death. Moreover, this protein preserved human erythrocytes and lymphocytes integrity after 24-48 h of exposure, suggesting the fractionation potentiated the anti-biofilm activity of the *C. baccatum* crude extract while absolutely reduced its cytotoxicity. Our results corroborate to the search of new non-antibiotic strategies to combat microorganisms with a reduced pressure for resistance development.

Keywords

Anti-biofilm, peptides, 2S sulfur-rich seed storage protein 2-like, *Capsicum*, spin-coated, cytotoxicity.

Introduction

Bacterial resistance to antibiotic and host immunologic deficient are the first cause of death into intensive-care units worldwide. The rapid emergence of resistance to antibiotics occurs due to mutations, horizontal gene transfer and also results from acquisition of the high antibiotic tolerance displayed by bacterial when growing as biofilm communities on the surface. Biofilm has been considered an important virulence factor, which is present in 80% of human infections as endocarditis, osteomyelitis, chronic sinusitis, urethritis, periodontitis, characterizing it as a severe public health problem (Macedo e Abraham, 2009; Madeo e Frieri, 2013; Travier *et al.*, 2013).

Moreover, the biofilm is a complex matrix, composed of extracellular polymeric substance (EPS) wrapping microorganisms communities, irreversible adhered to a biotic or abiotic surface. Micro colonies are the beginning of mature biofilm and its structure and composition can react or adsorbed with external agents mediating the adhesion to surfaces and also promoting physical protection against the antibiotics or the immune system response (Davies, 2003; Romling e Balsalobre, 2012; Haussler e Fuqua, 2013). Accordingly, adhesion and colonization are required for the establishment of bacterial infection and pathogenesis. Thus, the bacteria may undergo specific molecular changes to establish biofilms and adhering on implanted devices as probes, prostheses, catheters or on damaged tissues, impacting importantly on the patients' denouement and costs of the health system (Arciola *et al.*, 2012; Lim *et al.*, 2013).

In this context, *Staphylococcus epidermidis* is a well-established specie for biofilm formation and development models (Mulcahy *et al.*, 2008). Although *S. epidermidis* is not usually pathogenic, immunocompromised patients are more susceptible to its infection whereas resident bacteria can become opportunistic, attaching to the biomaterial surface and forming resistant biofilms (Chusri *et al.*, 2012; Laverty *et al.*, 2013). This ability to form biofilms on devices is a major virulence factor of *S. epidermidis* (Otto, 2008).

Plant-derived compounds have gained extensive interest in the search for alternatives to control microorganisms (Essawi e Srour, 2000). These compounds are widely accepted due to their use in folk medicine for the prevention and treatment of diseases and infections (Guarrera, 2005). The genus *Capsicum* comprises more than 200 varieties, and their fruits are broadly diverse in relation to size, shape, flavor and sensory heat. *Capsicum* species are native to the tropical and humid zones of Central and South

America and belong to Solanaceae family, which includes peppers of important economic value (Govindarajan, 1986; Menichini *et al.*, 2009). They are commonly used as spice or food, as well as remedies in Indian, Native American and Chinese medicinal traditions to treat arthritis, rheumatism, stomach ache, skin rashes, dog/snake bite and wounds (Meghvansi *et al.*, 2010).

Additionally, pepper fruits are a remarkable source of antioxidant compounds, like vitamins, carotenoids, capsaicinoids and phenolic compounds (Kappel *et al.*, 2008). The *Capsicum* genus possesses some peptides as proteases inhibitors and antimicrobials peptides related to natural defense of the plant (Fernando *et al.*, 2011; Dias *et al.*, 2013; Carrillo-Montes *et al.*, 2014). The *Capsicum* genus also displays anti-inflammatory, antioxidant, antihypertensive, hypoglycimiant and hypocholesterolemic proprieties however, the pharmacologic potential and chemical composition of *C. baccatum*, specifically, has been underexplored (Ahuja *et al.*, 2007; Aizawa e Inakuma, 2009; Srinivasan *et al.*, 2009; Galvez Ranilla *et al.*, 2010; Liu e Nair, 2010; Zimmer *et al.*, 2012a; Zimmer *et al.*, 2012b). So, recently, we demonstrated the activity of aqueous seeds extract of *C. baccatum* (AqS), which was able to decrease 80% and 60% of biofilm formation by *S. epidermidis* and *Pseudomonas aeruginosa*, respectively, indicating that *C. baccatum* is potential source of anti-biofilm compounds (previous results / data not shown).

Considering the importance of anti-virulence approaches, the present study aimed to investigate the ability of purified fractions of *C. baccatum* to control and prevent the biofilm formation by *S. epidermidis*. In addition, a purified fraction was analyzed by HPLC-DAD-MS and MALDI-TOF, screened toxicologically *in vitro* and evaluated as coating agent, corroborating to the search of nonantibiotic strategy against the drug-resistance war.

Materials and methods

Chemicals

All reagents were purchased from Sigma Chemical Company® (St. Louis, MO, USA), which were analytical or HPLC grade. All solvents were purchased from Vetec® AG (Rio de Janeiro, Brazil). The matrices 2,5-dihydroxybenzoic acid (DHB) and sinapinic acid (SA) were purchased from Bruker Daltonics.

Plant material and extraction

C. baccatum var. *pendulum* (Willd.) Eshbaugh (Solanaceae) fruits were obtained from a cultivated area in Turuçu, Rio Grande do Sul (RS), Brazil. A voucher specimen (number P278) was identified and deposited at the Herbarium of Brazilian Government Research Institute EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária, Pelotas, RS, Brazil). The seeds were separated from fresh fruits and booths; seeds were dried in a circulating air oven (40°C) and they were powdered. The extracts were obtained according to Zimmer and collaborators (2012). Briefly, the seeds of *C. baccatum* were submitted to successive extractions in a Soxhlet apparatus using dichloromethane, *n*-butanol and water until complete exhaustion. The interest aqueous extract was lyophilized to obtain the aqueous extract from seeds (AqS).

Purification of aqueous extract from seeds (AqS)

AqS were solubilized in water and subjected to a column chromatography (3 cm of diameters) using Sephadex™ LH-20, 40 g for 700 mg of sample. Only water was used as the eluent system and aliquots of 10 ml were collected, resulting fourteen fractions (F1-F14).

Bacterial strain and culture conditions

S. epidermidis ATCC 35984, was cultivated in Muller Hinton agar at 37ºC overnight. The inoculum concentration was adjusted to an optical density at 600 nm, equivalent to 10^8 CFU/ml.

Sample preparation for microbiological assays

AqS and their fractions were freshly dissolved directly in milli-Q water and filtered through a 0.2 µm pore membrane, before each assay. The AqS was tested in the concentration of 2 mg/ml and the fractions in the concentrations of: 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml.

Antibiofilm and antibacterial assays

A protocol developed by Zimmer and collaborators (2013) was applied to evaluate the biofilm formation, using standard sterile 96-well polystyrene flat bottom microtiter plates (Costar 3599 Corning, Inc. NY, USA). It was added 50 µl of LB broth into each well, 100 µl of extract or fraction and 100 µl of bacterial suspension (equivalent to 10^8 CFU/ml). The control sample (untreated) received water instead of extract or fraction. Then, the microplates were statically incubated at 37°C for 24 h. After this period, the contents of the wells were removed and they were washed with sterile saline 0.9% three times. The biofilm layer formed was fixed at 60°C for 1 h. The biofilm was stained with crystal violet 0.4% for 15 min, the dye excess was removed with running water and the adhered dye solubilized with ethanol (Merk, Germany). Finally, the absorbance (570 nm) was measured (Spectramax M2e Multimode Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Bacterial growth was monitored by absorbance at 600 nm and

rifampicin (16 µg/ml into the well) was used as positive control for *S. epidermidis* growth.

Antibacterial kinetics

It was developed using a drop plate method adapted from Chen et al (2003), for simultaneous colony counting and most probable number (MPN) enumeration. Samples were replaced at 0, 1, 3, 6, 12, 24 h after incubation (37°C as previous described), applying sterile saline 0.9 % in a "4 x 4" drop plate, in Mueller Hinton (MH) agar (Oxoid Lda., England), with 10^{-2} – 10^{-14} dilutions, in duplicate (N = 2). The OD_{600} was also measured at the same times. Only the active fraction (2.0 mg/ml) was evaluated and rifampicin (16 µg/ml) was used as death control. The results are expressed as log10 of cells number per incubation time.

Coating preparation, technique and characterization

Solutions of AqS and the most active fraction (F9) were prepared using methanol 70% and filtered through a 0.2 µm pore membrane. Slides of Permanox™ (3 x 2.5 cm) were coated once and three times with 2.0 mg/ml and 10.0 mg/ml of sample, separately. Methanol was used as no treated control. In the Spin Coater Laurell Model WS-650-MZ-23NPP/LITE, 300 µl of AqS or active fraction solutions were deposited on Permanox™ slide. It was accelerated 5 s up to angular velocity 500 rpm and subsequently accelerated to 4000 rpm for 40 s. To remove the remaining solvent and to promote film annealing the slides were head-treated at 40ºC in the stove during 4 hours. After the coat process, the slides were sterilized for UV light for 20 minutes, cut to 10 x 25 mm slides and used in anti-biofilm and scanning electron microscopy evaluations (as follow).

To characterize the Permanox coated samples, the water contact angle (WCA) was measured using sessile drop technique and Milli-Q water. The drop images were captured and analyzed by a Theta Lite Optical Tensiometer (Attension). The contact angles are a mean of five measurements of different points of each sample, as described by Trentin and collaborators (2015).

Scanning electron microscopy (SEM)

It was employed the method used for Zimmer *et al.* (2013). Briefly, *S. epidermidis* ATCC 35984 biofilm was cultured as previous described. One group received a peace of no coated Permanox™ coverslip inside (as no treated and no solvent control), others received a peace of coated sample with: methanol (as a solvent control), AqS and active fraction (coated once and three times at 2.0 mg/ml and 10.0 mg/ml concentrations). The no coated control received LB broth, the bacteria and fraction (F9)/extract into the medium and the coated samples received LB broth and the bacteria (24 h at 37°C). After, the coverslips samples were washed with sterile NaCl 0.9% and biofilm was fixed with 2.5% glutaraldehyde (prepared in 100 mM cacodylate buffer pH 7.2). Afterwards, they were washed with 100 mM cacodylate buffer (pH 7.2) and dehydrated with increasing concentrations of acetone, dried with CO_2 as critical point (Bal-Tec 030 apparatus / CPD 030 Balzers, Liechtenstein) and placed on a mounting base. Finally, they were coated with carbon and examined under a scanning electron microscope (model JEOL Instrument JSM-6060) at 10 kV.

Hemolysis test

This test used human venous blood and all the donors were healthy researchers and students as previous described for Rocha et al (2012). All the consents, procedures and documents were approved by Ethical Committee of Universidade Federal do Rio Grande

do Sul (UFRGS) under authorization number 666.655/2014. The active fraction (F9) was tested at 2.0 mg/ml and AqS at 2.0 and 4.0 mg/ml concentrations. We used 1% Triton™ as reference sample (100% of hemolysis) and phosphate-buffered saline (PBS) and erythrocytes as negative control (no hemolytic). The tested samples were statically compared with the reference and negative samples.

Lymphocytes test / LDH

This test used human venous blood (sterile tube containing heparin) and all the donors were healthy. All the consents, procedures and documents were approved by Ethical Committee of UFRGS under authorization number 666.655/2014. The lymphocytes were separated immediately by Histopaque™ gradient centrifuge and washed three times with RPMI 1640, re-suspended at 10^6 cells/ml and cultured in RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS) (Gibco BRL, Grand Island, NY), penicillin (100 IU/ml), streptomycin (100 UI/ml) (Sigma Chemical Co., St. Louis, MO) and amphotericin B (2.5 µg/ml) (Gibco BRL, Grand Island, NY). The lymphocytes were cultured (100,000 cells/well), 300µl in 24-well polystyrene flat bottom plates and added 300µl of the following treatments: F9 (active fraction) at 2 mg/ml, AqS at 2 and 4 mg/ml, 1% Triton™ as death control and RPMI medium as live control, all in quintuplicate. The samples were solubilized only in RPMI 1640 supplemented with 10% FBS medium and filtered. The cells were maintained at 37°C, with 5% CO_2, for 24 and 48 h. Cell morphology was verified by optical microscopy and by counting, determined using flow cytometer FACSVerse™, with blue laser 488 nm and flow sensor (BD Biosciences, San Jose, CA, EUA) (BD FacSuite™ Software). It was considered cytotoxic the samples that present a minimum reduction of 50% of cells counting (Mendez *et al.*, 2008; Paim *et al.*, 2011). For LDH determination, the supernatant of these treated cells was used according the enzymatic assay employed LDH kit available from Laborclin™ (São José do Rio Preto,

São Paulo, Brazil). The results were expressed as percentage, considering the Triton treatment as 100% of LDH releasing.

Liquid Chromatography-Tandem Mass Spectrometry Analysis

The analyses were performed on as Shimadzu UFLC-20AD system coupled to Diode Array Detector (DAD) and an ESI-ion trap mass spectrometer (AmaZon SL, Bruker Daltonics). An Luna C18 column (5 µm, 4,6 mm x 250 mm, Phenomenex) was applied to chromatography analyses. The mobile phase was acetonitrile (solvent B) and water (solvent A), both containing 0.1% formic acid. The flow rate was 0.6 ml min-1 and the injection volume was 20 µl. The elution profile was the following: 2% B (0 to 3 min), 2 to 20% B (3 to 22 min), de, 20 to 30% B (22 to 30 min), 30 to 100% B (30 to 40 min), 100% B (40 to 45 min). The LC flow was split 3:7. The MS conditions were the following: capillary voltage of 3500 V, the nebulizer gas (nitrogen) pressure was set to 50 psi, a dry gas flow of 9 l/min and dry temperature was 300 °C. MS data were acquired in negative and positive ion mode. The samples were analyzed by mass range *m/z* 0-1200 (Fig. 1S and 2S, Supplementary Material).

MALDI-MS and MS/MS analyses

UltrafleXtreme MALDI-TOF/TOF equipment (BrukerDaltonics, Bremen, Germany) was used for the analyses. For analyses of the fractions, the matrixes 2,5-dihydroxibenzoic acid (DHB) and sinapinic acid (SA) was evaluated, but the better results were obtained with DHB, which is used for all protein analyses. The samples were analyzed by reflector (700 to 5000 Da and 4 to 20 kDa) and linear mode (4-20, 20-50 and 50-100 kDa). The samples were dissolved in acetonitrile (ACN):water with 0.1% trifluoroacetic acid (20:80, v/v) and the DHB matrix (20 mg/ml^{-1}) in ACN:H_2O (30:70, v/v) with 0.1% trifluoroacetic acid. The sample solution and the matrix (1:1 v/v) were mixed in equal amounts and,

spotted onto a ground stainless steel MALDI target (1 µl). The experimental conditions used were: pulsed ion extraction of 360 ns, laser frequency of 1000 Hz, positive ion mode and 5000 shots were averaged to record a mass spectrum.

The peptides, obtained from the digestion with trypsin, were analyzed by MALDI using a saturated solution of α-cyano-4-hydroxycinnamic acid (CCA) prepared in ACN:H_2O (30:70, v/v) with 0.1% trifluoroacetic acid. The samples were dissolved in ACN:water added 0.1% trifluoroacetic acid (30:70, v/v). The sample solution and the matrix (1:1 v/v) were mixed in equal amounts and, spotted onto a ground stainless steel MALDI target (1 µl). The peptide fractions were analyzed by MS and MS/MS. For MS analyses, the experimental conditions used were: pulsed ion extraction of 100 ns, laser frequency of 1000 Hz, reflectron mode, positive ion mode and 600 shots were averaged to record a mass spectrum. The ions selected were accelerated to 19 kV on the LIFT cell for MS/MS analyses.

External calibration were performed using peptide calibration standards (angiotensin II and I, substance P, bombesin, ACTH clip 1-17, ACTH clip 18-39 and somatostatin) and proteins standards I (insulin, ubiquitin I, cytochrom C and myoglobin) and II (trypsinogen, protein A and albumin-bovine (BSA) from Bruker Daltonics.

Database identification

MS/MS spectra were analysed and converted to mfg files using Mascot Distiller (MATRIX SCIENCES, London, UK). Protein identification was performed by database searches with a local Mascot Server v 2.3 (MATRIX SCIENCES, London, UK) against the proteome sequence of *Capsisum anuum* (Kim *et al.*, 2014). Parameters used for identification allowed a maximum of one missed tryosin cleavage, carbamidomethylation of cysteine, possible oxidation of methionine, peptide and MS/MS tolerance of 0.25 Da,

and a significance threshold set at $p < 0.05$. The sequences from MASCOT significant hits were used for annotation using the NCBI Blastp program and NCBI Conserved Domain Database.

Results

In the present study we report the anti-biofilm prospect of 2S sulfur-rich seed storage protein 2-like, a purified fraction (F9) from *C. baccatum,* in the search of antivirulence agents against clinically relevant bacteria.

Accordingly, the AqS was fractionated in 14 fractions (F1-F14) using Sephadex LH-20™ and all the fractions were screened for anti-biofilm and antibacterial activities against *S. epidermidis* ATCC 35984 as bio guided model of choice, at the concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml, in 96-well plates (data not shown).

In this screening, according the dose-response curve, the most noticeable activity was demonstrated by the highest concentrations tested: 2.0 and 4.0 mg/ml, for the fraction F9. The other fractions apparently had lower activity when compared to F9 (data not shown).

Once identified the main fraction (F9), with 1.4% of extraction yield, responsible for the activity and determined the best concentration at the dose-response, it was done the elucidation of the F9 employing MALDI-MS and LC-DAD-ESI-MS analyses. The identification and the discussion were based on F9 results, the most active fraction. Its analyses by LC-DAD-ESI-MS did not reveal secondary metabolites of low molecular weight (Figure 1S and 2S, Supplementary Material). In addition, it did not show ions up to *m/z* 4000 from MALDI-MS analyses, but many ions were observed in the range 4 to 20 kDa (Figure 1). The ion *m/z* 4305 showed the highest relative ion intensity in the mass spectrum of F9, besides the other intense ions were observed at *m/z* 4220, 4364, 9372 and

9415. In addition, some ions with lower relative ion intensities were detected from F9 in this same mass window. The fraction F9 was also analyzed by MALDI-MS using different mass range to determine the presence of other proteins with molecular weight very high. However, no ions were observed in mass range 20-100 kDa (Figure 3S and 4S, Supplementary Material). So the active fraction F9 was submitted electrophoresis gel and subsequently for digestion with trypsin to obtain the spots (P1, P2 and P3). The digested peptides were analyzed by MALDI-MS and MS/MS for the identification of protein using the proteome of *C. annum* as database. The three spots were identified as a protein belonging to the Alpha-Amylase Inhibitors (AAI), Lipid Transfer (LT) and Seed Storage (SS) Protein family, according to the NCBI CDD. The search for ortologs in Viridiplantae using NCBI Blastp led to the identification of 2S sulfur rich seed storage proteins from *Solanum* sp (Table 1).

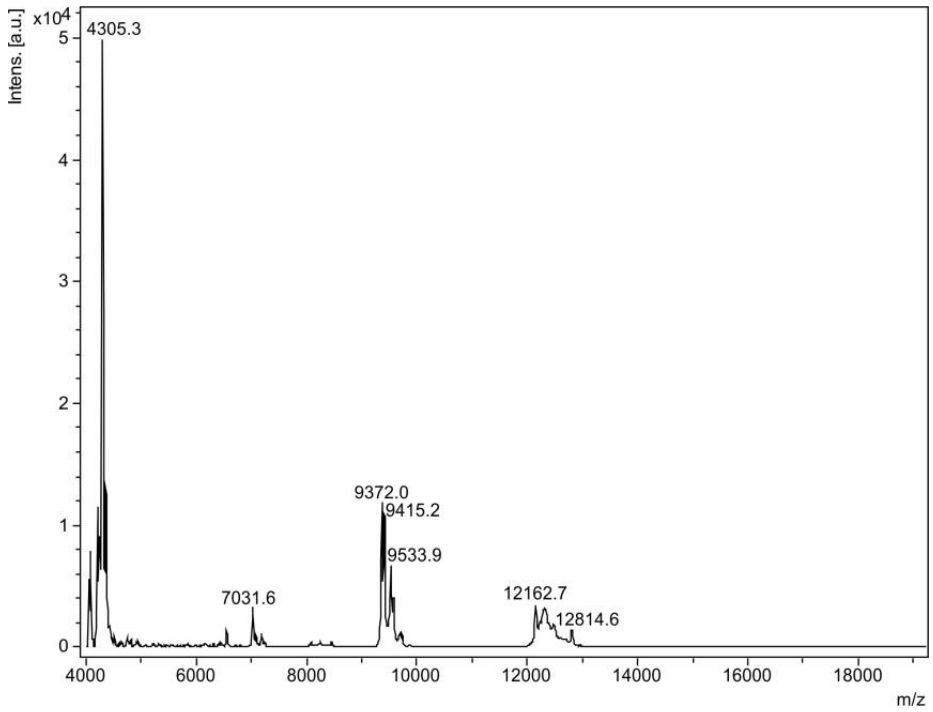

Figure 1. MALDI-MS mass spectra (positive ion mode) of fraction F9 obtained from the seeds of *C. baccatum.*

Table 1: Identification of proteins by MALDI-MS/MS

Peptide	MASCOT Identification*		Blast best Hit ID			
	ID	Score*	ID	Description	Pos. (%)	e-value
P1	Capana01g000302	132	XP_009621418.1	2S sulfur-rich seed storage protein 2-like (*Nicotiana tomentosiformis)*	77	2,00E-64
P2	Capana01g000310	217	XP_006358919.1	2S sulfur-rich seed storage protein 2-like (*Solanum tuberosum)*	83	3,00E-42
P3	Capana01g000309	65	XP_006358919.1	2S sulfur-rich seed storage protein 2-like (*Solanum tuberosum)*	83	3,00E-42

*For the *Capsicum* proteome database in Mascot, scores > 28 indicate identity or extensive homology ($p<0.05$)

The 2S sulfur-rich seed storage protein 2-like (2S sulfur-rich s.s.p. 2-like) was evaluated in anti-biofilm formation assay in comparison with the AqS. These results are shown in Figure 2. Biofilm formation was strongly prevented (96%) after 24 h of exposure by bacteria in relation to untreated cells. And, compared to the AqS (70%), the 2S sulfur-rich s.s.p. 2-like potentiated this effect.

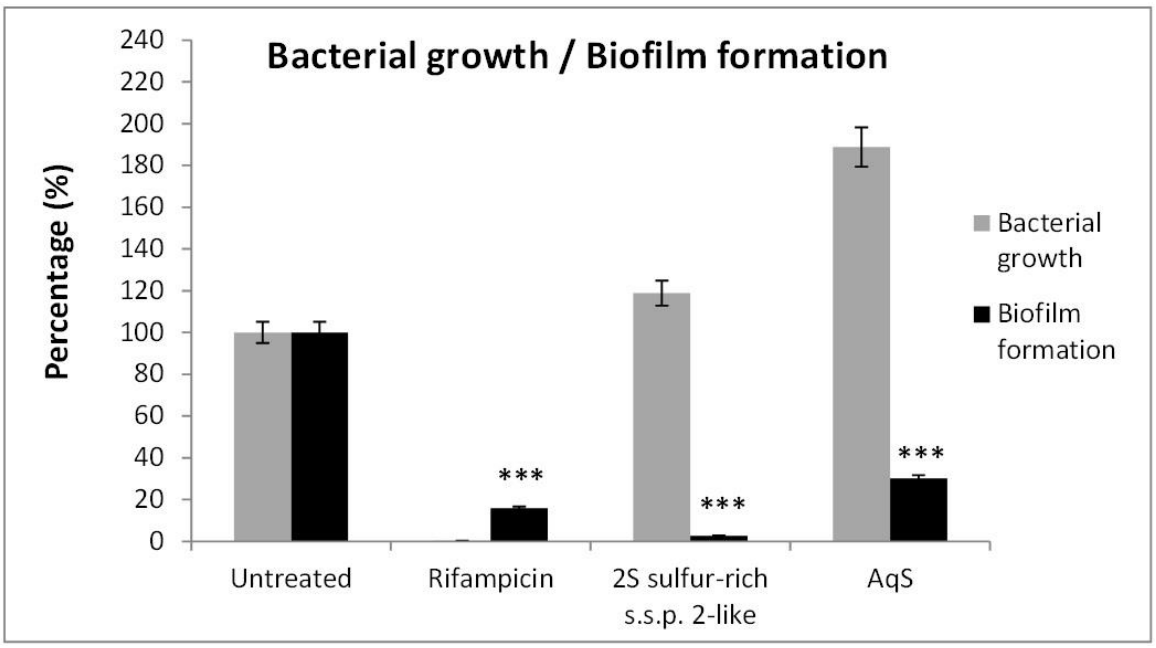

Figure 2. Bacterial growth and biofilm formation by *S. epidermidis* ATCC 35984, exposed to 2S sulfur-rich s.s.p. 2-like at 2 mg/ml or AqS (aqueous extract from the seeds) at 2 mg/ml, from *C. baccatum*. Untreated sample was used as positive (growth/biofilm) control and Rifampicin (16 µg/mL) as negative control. The absorbance results obtained were converted to percentage (%) values where the untreated sample is 100% of growth and biofilm formation. ***Represents statistical difference when compared to Untreated sample using the ANOVA variance analysis followed by Tukey's post hoc and the statistical significance was accepted for p values <0,05.

Excellently, 2S sulfur-rich s.s.p. 2-like did not inhibit planktonic growth of the bacteria in the concentration tested as well as the AqS, shown in the OD_{600} measurement (Figure 2). Therefore, to confirm the absence of effect of 2S sulfur-rich s.s.p. 2-like upon *S. epidermidis* growth according to incubation time, a kinetic assay was performed (Figure 3). There was not decrease in the bacterial growth in any period of exposure (0, 1, 3, 6, 12, 24 h) to 2S sulfur-rich s.s.p. 2-like. Instead of it, 2S sulfur-rich s.s.p. 2-like-treated bacteria had similar kinetic performance to untreated cells. A completely different dynamic profile was observed to rifampicin-treated bacteria that decreased the counting cells until the absolutely absence of them. This way, we could demonstrate that 2S sulfur-rich s.s.p. 2-like shows no antibacterial activity against *S. epidermidis*.

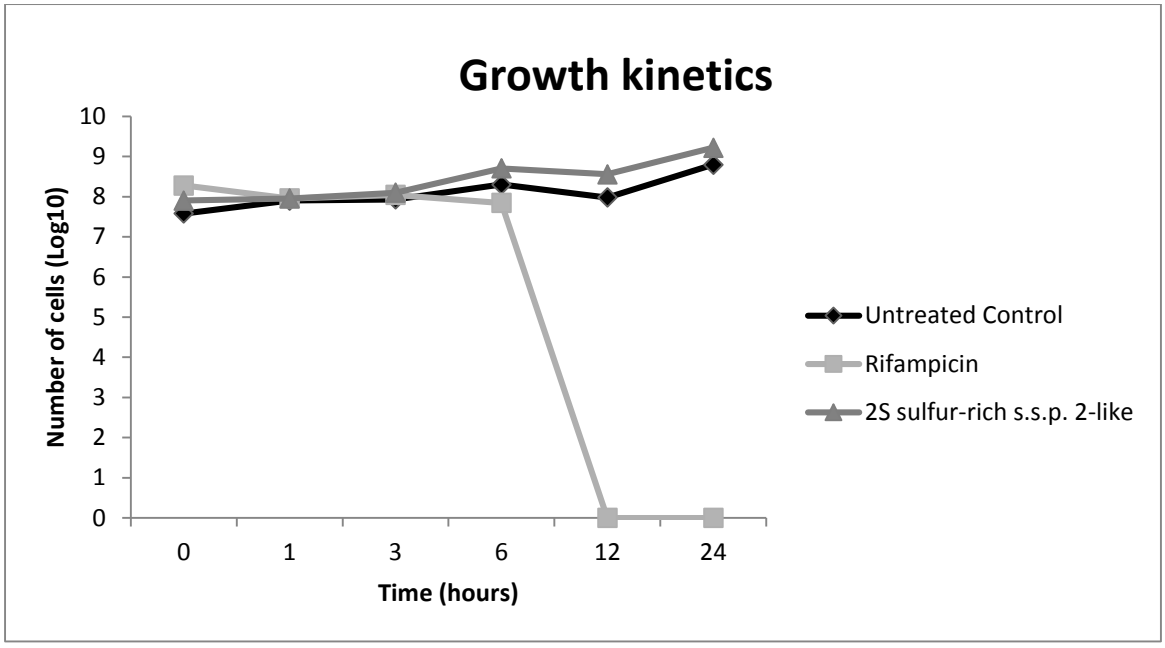

Figure 3. Growth kinetics of *S. epidermidis* ATCC 35984 exposed to 2S sulfur-rich s.s.p. 2-like at 2 mg/ml, from *C. baccatum,* for simultaneous colony counting and most probable number (MPN) enumeration, showed as number of cells (Log10). Rifampicin (16 µg/ml) was used as negative control.

To verify the promising prevent bacterial adhesion and biofilm related we analyzed by SEM, Permanox™ slides after surface modification with the incorporation of 2S sulfur-rich s.s.p. 2-like or AqS for Spin-Coating technique (Figure 4). The slides coated with 2S sulfur-rich s.s.p. 2-like monolayer at 2.0 mg/ml, strongly decreased bacterial adhesion compared to the controls. The slides coated with 2S sulfur-rich s.s.p. 2-like three layers at 2.0 mg/ml, showed only individual cells, there was no presence of matrix. Thus, when the slides were coated with 2S sulfur-rich s.s.p. 2-like, once or three layers, at the 10.0 mg/ml concentration, both showed completely absence of matrix or biofilm. Moreover, the slides coated with AqS appear to develop a similar response to the 2S sulfur-rich s.s.p. 2-like. In the opposite, the slides coated just with the vehicle (methanol, used to solubilize the samples) did not protect the Permanox™ against bacterial adhesion and biofilm formation as well as no coated controls.

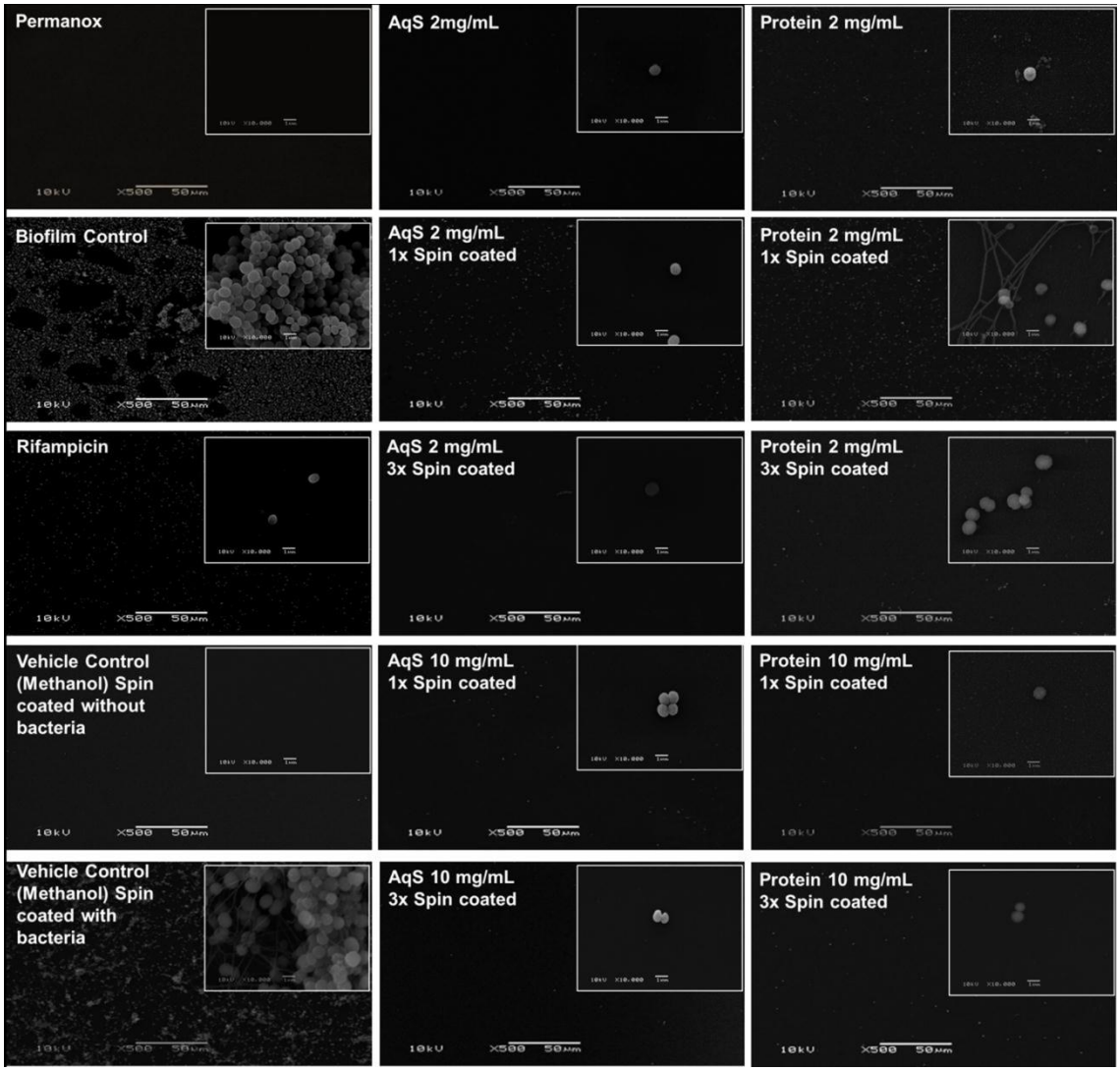

Figure 4. Scanning electron microscopy (SEM) images of Permanox slides after surface modifications: Protein (incorporation of 2S sulfur-rich s.s.p. 2-like, once or three times at 2 or 10 mg/ml), AqS (incorporation of aqueous extract from seeds, once or three times at 2 or 10 mg/ml) or Methanol (incorporation of methanol as vehicle control, with/without bacteria) for Spin-Coating technique. And, the controls groups without surface modifications: Permanox™, Biofilm Control (as untreated control) and Rifampicin. Scale bars: 500 and 10,000 x magnification.

Furthermore, with the images of the SEM was possible to estimate the quality of the coated layers over the Permanox™. There were no disrupted spaces and the layer seemed to be uniform over entire surface visualized. The 2S sulfur-rich s.s.p. 2-like and AqS coated surfaces had a water contact angle (WCA) of about 75º, both, decreasing up to 18º and 6º, respectively when coated three times with 10 mg/ml, while non-coated and 70% methanol coated surfaces kept hydrophobic, with WCA of 87 and 92, respectively (Figure

5). These measurements showed a decrease in the contact angle of coated surfaces (2S sulfur-rich s.s.p. 2-like / AqS), concentration-dependent manner (consequently layers-dependent too), indicating a higher hydrophilic characteristic than non-coated controls.

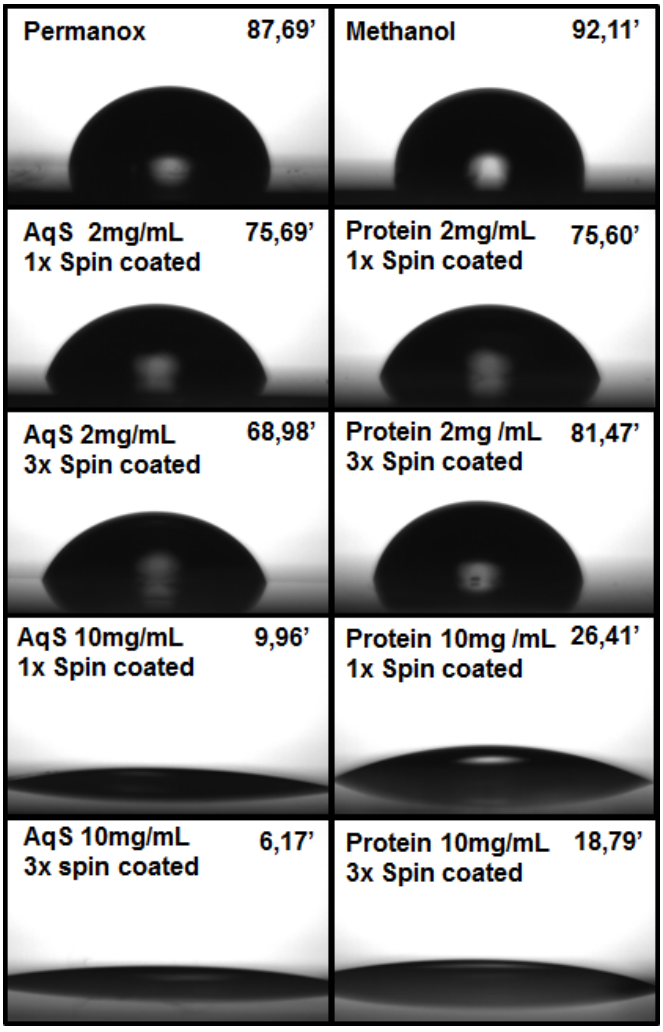

Figure 5. Water contact angle (WCA) of Permanox slides coated with Protein (2S sulfur-rich s.s.p. 2-like) or AqS (aqueous extract from seeds) from *C. baccatum*. The WCA are a mean of five measurements of different points of each sample, captured and analyzed by a Theta Lite Optical Tensiometer.

To preliminary evaluate the toxicity of the 2S sulfur-rich s.s.p. 2-like and AqS, a simple model to assess injury in human erythrocytes was carried out (Figure 6). At 2.0 mg/ml, 2S sulfur-rich s.s.p. 2-like was not hemolytic, preserving the erythrocytes integrity similar by as PBS, the negative control, although AqS at the concentrations tested (2.0 and 4.0

mg/ml) showed to be as hemolytic as Triton™, the positive control. The lymphocytes results assayed for 2S sulfur-rich s.s.p. 2-like (2.0 mg/ml) did not present cytotoxic effects (< 50% of reduction on cells counting) after 24 h of exposure (7.7% reduction of cells counting), neither after 48 h, nevertheless it showed a decrease of cell counting (42.3% reduction of cells counting) (Figure 7). In contrast, AqS samples at 2.0 and 4.0 mg/ml, presented cytotoxic effects with a considerable reduction of cells counting, 85.4% and 99.8% after 24 h and 82.7% and 99.48% after 48 h of exposure, respectively (Figure 7). The LDH assay indicate both 2S sulfur-rich s.s.p. 2-like and AqS did not affected the lymphocytes membrane integrity (Triton was 100% of LDH quantification reference, no treated control with 17.3%, AqS 2 mg/ml with 16%, AqS 4 mg/ml with 20% and 2S sulfur-rich s.s.p. 2-like with 14.7%).

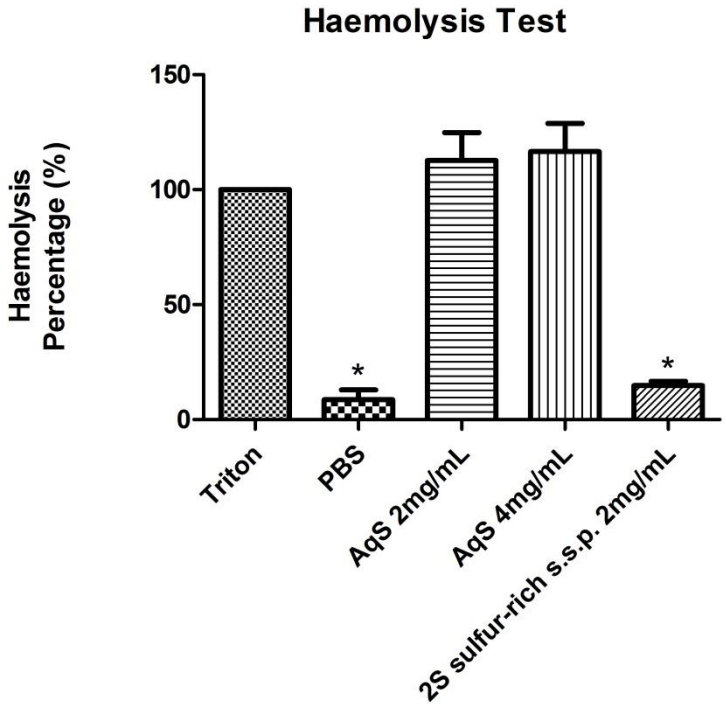

Figure 6. Haemolysis test by human erythrocytes exposed to 2S sulfur-rich seed storage protein 2-like at 2 mg/ml and AqS (aqueous extract from the seeds), at 2 and 4 mg/ml, from *C. baccatum*. The tested samples were statically compared to reference sample, 1% Triton (100% of hemolysis) and to the negative control, phosphate-buffered saline (PBS) (no hemolytic). * Represents statistical difference when compared to the reference, 1% Triton using the ANOVA variance analysis followed by Tukey's post hoc and the statistical significance was accepted for p values <0,05.

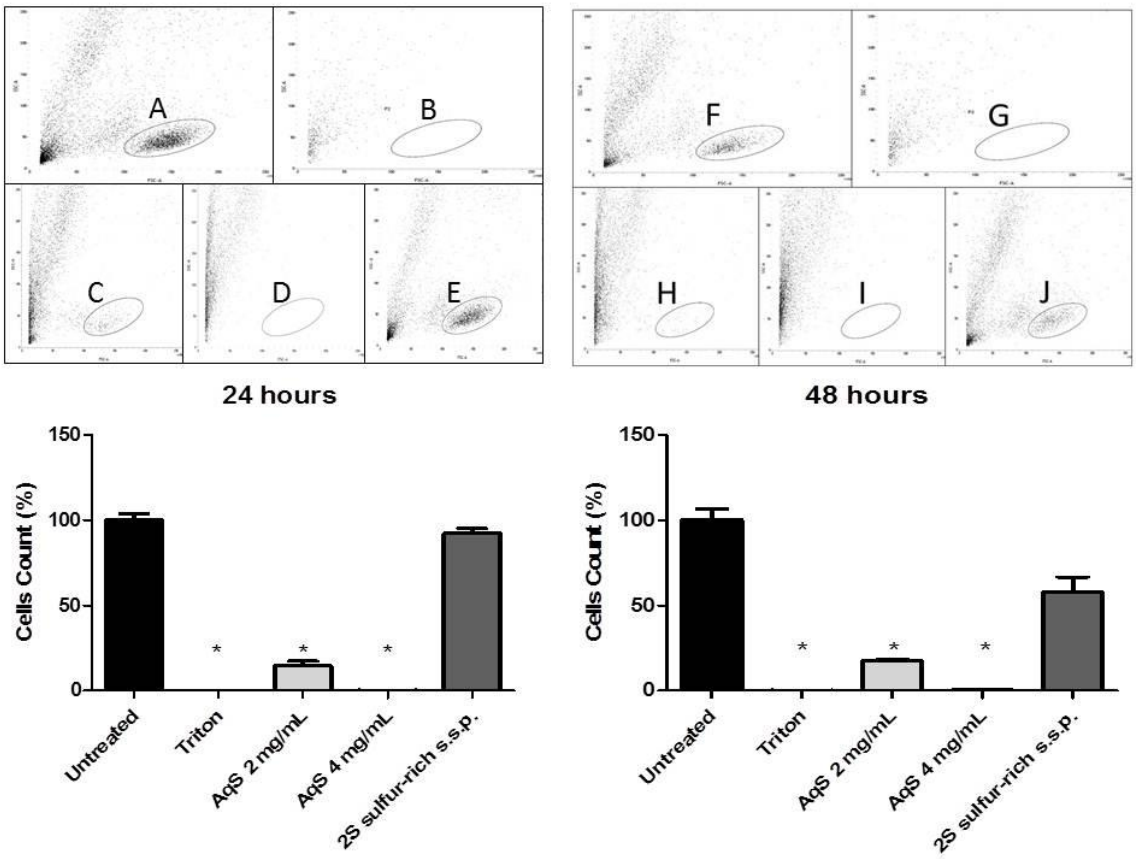

Figure 7. Lymphocytes test by human isolated cells, after 24 and 48 h of exposure to 2S sulfur-rich s.s.p. 2-like at 2.0 mg/ml or AqS (aqueous extract from the seeds) at 2.0 and 4.0 mg/ml, from *C. baccatum*. The 1% Triton was used as negative control (death) and RPMI 1640 as positive control (untreated/live). Images obtained by flow cytometer are shown and A-E represents the samples exposed to 24 h of treatment and F-J represents the samples exposed to 48 h. (A/F) Untreated, (B/G) negative control, 1% Triton, (C,D/H,I) AqS treated cells at 2.0 and 4.0 mg/ml, respectively and (E/J) 2S sulfur-rich s.s.p. treated cells at 2.0 mg/ml. * Represents a minimum reduction of 50% of cells counting when compared to untreated sample and it was considered cytotoxic.

Discussion

Considering the previous results obtained with aqueous seeds extract (AqS) from *C. baccatum* which significantly inhibited biofilm formation by *S. epidermidis* and *P. aeruginosa* (previous results), in this study we aimed to identify the active compounds from AqS and evaluate its potential as antivirulence coat agent as well as its cytotoxicity *in vitro*.

The most active fraction was F9 that after 24 h of treatment strongly prevented the biofilm formation (Figure 2) of *S. epidermidis*, demonstrating that the bioguided fractionation from AqS potentiated considerably its effect.

The extracts from fruits of *C. baccatum*, a pepper widely consumed in Brazil, shows antioxidant, anti-inflammatory, antifungal properties and others (Kappel *et al.*, 2008; Zimmer *et al.*, 2012a), but there is scare biological and chemical information about the extracts obtained from seeds. So, the fractions obtained from seed extract firstly were analyzed by LC-DAD-ESI-MS, but secondary metabolites were not observed using this analytical tool. In addition, they were also analyzed by MALDI-MS to detect and identify compounds with high molecular weight. The MALDI-MS profiling of F9, the most active fraction, exhibited an array of ions from 4 kDa to 13 kDa and no ion was observed from mass window ranging from 13 kDa to 100 kDa. Therefore, the whole chemical data obtained from F9 suggested the activity is related to presence of proteins. So, it was submitted to electrophoresis and digestion with trypsin, and the peptides were analyzed by MALDI-MS/MS to identify the main proteins. They were identified as 2S sulfur-rich s.s.p. 2-like. The NCBI bank describes the 2S sulfur-rich s.s.p. 2-like into the domain of Alpha-Amylase Inhibitors (AAIs) and Seed Storage (SS) Protein subfamily. They are mainly present in the seeds of a variety of plants. AAIs play an important role in the natural defenses of plants against insects and pathogens such as fungi, bacteria and viruses. AAIs impede the digestion of plant starch and proteins by inhibiting digestive alpha-amylases and proteinases. Also included in this subfamily are SS proteins such as 2S albumin, gamma-gliadin, napin, and prolamin. Osborne (1924) classified the SS proteins into groups on the basis of their extraction and solubility in water (albumins), dilute saline (globulins), alcoholhater mixtures (prolamins), and dilute acid or alkali (glutelins). All SS proteins have a number of common properties as they are synthesized

at high levels in specific tissues and at certain stages of development, some of which are rich in sulfur amino acids and others of which are poor and their fractions are mixtures of components that exhibit polymorphism both within single genotypes and among genotypes of the same species. They are widely distributed in dicot seeds and despite differences in their subunit structure and synthesis, all the 2S albumins are compact globular proteins with conserved cysteine residues (Shewry *et al.*, 1995).

There are no previous studies demonstrating the relationship between 2S sulfur-rich s.s.p. 2-like and the anti-biofilm activity. This is the first report using *Capsicum* purified protein to investigate it. Plants are constantly exposed to a great variety of potentially pathogenic organisms and there are at least two mechanisms of pathogen resistance in plants: structures and compounds synthesized during the plant normal development (resistance factors) and the mechanism activated only after contact with the pathogen (induced resistance factors). After the interaction between the products of pathogenic organisms and the plant, it becomes the establishment of a series of alterations which include the activation and synthesis of defense peptides and proteins (Castro e Fontes, 2005).

Peptides are a common chemical composition in the seeds from *Capsicum* genus as well as Antimicrobial peptides (AMPs), members of lipid transfer protein (LTPs), members of the thionin family and members of the plant defensins family (Lee *et al.*, 2004; Dias *et al.*, 2013). These peptides serve as an ancient defense mechanism against a wide range of microorganisms including bacteria, protozoa, yeast, fungi and viruses that easily come into contact with the host through the environment. AMPs are important components of the first line defense of innate immune systems in various species, including mammals, amphibians, insects and plants. Protease Inhibitors (PIs) are generally considered to act as storage proteins (nitrogen source) and are ubiquitously expressed in seeds and tubers or inducible expressed in response to pest and pathogen attack (Ribeiro *et al.*, 2007; Ribeiro

et al., 2012; Ribeiro *et al.*, 2013). PI-II, have been recently identified in several medical applications as anticarcinogenic, chemoprevention agents of human prostate cancer, anti-inflammatory, radioprotector, and bioprotector of other bioactive compounds (Carrillo-Montes *et al.*, 2014). A few reports suggest that protein fractions plant-derived can exhibit antiadhesive properties (Lengsfeld *et al.*, 2004; Wittschier *et al.*, 2007; Bensch *et al.*, 2011). AMPs represent a potential promising source for the development of new alternative agents to combat resistant bacteria. Their actions are related to physical properties and it is more difficult for bacteria to develop resistance to such peptides. Furthermore, the literature shows that some others peptides isolated from different sources have been investigated for their anti-biofilm activity against several bacterial strains, including cathelicidin (Amer *et al.*, 2010; Kanthawong *et al.*, 2012), histatin, mucins (Wei *et al.*, 2006; Da Silva *et al.*, 2012), magainins (Beckloff *et al.*, 2007; Neiva *et al.*, 2013), pleurocidin (Choi e Lee, 2012), and lactoferrin (Dashper *et al.*, 2012). Like dermaseptins, these peptides are cationic and unstructured and fold into amphipathic αhelices upon contact with membranes (Zaïri *et al.*, 2014).

Considering the ability of microorganisms to form an extracellular polymeric matrix, to aggregate and to attach to surfaces, they have an evolutionary advantage which is phenotypically beneficial to increase survival and symbiotic relationships to be established within the biofilm environment. Our results with 2S sulfur-rich s.s.p. 2-like as anti-biofilm agent showed the capable to prevent this attachment and consequently discontinue this phenotypical advantageous. In staphylococci, these important bacterial survival mechanisms are defined by two quorum-sensing systems: the *agr* and the *luxS.* The attachment to an abiotic surface is dependent on the physicochemical characteristics of the material and bacterial surfaces through hydrophobic or electrostatic interactions and the capsular polysaccharide adhesion, a nonspecific adhesion, is responsible for this

process, whereas slime-associated antigen is linked to accumulation and biofilm maturation. A secondary attachment of *S. epidermidis* is improved by the presence of a specific cell adhesion (AtlE), which binds to plasma proteins such as vitronectin. Furthermore, teichoic acids are the greatest source of polyanionic charge on the staphylococcal bacterial cell envelope, being an important component of the matrix. Biofilm maturation comprises adhesive processes that link bacteria together during proliferation and disruptive processes that form channels in the biofilm structure (necessary for nutrients to reach cells in deeper biofilm layers). The importance of biofilm detachment for infection resides in the dissemination of infection and may cause acute, nonbiofilm infections, such as sepsis (Laverty *et al.*, 2013; Otto, 2013). Host defenses mechanisms against bacteria organized in biofilms are not completely understood and are still under investigation. There are some studies that investigate the role of polymorphonuclear neutrophils (PMNs) against staphylococcal biofilms (Wagner *et al.*, 2003; Wagner *et al.*, 2006), giving evidence to the infiltration of these cells in biofilm, to their different effects in destruction of either *S. aureus* or *S. epidermidis* biofilms, and also to the damage that these cells cause to the host tissues at the site of infection (Arciola *et al.*, 2012).

Several alternative nonbiocidal strategies that target molecular related to virulence factors have been proposed (Travier *et al.*, 2013). In this context, the 2S sulfur-rich s.s.p. 2-like demonstrated to prevent biofilm formation without inhibitory effect against *S. epidermidis,* according to a kinetic assay (Figure 3).

Moreover, these approaches include: reducing adhesion to prevent biofilm formation and related infections as modification of the biomaterial surface to confer anti-adhesive properties (coatings). In addition, the Permanox™ slides coated with 2S sulfur-rich s.s.p. 2-like once layer at 2.0 mg/ml, decreased biofilm and with three layers, completely

prevented its formation. When the slides were coated once or three layers, at the 10.0 mg/ml, both showed completely absence of matrix or biofilm formation showing to be effective as anti-adhesive agent (Figure 4). In accordance, the changes in contact angles measurements of the substrates, contribute to verify the efficacy of the coating process indicating the deposition of the bioactive compound. Attachment to an abiotic surface, such as the plastic surface of an indwelling medical device, is dependent on the physicochemical characteristics of the device and bacterial surfaces. This type of attachment is thus driven mostly by hydrophobic or electrostatic interactions (Otto, 2013). The higher hydrophilic surface character obtained on the coated material, contributed with the reduction on bacterial adhesion (Nostro *et al.*, 2012). Despites the many existing theoretical models trying to describe bacterial adhesion, the degree of hydrophobicity of the staphylococcal cell surface and that of the matching biomaterial surface are generally considered highly important for the first attachment (Legeay *et al.*, 2006; Arciola *et al.*, 2012).

A recently study of our research group demonstrated that crude ethanol and butanol extracts from fruits of *C. baccatum* have a level of pharmacological safety for oral administration, during 60 days in mice. In fact, animals showed normal locomotor ability, blood cells counts, lipid and glucose homeostasis, as well as tissue-specific markers of functionality/damage (Zimmer *et al.*, 2012b). However, AqS was hemolytic and toxic against lymphocytes cells *in vitro.* Although, 2S sulfur-rich s.s.p. 2-like was not cytotoxic and preserved the human erythrocytes and lymphocytes after 24 h of treatment. After 48 h, the peptides few decreased the lymphocytes counting number but it was not significant and probably occurred due to *in vitro* situation with the absence of an elimination system to minimize it. The type of cell death was estimated using LDH release assay, a key signature of necrosis, indicating that both treatments were not

necrotic due to the low release of LDH compared to Triton-treated cells. They showed no plasma membrane perturbations related to 2S sulfur-rich s.s.p. 2-like and AqS treatments (they were similar to no-treated cells). These effects contribute to the successful of the purification process of F9 that potentiated the anti-biofilm activity of AqS and absolutely reduced its cytotoxicity.

Finally, to succeed in the combat against bacterial resistance, is necessary to aim the efforts in each specific pathogen and virulence mechanisms considering bio-interactions as well as related to infection-resistant medical materials. Furthermore, the identification of novel molecules designed specifically to target mechanisms involved in biofilm formation or tolerance towards to novel therapies designed to be combined with antibiotics against bacterial biofilm-associated infections. Accordingly, 2S sulfur-rich s.s.p. 2-like isolated from *C. baccatum* var. *pendulum* presents and supports a new prime structure as anti-biofilm agent. Its structure can be engineered with relative ease due to peptides chemistry allows a multitude of modifications that are relatively time and cost effective, corroborating to the search of new non-antibiotic strategies against the drug-resistance war.

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Supplementary Material

2S sulfur-rich seed storage protein 2-like, from Red pepper *Capsicum baccatum* var. *pendulum* (Solanaceae), are potential ant-biofilm agent as antivirulence therapy against clinical relevance bacteria

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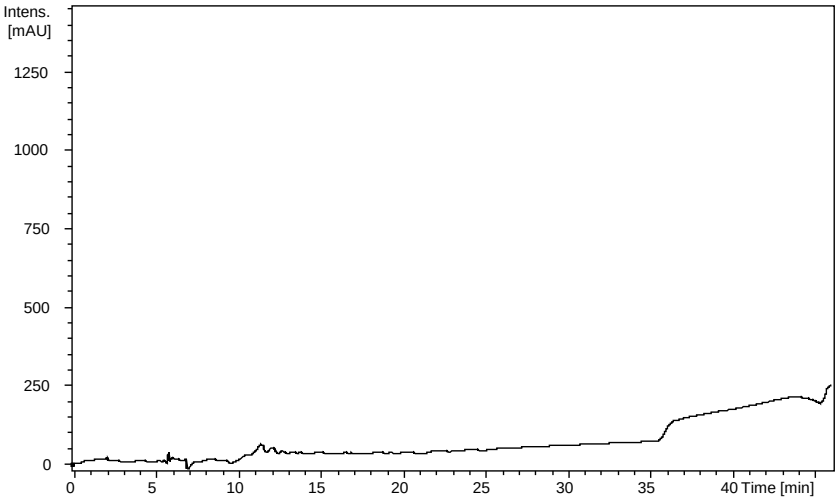

Figure 1S. LC-DAD profile (λ= 230 nm) of the fraction F9.

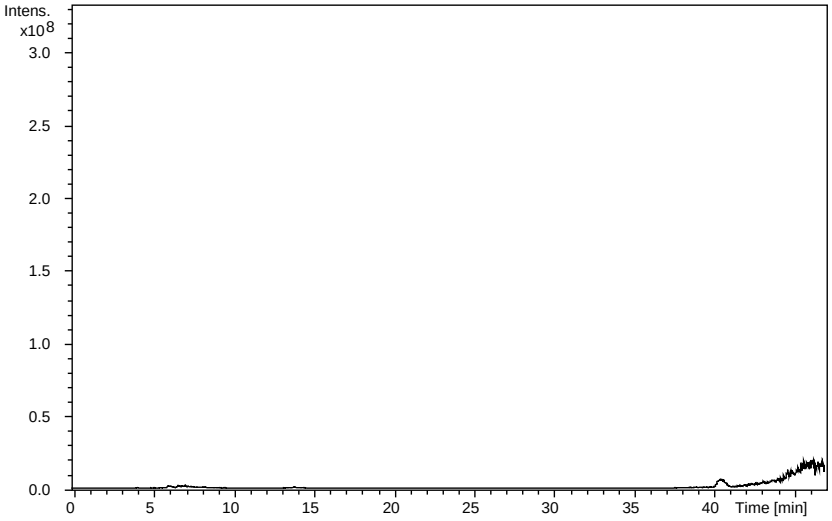

Figure 2S. LC-MS profile of the fraction F9 (positive ion mode).

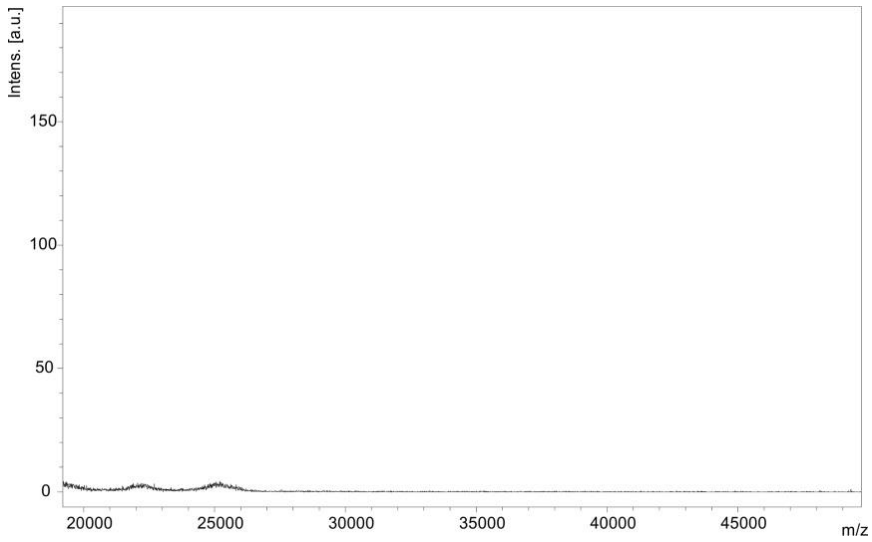

Figure 3S. MALDI-MS of fraction F9 obtained from *Capsicum baccatum* (linear mode, 20-50 kDa).

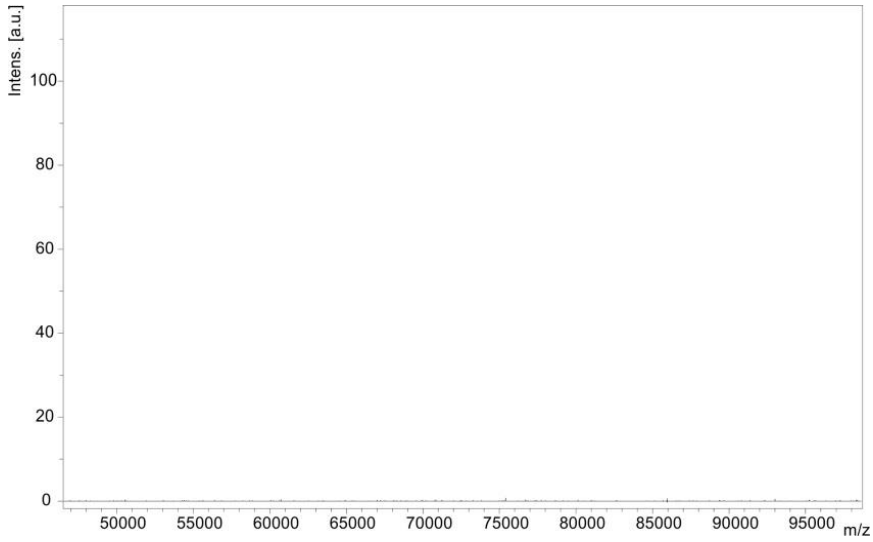

Figure 4S. MALDI-MS of fraction F9 obtained from *Capsicum baccatum* (linear mode, 45-100 kDa).

Discussão

Este é o primeiro estudo a demonstrar o potencial antibiofilme do extrato e de produtos isolados da pimenta vermelha *C. bacattum* var. *pendulum*, frente a bactérias de relevância clínica, como proposta de agentes antivirulência.

Muitos esforços estão sendo feitos para entender as bases moleculares da formação de biofilme em *Staphylococcus,* principalmente *S. epidermidis,* em função de este micro-organismo ser a causa mais frequente de infecções associadas a biofilme, que resultam em sepse (Laverty *et al.*, 2013; Otto, 2013). Nesse sentido, a matriz do biofilme além de conferir estabilidade mecânica para o biofilme, torna irreversível a adesão da bactéria a superfícies. Alguns estudos lançados na 6ª Conferência Internacional em Biofilme em 2012 debateram a importância da composição dessas estruturas através de análises bioquímicas e genéticas das matrizes, da morfologia rugosa das colônias formadas por elas, entre outros vários parâmetros, na prerrogativa de corroborar com a pesquisa de novos fármacos capazes de impedir a formação do biofilme ou mesmo desorganizar essas estruturas impedindo a infecção (Haussler e Fuqua, 2013).

Neste contexto, as plantas produzem muitos compostos que são biologicamente ativos, tanto para o seu crescimento e desenvolvimento normal, quanto em resposta ao ataque de patógenos e ao estresse (Trentin *et al.*, 2011). O extrato aquoso das sementes de *C. baccatum* (AqS, 2 mg/mL), quando testado frente a *S. epidermidis* e *P. aeruginosa*, foi o mais promissor entre os avaliados, não apresentando atividade antimicrobiana, além de inibir fortemente o biofilme em 80% e 60%, respectivamente. Dessa forma, é de conhecimento que a inibição da adesão a um substrato acontece mais facilmente do que a inibição do crescimento de um biofilme já estabelecido (maduro), portanto, o pré-tratamento de superfícies com derivados de plantas que possuem atividade antibiofilme, produz uma película desfavorável para a bactéria, reduzindo assim a sua adesão a essas superfícies (Sandasi *et al.*, 2010).

Nesse sentido, nossos ensaios fitoquímicos apontaram para a presença de aminoácidos/proteínas, polifenóis, taninos e terpenos no AqS. De acordo com a literatura, algumas proteínas de origem vegetal, incluindo isolados de *Capsicum*, têm sido relacionadas a importantes atividades antimicrobiana, antifúngica e anti adesivas (Lee *et al.*, 2004; Lengsfeld *et al.*, 2004; Ribeiro *et al.*, 2007; Fernando *et al.*, 2011; Dias *et al.*, 2013); os polifenóis apresentam efeito antimicrobiano sobre micro-organismos em biofilmes, incluindo o *S. epidermidis* (Ferrazzano *et al.*, 2009; Sampaio *et al.*, 2009; Prabhakar *et al.*, 2010; Schito *et al.*, 2011; Trentin *et al.*, 2011); aos taninos também já foi atribuída propriedades anti adesivas (Wittschier *et al.*, 2007); e os terpenos exibem atividade antimicrobiana (excluindo óleos voláteis) (Sparg *et al.*, 2004; Zhang *et al.*, 2008; Popova *et al.*, 2009).

Na sequência do estudo, uma vez estabelecido o AqS como o extrato mais ativo, realizamos o processo de fracionamento bioguiado na busca pela fração mais ativa (AF). Selecionamos para tal, o *S. epidermidis* ATCC 35984, por se tratar de um modelo bem estabelecido de formação e desenvolvimento de biofilme (Mulcahy *et al.*, 2008) e pela habilidade de formação de biofilmes em biomateriais ser apontada como o seu maior fator de virulência (Otto, 2008). Além disso, o AqS demonstrou resultados mais promissores frente a essa bactéria.

A AF obtida inibiu em mais de 96% a formação do biofilme de *S. epidermidis*, mantendo a ausência de atividade antimicrobiana e evidenciando uma potenciação do efeito do AqS. Ambos, AqS e AF não inibiram o crescimento de *S. epidermidis*, indicando que o mecanismo envolvido na inibição de biofilme é independente da morte bacteriana. Deste modo, a redução na adesão bacteriana e na formação de biofilme por uma via que não envolva a morte celular é uma característica marcante e contempla um novo conceito em terapias antivirulência, com o objetivo de tornar os micro-organismos mais susceptíveis a agentes antimicrobianos e ao sistema imunológico.

Várias estratégias alternativas não biocidas, que têm como alvo molecular principalmente os fatores de virulência, têm sido propostas para a inativação do

biofilme, assim como o recobrimento de biomaterias com agentes de propriedade anti adesivas (Arciola *et al.*, 2012). Visando essa proposta, nós recobrimos lâminas de Permanox® com uma única camada de AqS ou AF, ou com três camadas, nas concentrações de 2 e 10 mg/mL, e avaliamos a sua capacidade de impedir a formação de biofilme nesse material quando exposto ao modelo de *S. epidermidis*. Para isso, as lâminas foram recobertas através da técnica de *Spin-Coating* e avaliadas posteriormente por imagens de Microscopia Eletrônica de Varredura (MEV). Por conseguinte, as atividades demonstradas para AqS e AF foram confirmadas, pois foi possível identificar que ambos diminuíram significativamente a formação de biofilme, protegendo as lâminas de Permanox™ contra a adesão bacteriana. Em especial, quando recobertas três vezes (2 mg/mL) e com uma única camada (10 mg/mL), foi observado a ausência total de matriz e consequentemente a inexistência de formação de biofilme (Flemming e Wingender, 2010).

Nossos ensaios citotoxicológicos iniciais apontam que o isolamento da AF foi efetivo não só na potenciação do efeito biológico, mas também no aspecto toxicológico. Os resultados evidenciaram que o AqS foi tóxico às células sanguíneas gerando hemólise, bem como reduziu significativamente o número de linfócitos em ambas as concentrações testadas (2 e 4 mg/mL) após 24 e 48 h. Já a AF manteve os eritrócitos humanos intactos e não apresentou toxicidade sobre os linfócitos em 24 h de tratamento na concentração de 2 mg/mL. Embora após 48h de tratamento houve uma redução no número de linfócitos, essa redução não foi significativa. Consideremos ainda, o fato de que em condições experimentais *in vitro* não existem sistemas de depuração e depleção. Dessa maneira, o isolamento da AF além de potencializar o efeito antibiofilme do AqS, reduziu significativamente a sua toxicidade.

Ainda, a fim de realizar a elucidação da composição química da AF, realizamos uma gama de análises espectrométricas na busca direta por massas e perfis de fragmentação dessa fração em massas MALDI-MS e MALDI-MS/MS. Encontramos primeiramente massas compatíveis com proteínas e na sequência,

submetemos a fração a um processo de separação por eletroforese e digestão com tripsina. Após, repetimos as análises de massas dos peptídeos obtidos e identificamos os *hits* significativos no MASCOT e analisamos esses resultados no programa *NCBI Blastp* e *NCBI Conserved Domain Database,* utilizando o proteoma de *C. annum* como *Database*. Identificamos assim uma proteína pertencente à família dos Inibidores da Alfa-Amilase (AAI), de Transferência de Lipídeos (LT) e de Armazenamento nas Sementes (SS), de acordo com o *NCBI CDD*. A busca por *ortologs* em *Viridiplantae*, usando *NCBI Blastp*, levou à identificação da proteína *2S sulfur-rich seed storage protein 2-like* de *Solanum* sp, com alto índice de homologia.

Essas proteínas estão presentes principalmente nas sementes de uma variedade de plantas, principalmente em dicotiledôneas. As AAI desempenham um papel importante na defesa natural das plantas contra insetos e patógenos tais como fungos, bactérias e vírus, além de impedirem a digestão do amido e de proteínas nessas plantas. Todas as proteínas SS têm algumas propriedades comuns e são sintetizadas em níveis elevados em tecidos específicos, em certas fases do desenvolvimento, sendo que alguns aminoácidos são ricos em enxofre e outros não (Shewry *et al.*, 1995).

As plantas estão em constante presença de uma grande variedade de patógenos e para se defender sintetizam fatores de proteção ou fatores de proteção induzida, como a ativação ou síntese de peptídeos e proteínas de defesa (Castro e Fontes, 2005). Esses peptídeos são componentes químicos comuns nas sementes do gênero *Capsicum*, assim como os peptídeos antimicrobianos (AMPs), membros de proteínas de transferência lipídica (LTPs), membros das famílias das tioninas e das defensinas ou ainda dos inibidores de proteases (PIs) (Lee *et al.*, 2004; Ribeiro *et al.*, 2007; Ribeiro *et al.*, 2012; Dias *et al.*, 2013; Ribeiro *et al.*, 2013). Algumas aplicações farmacológicas têm sido identificadas para esses peptídeos, em especial os PIs, como anticarcinogêncico, químiopreventivo para câncer de próstata, anti-inflamatório e radioprotetor (Carrillo-Montes *et al.*, 2014). Mas o mais relevante no nosso contexto é que alguns trabalhos sugerem que essas proteínas derivadas de

plantas podem exibir atividades anti adesivas assim como as demonstradas neste estudo (Lengsfeld *et al.*, 2004; Wittschier *et al.*, 2007; Bensch *et al.*, 2011).

Um resumo das atividades realizadas e aqui discutidas está apresentado na figura 3, em forma de uma linha do tempo.

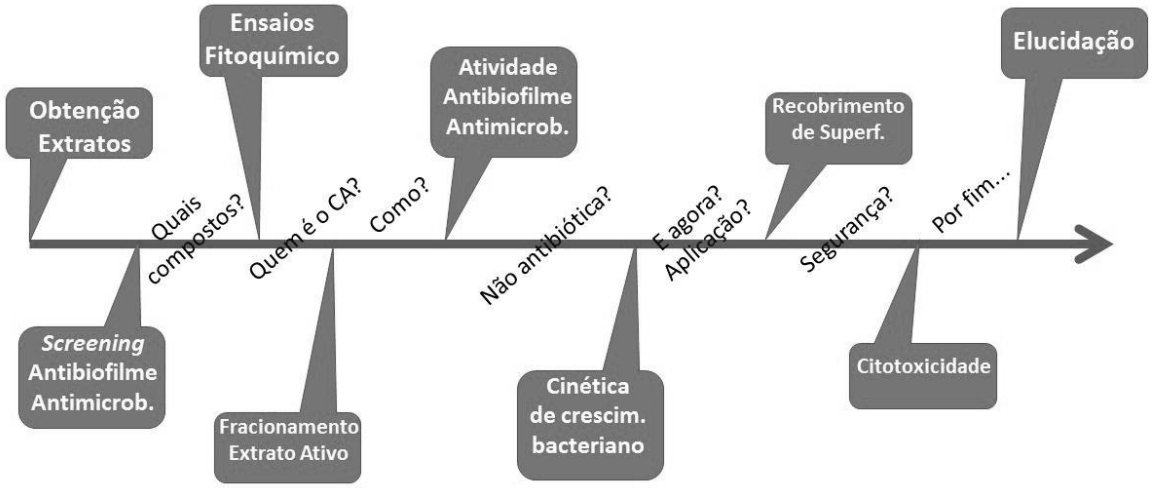

Figura 3. Ilustração em linha do tempo, das atividades e dos questionamentos que guiaram o desenvolvimento desse trabalho de pesquisa.

Conclusões

A resistência bacteriana aos antibióticos e o sistema imunológico deficiente do hospedeiro são a primeira causa de morte em unidades de tratamento intensivo em todo o mundo. A rápida emergência de resistência a antibióticos ocorre devido a várias condições, como adquirida através de mutações ou a transferência de genes horizontal, e resulta também da aquisição de alta tolerância a antibióticos exibida pelas comunidades bacterianas em biofilmes que crescem sobre a superfície de implantes médicos contaminados (Madeo e Frieri, 2013; Scopel *et al.*, 2013; Travier *et al.*, 2013).

Dessa maneira, com grande relevância, identificamos no extrato aquoso de *C. baccatum* e em produtos isolados, como a fração purificada de proteínas *2S sulfur-rich seed storage protein 2-like*, novos agentes com ação antibiofilme que podem colaborar dificultando o rápido desenvolvimento de resistência bacteriana, como terapia anti-virulência. Demonstramos, portanto, que o recobrimento de materiais com *2S sulfur-rich seed storage protein 2-like*, pode contribuir como uma nova estratégia não biocida na redução da contaminação de implantes médicos, diminuindo a exposição dos pacientes a essa importante fonte de infecções. Ainda, o seu isolamento foi efetivo também quanto à baixa toxicidade apresentada em relação ao extrato, nos ensaios de citotoxicidade frente a eritrócitos e linfócitos humanos, o que indica possíveis indícios de segurança farmacológica.

Considerando esse impactante contexto, este é o primeiro estudo a avaliar e reportar a atividade antibiofilme de *C. baccatum var. pendulum* (Solanaceae). Nossos resultados fundamentam o potencial farmacológico dessa importante espécie, amplamente difundida e de grande valor econômico, como inibidor da formação de biofilme e encorajam a busca por novas atividades. Assim, apontamos que *C. baccatum* é uma importante fonte natural e ethnofarmacológica na busca de produtos bioativos inovadores.

Em consonância com os resultados obtidos até o momento, a identificação da molécula ativa com ação antibiofilme, *2S sulfur-rich seed storage protein 2-like*, nos estimula na continuidade dessa pesquisa, pois, a sua estrutura proteica pode ser facilmente engenhada em função da química dos seus peptídeos permitirem múltiplas modificações que são de relativo custo e tempo efetivo, corroborando com a busca de novas estratégias não antibióticas contra a resistência bacteriana.

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