UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Estratégias de combate à adesão de bactérias patogênicas e formação de biofilmes: prospecção de fitocompostos e modificações de superfícies visando uso biomédico

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Tese apresentada por **Danielle da Silva Trentin** para a obtenção de TÍTULO DE DOUTOR em Ciências Farmacêuticas

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“Os nossos pais amam-nos porque somos seus filhos, é um fato inalterável. Nos momentos de sucesso, isso pode parecer irrelevante, mas nas ocasiões de fracasso, oferecem um consolo e uma segurança que não se encontram em qualquer outro lugar.”

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Goethe

RESUMO

A maioria das bactérias não cresce como células individuais, mas em comunidades estruturadas como organismos pseudomulticelulares, ou biofilmes, estando presentes em praticamente todos os ecossistemas naturais e patogênicos. A adesão bacteriana à superfície e subsequente formação de biofilme promove mudanças metabólicas, fenotípicas e genotípicas que faz com que a sua erradicação seja extremamente difícil. Desta maneira, microrganismos que apresentam susceptibilidade a determinados antimicrobianos em testes laboratoriais convencionais, são na verdade altamente resistentes aos mesmos quando na forma de biofilmes. Bactérias na forma de biofilmes estão associadas com aproximadamente 80% das infecções médicas, ganhando destaque naquelas relacionadas a implantes médicos. Com o aumento da expectativa de vida humana, maior é a necessidade de substituição e reparo de funções biológicas e, portanto é estimado um aumento no número de pessoas hospitalizadas e que irão receber implantes biomédicos. Esses materiais, independente do seu nível de sofisticação, estão suscetíveis ao risco de colonização microbiana e infecção. O presente trabalho, conduzido de forma multidisclinar, utilizou microrganismos patogênicos e superfícies modelo, para demonstrar provas de conceito com relação a duas estratégias para o combate da formação de biofilmes de bactérias: (i) a busca por fitocompostos com atividade antiformação de biofilmes, guiado por relatos etnofarmacológicos, e o posterior recobrimento de superfície polimérica com estes compostos e, (ii) a modificação de propriedades de superfícies, através da técnica de plasma iônico, para a obtenção de superfícies antiadesivas. Assim, 45 extratos aquosos foram obtidos de 24 plantas utilizadas na medicina tradicional do bioma Caatinga. O rastreamento de atividade antibiofime e antibacteriana (nas concentrações de 0,4 e 4,0 mg/mL) evidenciou o alto potencial antibiofilme de extratos contra *Staphylococcus epidermidis* ATCC 35984 e indicou três plantas com atividade antimicrobiana para *Pseudomonas aeruginosa* ATCC 27853. Subsequentemente, o estudo foi focado na purificação dos compostos bioativos de quatro plantas: *Pityrocarpa moniliformis*, ativa contra *S. epidermidis* e, *Anadenanthera colubrina*, *Commiphora leptophloeos* e *Myracrodruon urundeuva*, ativas contra *P. aeruginosa*. O fracionamento bioguiado e a caracterização química das frações por MALDI MS MS demonstraram que os

compostos ativos nos quatros casos pertencem à classe dos taninos. Estruturas complexas de proantocianidinas (composta principalmente por profisetinidina para *A. colubrina* e por prorobinetinidina para *C. leptophloeos*), e de taninos hidrolisáveis (constituído por unidades de ácido gálico em *M. urundeuva*) foram identificadas. Estes taninos inibiram a formação de biofilme de *P. aeruginosa* através da ação bacteriostática, causando danos de membrana e excesso na proliferação de vacúolos bacterianos, embora a membrana de eritrócitos tenha sido preservada. Com relação à *P. moniliformis*, proantocianidinas ricas em prodelfinidina (0.125 mg/mL) foram os compostos responsáveis pela completa inibição da formação de biofilme de *S. epidermidis*, sem afetar a viabilidade do microrganismo. Como demonstrado por diversas técnicas, os resultados indicam que tanto a superfície bacteriana (*S. epidermidis*) quanto a superfície dos materiais testados (vidro e poliestireno) são espontaneamente recobertos pelas proantocianidinas, tornando-as superfícies hidrofílicas. Através da técnica de "spin coating", a superfície polimérica foi recoberta com estas proantocianidinas, convertendo-se em uma superfície fortemente hidrofílica. A habilidade de prevenir a adesão de *S. epidermidis* foi mantida e o material se mostrou compatível com as células epiteliais de mamíferos, indicando o grande potencial destes produtos naturais como agentes funcionais de recobrimento de superfícies. No outro enfoque deste estudo, a modificação de propriedades de superfícies via descarga de plasma dos gases N_2/H_2 produziu, de maneira rápida e bastante efetiva, superfícies de poliestireno capazes de impedir a adesão de bactérias altamente resistentes aos antimicrobianos. Através de espectroscopia de raio X, verificou-se que uma concentração de nitrogênio de 8,8% e a componente polar da energia de superfície superior a 15 mJ/m^2, são necessários para reduzir a adesão de bactérias que apresentam superfície hidrofílica (como enterobactérias produtoras de carbapenemase e MRSA), enquanto que cepas hidrofóbicas (exemplificadas pelo *S. epidermidis*) mantiveram a capacidade de aderir e formar biofilmes. As interações respulsivas explicam os efeitos antiadesivos obtidos, tanto no recobrimento de superfícies por proantocianidinas quanto no tratamento por plasma iônico.

Palavras-chave: adesão, biofilme, Caatinga, extratos aquosos, taninos, modificação de superfícies, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*

ABSTRACT

Strategies to combat adhesion and biofilm formation of pathogenic bacteria: phytocompounds prospecting and surface modifications aiming biomedical use

Most bacteria do not grow as individual cells, but in communities structured as pseudomulticelulares organisms, or biofilms, being present in virtually all natural ecosystems and pathogens. The bacterial adhesion to surfaces and subsequent biofilm formation promotes metabolic, phenotypic and genotypic changes, which makes their eradication extremely difficult. Thereby, microorganisms that exhibit susceptibility to antimicrobials during conventional laboratory tests are in fact highly resistant to them when in the form of biofilms. Bacteria living as biofilms are associated with approximately 80% of all medical infections, mainly those related to indewelling devices. With increasing life expectancy, greater is the need for replacement and repair biological functions and, therefore, it is estimated an increasing number of hospitalized people and who will receive biomedical implants. However, regardless of the level of material sophistication, all of them are susceptible to the risk of microbial colonization and infection. This study, conducted in a multidisclinar way, employed pathogenic microorganisms and surface models to demonstrate proofs of concept regarding two strategies to combat bacterial biofilm formation: (i) the search for phytocompounds having antibiofilm formation activity, guided by ethnopharmacological reports, and further the polymer surface coating with these compounds, and (ii) the modification of surface properties, by the ionic plasma discharge technique to obtain anti-adhesive surfaces. Thus, 45 aqueous extracts were obtained from 24 plants used in the tradicinal medicine of the Caatinga biome. The screening of antibiofim and antibacterial activities (at concentrations 0.4 and 4.0 mg/mL) showed the high antibiofilm potential of the extracts against *Staphylococcus epidermidis* ATCC 35984 and indicated three plants with antimicrobial activity against *Pseudomonas aeruginosa* ATCC 27853. Subsequently, the study was focused on the purification of bioactive compounds from four plants: *Pityrocarpa moniliformis*, active against *S. epidermidis* and, *Anadenanthera colubrina*, *Commiphora leptophloeos* and *Myracrodruon urundeuva*, active against *P. aeruginosa*. The bioguided fractionation and the chemical

characterization of the fractions by MALDI MS MS showed that the active compounds in the four cases belong to the class of tannins. Complex structures of proanthocyanidins (mainly composed profisetinidin for *A. colubrina* and prorobinetinidin for *C. leptophloeos*), and hydrolysable tannins (consisting of gallic acid units in *M. urundeuva*) were identified. These tannins inhibited biofilm formation of *P. aeruginosa* through bacteriostatic action, causing membrane damage and excess on the proliferation of bacterial vacuoles while the erythrocyte membrane was preserved. With respect to *P. moniliformis*, proanthocyanidins riched in prodelphinidin (0.125 mg/mL) were the compounds responsible for the complete inhibition of *S. epidermidis* biofilm formation, without affecting the viability of the microorganism. As demonstrated by various techniques, the results indicated that both surfaces, of the bacterium (*S. epidermidis*) and of the tested materials (glass and polystyrene), were spontaneously covered by proanthocyanidins, becoming hydrophilic surfaces. Using spin coating technique, the surface was coated with these proanthocyanidins, making the surface highly hydrophilic. The ability to prevent adherence of *S. epidermidis* was maintained and the material proved to be compatible with mammalian epithelial cells, indicating the potential usefulness of these natural products as functional agents for coating surfaces. In another approach of this study, the modification of surface properties via plasma discharge using N_2/H_2 gases mixtures produced, by a quickly and effectively way, polystyrene surfaces able to prevent the adhesion of bacteria highly resistant to antibiotics. Through X-ray spectroscopy, it was found that a nitrogen concentration of 8.8% and the polar component of surface energy greater than 15 mJ/m^2 are needed to reduced adhesion by bacteria exhibiting hydrophilic surface (such as Enterobacteriaceae carbapenemase-producing and the MRSA), while hydrophobic strains (exemplified by *S. epidermidis*) had the capacity to adhere and to form biofilms. The respulsive interactions could explain the anti-adhesive effects obtained in the coating of surfaces by proanthocyanidins as well as in the ionic plasma treatments.

Keywords: adhesion, biofilm, Caatinga, extracts, tannins, surface modification, *Staphylococcus epidermidis, Pseudomonas aeruginosa*

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I - INTRODUÇÃO

Infecções, causadas por agentes etiológicos amplamente resistentes aos antimicrobianos, representam um dos grandes desafios atuais da saúde pública, acarretando em altas taxas de morbi-mortalidade, aumento no tempo de internação e nos gastos do sistema de saúde (MARAGAKIS *et al*., 2008; FRENCH, 2010; NEIDELL *et al*., 2012; PRIMO *et al*., 2012). Mais recentemente, o crescente uso de biomateriais implantáveis elevou a expectativa de vida humana no reestabelecimento de inúmeras funções vitais, mas concomitantemente, exacerbou este problema, sendo que as infecções associadas a biomateriais passaram a ser reconhecidas como um dos maiores problemas clínicos (BUSSCHER *et al*., 2012). De acordo com o órgão norte-americano "National Institutes of Health", aproximadamente 80% de todas as infecções no mundo estão associadas a biofilmes (DAVIES, 2003), especialmente envolvendo dispositivos médicos (DAROUICHE, 2001; BRYERS, 2008).

Na década de 90, a importância dos biofilmes bacterianos foi destacada por Costerton e colaboradores (1999), os quais salientaram que a formação de comunidades sésseis bacterianas e a sua inerente resistência aos antimicrobianos constituem a causa de muitas infecções crônicas e persistentes. O complexo mecanismo necessário para formação de biofilmes ainda é foco de investigação, havendo o envolvimento de diferentes circuitos regulatórios e mediadores químicos bastante peculiares para cada tipo e espécie bacteriana. Entretanto, baseado em modelos experimentais "in vitro", a formação de biofilme classicamente constitui-se de um processo de quatro etapas: (1) a adesão inicial de células bacterianas a um substrato; (2) agregação celular e acumulação em múltiplas camadas; (3) maturação do biofilme; (4) desprendimento de células do biofilme para o estado planctônico e início de novo ciclo (COSTERTON *et al*., 2005; HALL-STOODLEY e STOODLEY, 2005). A adesão inicial bacteriana é, portanto, o passo crucial para o complexo desenvolvimento dos biofilmes – estilo de vida bacteriano caracterizado por mudanças metabólicas, fenotípicas e genotípicas que os tornam difíceis de erradicar - e um alvo atraente para o controle dos mesmos (LEWIS, 2008).

Com o objetivo de prevenir a adesão bacteriana a substratos abióticos, modificações nas características do material constituem uma das abordagens mais

promissoras. Assim, a modificação de propriedades físico-químicas (rugosidade, carga, composição química, hidrofobicidade) de diversos materiais utilizados na fabricação de biomateriais, como polímeros e metais, através de técnicas de engenharia de superfície representam uma interessante estratégia para se alcançar superfície antiadesivas (PONCIN-EPAILLARD e LEGEAY, 2003; BAZAKA *et al*., 2011). Uma segunda abordagem é a modificação de superfícies com compostos bioativos. Alguns estudos vêm demostrando bons resultados com a imobilização de moléculas antimocrobianas (HICKOK e SHAPIRO, 2012), entretanto, com o aumento da resistência bacteriana e o declínio no desenvolvimento de novos fármacos antimicrobianos (DOUTHWAITE, 2010), a utilização contínua destes agentes é controversa uma vez que pode induzir a pressão seletiva para a resistência. A modificação de superfícies com compostos capazes de suprimir a adesão bacteriana sem intervir no crescimento e preservar a adesão de células mamíferas é, portanto, de extrema importância.

Neste contexto, os produtos naturais, oferecem uma diversidade de moléculas bioativas estruturalmente distintas, não necessariamente a entidade fármaco final, as quais têm sido utilizadas como uma importante fonte de agentes terapêuticos inovadores e eficazes ao longo da história humana, desempenhando um papel extremamente importante na descoberta e no processo de desenvolvimento de medicamentos (NEWMAN e CRAGG , 2012). Muitas plantas, por sua vez, são tradicionalmente utilizadas para inibir o crescimento microbiano (COWAN, 1999; RIOS e RECIO, 2005), fato que sugere uma possível ação biológica e indica uma maior tolerância e segurança frente a entidades químicas novas para uso humano (PATWARDHAN e VAIDYA, 2010). Estas características demonstram o valor do conhecimento etnofarmacológico como ferramenta para direcionar a busca de compostos ativos. A Caatinga, bioma exclusivamente brasileiro, possui uma rica flora com diversas indicações medicinais ainda pouco investigada cientificamente (CARTAXO *et al*., 2010) e, é caracterizada por se localizar em uma região com baixa pluviosidade e altas temperaturas anuais. Este ambiente extremo pode conduzir a uma produção de metabólitos secundários diferenciados, favorecendo a biosíntese de fenólicos devido a constante exposição à radiação solar (ALMEIDA *et al*., 2012).

Cientes de tal problemática, o governo federal lançou editais para o desenvolvimento de novas tecnologias e para a formação de recursos humanos dentro da área de nanobiotecnologia. Nosso grupo encontra-se atualmente inserido em um projeto da REDE NANOBIOTEC-BRASIL/CAPES. A REDE permite a interação de grupos interdisciplinares, incluindo a cooperação com a UFPE – Recife (responsável pelo estudo botânico e etnofarmacológico, bem como a coleta e o processamento do material vegetal das plantas da Caatinga, que são utilizados neste trabalho), com o Laboratório de Superfícies e Interfaces Sólidas de Instituto de Física - UFRGS (colaborando com o tratamento de superfícies com descarga de plasma iônico e a caracterização físico-química de supefícies, empregados neste estudo) e com a UEA – Manaus, o que possibilita o desenvolvimento de um trabalho multidisciplinar e fornece a oportunidade de melhor contribuição no entendimento das interações entre superfícies físicas e biológicas.

As análises de MALDI MS/MS foram realizadas no Núcleo de Pesquisa em Produtos Naturais e Sintéticos da Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo.

Nesta tese os resultados estão apresentados na forma de 6 manuscritos organizados conforme as instruções dos respectivos periódicos. A fim de facilitar a leitura, as tabelas e figuras foram inseridas no texto de cada um dos manuscritos.

II – REVISÃO DO TEMA

II - 1. Aspectos gerais da adesão bacteriana e formação de biofilmes

Diversidade metabólica e a capacidade de adaptação a estresses ambientais são características fundamentais dos microrganismos. As bactérias existem em dois estados de vida básicos: como células planctônicas também conhecidas como células de vida livre ou como células sésseis também conhecidas como biofilmes. Acredita-se que células planctônicas são importantes para a rápida proliferação e propagação dos microrganismos para novos territórios, enquanto as células sésseis caracterizam a cronicidade. Biofilmes têm sido descritos em muitos sistemas desde que Antony van Leeuwenhoek, em 1675, examinou “pequenos animais” no seu próprio dente, mas a teoria geral da existência de biofilmes só foi promulgada em 1978 (COSTERTON *et al.*, 1978). Desde então, estudos têm revelado que a maioria das bactérias não cresce como células individuais, mas em comunidades estruturadas como organismos pseudomulticelulares, ou biofilmes, estando presentes em praticamente todos os ecossistemas naturais e patogênicos (COSTERTON *et al.*, 1987; DAVEY e O'TOOLE, 2000; LÓPEZ *et al.*, 2010).

A adesão bacteriana, seja em uma superfície abiótica ou biótica, é o primeiro estágio na formação de biofilmes e é considerado um processo bastante complexo. Como regra geral, a adesão primária (ou adesão reversível) entre bactérias e superfícies abióticas ocorre mediada por interações físico-químicas não específicas, enquanto que a adesão a superfícies bióticas é acompanhada por interações moleculares mediadas por ligações específicas do tipo receptor-ligante, através de lectinas ou adesinas (DUNNE, 2002). Considerando superfícies abióticas, foco deste trabalho, a atração inicial das células bacterianas planctônicas à superfície ocorre aleatoriamente (por uma corrente de fluido sobre uma superfície através do movimento Browniano e força gravitacional) ou de modo dirigido via quimiotaxia e motilidade através de flagelos e pili (O’TOOLE e KOLTER, 1998). O estágio de adesão reversível é, portanto, ditado por interações físico-químicas não específicas de longo alcance entre a bactéria e o material, incluindo forças hidrodinâmicas, interações eletrostáticas, forças de van der Waals e interações hidrofóbicas (DUNNE, 2002; PAVITRA e DOBLE, 2008) (Figura 1). Uma vez que o organismo e a superfície

alcançam uma proximidade crítica (em torno de 1 nm), a determinação final de adesão depende da soma líquida de forças de atração ou repulsão gerada entre as duas superfícies. Estas interações eletrostáticas tendem a favorecer a repulsão, porque a maioria das bactérias e superfícies inertes são carregadas negativamente; por outro lado, as interações hidrofóbicas, parecem apresentar maior influência sobre o resultado da adesão primária. A repulsão líquida entre duas superfícies pode ser superada por interações do tipo ligações de hidrogênio ou por interações moleculares específicas mediadas por adesinas, como o pili (Figura 1). A longevidade de adesão primária depende, portanto, da soma total de todas essas variáveis, mas a química de superfície tende a deslocar o equilíbrio em favor da adesão ao prever que as substâncias orgânicas em solução irão se concentrar perto de uma superfície e que os microrganismos costumam se reunir em ambientes ricos em nutrientes (DUNNE, 2002). A fim de entender se a fixação bacteriana às superfícies é regulada pelas mesmas interações físico-químicas que determinam a deposição de partículas coloidais inanimadas, a utilização de modelos teóricos para prever este fenômeno vêm sendo considerada (KATSIKOGIANNI e MISSIRLIS, 2004, PAVITRA e DOBLE, 2008).

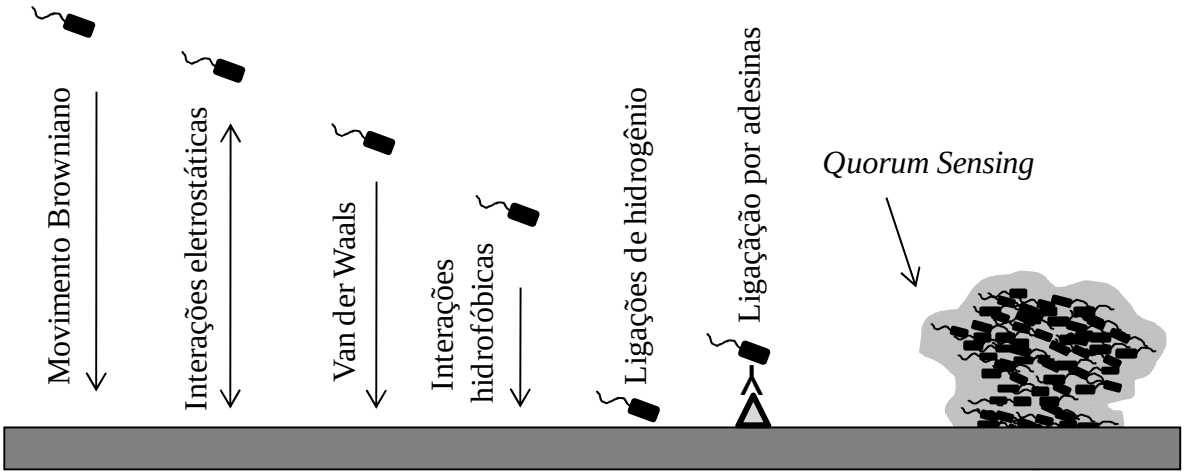

Figura 1: Interações envolvidas na colonização bacteriana à superfície abiótica (adaptada de PASCUAL, 2002).

Ao se considerar que a superfície do material em questão pode ser um biomaterial e que o mesmo será implantado em um hospedeiro, a adesão reversível entre a bactéria e a superfície pode ocorrer de maneira direta ou através de um filme condicionante (Figura 2). Este filme orgânico possui composição variável, de acordo

com o sítio de inserção, mas é constituído principalmente por proteínas, como albumina, imunoglobulina, fibrinogênio e fibronectina (HERRMANN *et al.*, 1988; GOTTENBOS *et al*., 2002.; ROCHFORD *et al*., 2012). Uma vez que uma superfície está condicionada, suas propriedades são permanentemente alteradas, de modo que a afinidade de um microorganismo por uma superfície original ou condicionada pode ser bastante diferente, explicitando a dificuldade imposta para controlar a adesão bacteriana à superfícies abióticas.

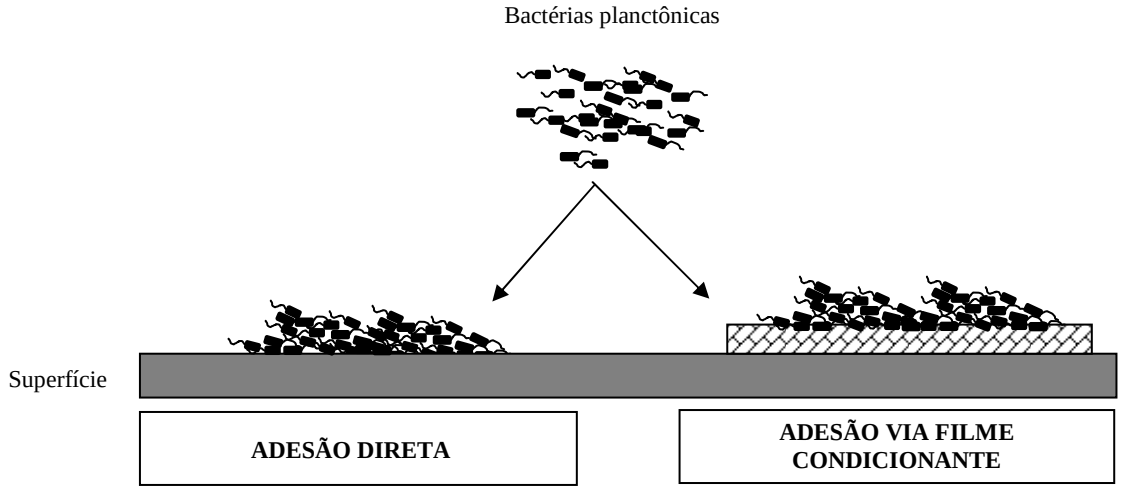

Figura 2: Adesão bacteriana direta (no momento ou próximo do momento da inserção de um implante) e via filme condicionante (após a inserção do implante).

Evidências indicam que após a adesão de células bacterianas, ocorre um aumento na produção, liberação e detectação de moléculas sinalizadoras que regulam respostas fenotípicas de formação de biofilme (DAVIES *et al.*, 1998; BJARNSHOLT e GIVSKOV, 2007; HODGKINSON *et al*., 2007). Conforme a densidade bacteriana aumenta, as moléculas autoindutoras podem acumular a um limiar de concentração e induzir a transcrição de genes específicos que regulam várias funções como motilidade, virulência, produção de matriz exopolissacarídica (EPS) e a formação de biofilmes. Este processo de comunicação encontrado em muitas bactérias patogênicas, que acopla a transcrição de genes específicos com a densidade celular bacteriana, é referido como *Quorum sensing* (*QS*) (WATERS *et al.*, 2005; GONZÁLEZ *et al.*, 2006, RUTHERFORD e BASSLER, 2012).

A segunda etapa da adesão bacteriana é a adesão secundária (ou adesão irreversível). Neste momento, os organismos fracamente ligados a superfície

consolidam o processo de adesão através da produção de EPS, a qual pode se complexar com materiais de superfície e/ou receptores específicos localizados no pili ou nas fímbrias. Durante esta fase de adesão, os microrganismos são capazes de se ligar a células da mesma ou de diferentes espécies, formando agregados sobre o substrato, os quais encontram-se firmemente ligados à superfície (STOODLEY *et al.*, 2002). Tipicamente, biofilmes maduros consistem de estruturas semelhantes a cogumelos, envoltos pelo EPS, permeados por canais de água. Estes canais funcionam como um sistema circulatório de entrega de nutrientes, da interface para o interior do biofilme, e de remoção de restos metabólitos (DAVEY e O'TOOLE, 2000; HALL-STOODLEY *et al.*, 2004). As bactérias por si mesmas representam uma fração variável (5-35%) do total do volume do biofilme, o restante do volume é de EPS (POZO e PATEL, 2007). Sob determinadas situações, por exemplo, quando o ambiente não se encontra mais favorável ou ainda devido a uma programação celular para a virulência, ocorre o desprendimento de células planctônicas ou até de grupos de células unidas pelo EPS podendo colonizar um novo local (HALL-STOODLEY e STOODLEY, 2005; BAYLES, 2007) (Figura 3).

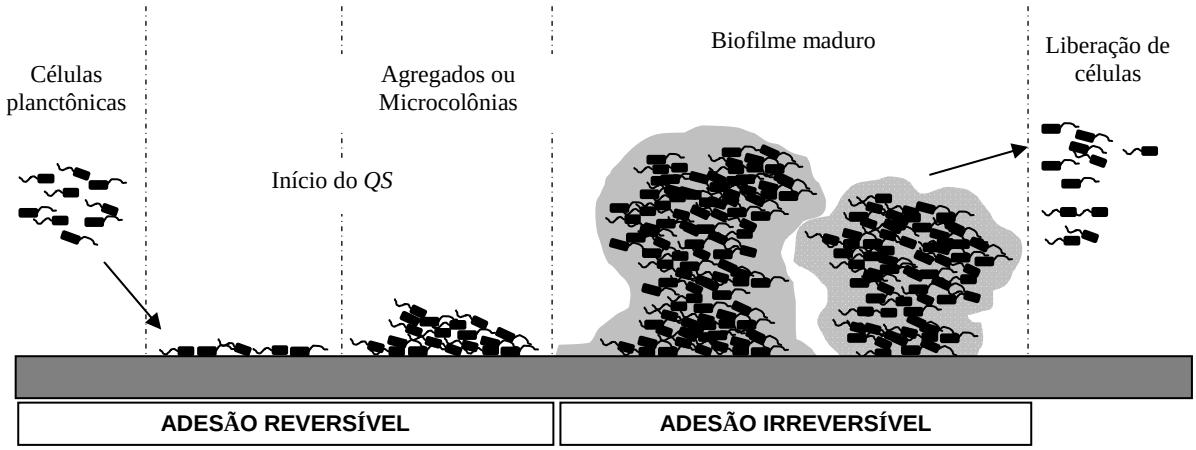

Figura 3: Estágios do desenvolvimento dos biofilmes (adaptada de MACEDO E ABRAHAM, 2009).

Desta maneira, a adesão bacteriana é um processo bastante complexo que envolve a interação multifacetada de três componentes: a bactéria, a superfície (biótica ou abiótica) e o microambiente em que eles se encontram (COSTERTON *et al.*, 1999; DAROUICHE, 2001; KATSIKOGIANNI e MISSIRLIS, 2004). Neste sentido, existem diversas variáveis que podem influenciar este processo, ilustrados na Figura 4.

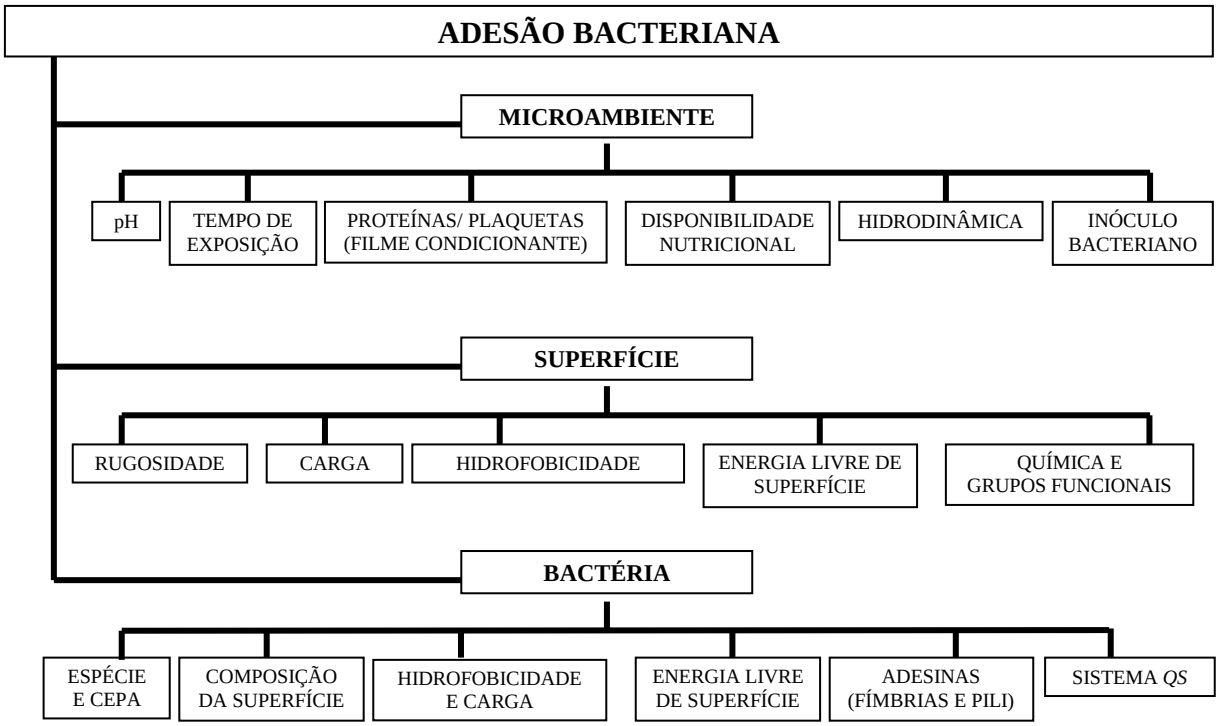

Figura 4: Parâmetros que influenciam na adesão bacteriana (adaptada de DAROUICHE, 2001).

II- 2. Importância clínica e impacto econômico das infecções associadas a biofilmes

A adesão bacteriana e a consequente formação de biofilme possuem um papel importante na patogênese, representando um grande obstáculo para a saúde humana, sendo causa comum de infecções persistentes (COSTERTON *et al.*, 1999; CHEN e WEN, 2011; MAH, 2012). De acordo com o órgão norte-americano "National Institutes of Health", biofilmes estão associados a aproximadamente 80% de todas as infecções médicas no mundo (DAVIES, 2003), incluindo endocardites, otites, prostatites, periodontites, conjuntivites, vaginites, infecções relacionadas à fibrose cística e como importantes colonizadores de implantes médicos, tais como cateteres venosos, arteriais e urinários, dispositivos intrauterinos, lentes de contato e próteses (DONLAN e COSTERTON *et al.*, 2002; FALAGAS *et al.*, 2009; HOIBY *et al.*, 2011).

Uma das mais importantes características dos biofilmes bacterianos é a sua resistência ao sistema imune do hospedeiro e aos agentes antimicrobianos. Bactérias

que vivem nestas comunidades são frequentemente de 10 a 1000 vezes mais tolerantes aos antimicrobianos do que quando na forma planctônica (DAVIES, 2003), indicando que alguns dos mecanismos envolvidos na resistência dos biofilmes aos antimicrobianos devem diferir dos mecanismos responsáveis pela resistência de bactérias planctônicas aos mesmos agentes (STEWART, 2002). Desta maneira, microrganismos que apresentam susceptibilidade a determinados antimicrobianos em testes laboratoriais convencionais, são na verdade altamente resistentes aos mesmos quando na forma de biofilmes. Como consequência, doenças envolvendo biofilmes são geralmente crônicas e difíceis de tratar. Diversos fatores têm sido postulados para explicar a baixa suscetibilidade aos antimicrobianos de células na forma de biofilmes (DONLAN e COSTERTON *et al.*, 2002; DAVIES, 2003; LAZAR e CHIFIRIUC, 2010), incluindo:

a) Baixa penetração de agentes químicos: O EPS reduz a capacidade de penetração de antimicrobianos em todas as áreas do biofilme. O EPS pode (i) atuar como barreira física para difusão, retendo grande parte dos agentes antimicrobianos e assim, reduzindo a quantidade do mesmo para agir sobre as células e, (ii) interagir quimicamente com estes agentes, sequestrando os antimicrobianos hidrofílicos e carregados positivamente, tais como os aminoglicosídeos (NICHOLS *et al.*, 1988). Além disso, bactérias na forma de biofilme resistentes a antimicrobianos tornaram-se suscetíveis ao tratamento após a dispersão ou desagregação do biofilme, uma observação que suporta a ideia de que a matriz de EPS pode proteger o biofilme através da limitação do transporte de agentes antimicrobianos (DAVIES, 2003).

b) Crescimento lento de células no interior do biofilme: As células bacterianas em biofilmes constituem populações heterogêneas com variadas taxas de crescimento em diferentes compartimentos do biofilme e variada suscetibilidade aos antimicrobianos (MAH *et al.*, 2001, STEWART e FRANKLIN, 2008). O desenvolvimento da tolerância aos antimicrobianos pode resultar da inibição da morte celular natural em uma subpopulação de células bacterianas, conhecidas como células dormentes ou “persisters”. Esta subpopulação que comumente encontra-se na base da estrutura dos biofilmes, onde há limitação de oferta de oxigênio, apresentando

reduzida taxa metabólica, a qual garante a sua resistência ao tratamento com antimicrobianos, visto que eles geralmente agem na fase de crescimento bacteriano, como síntese protéica, síntese de ácidos nucléicos e de parede celular. Desta forma, o tratamento antimicrobiano pode conduzir à erradicação da maioria da população suscetível, mas esta fração de células "persisters" não é atingida pela quimioterapia e atua, portanto, como um núcleo para re-infecção após a descontinuação terapêutica (STEWART, 2002; LEWIS, 2010; 2012) (Figura 5).

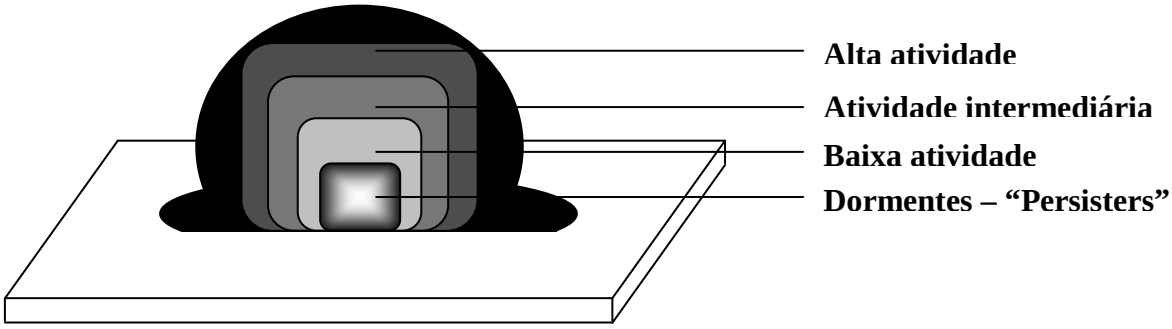

Figura 5: Atividade metabólica em uma microcolônia de biofilme (adaptada de DAVIES, 2003).

c) Transferência de genes de resistência: Biofilmes são idealmente adequados para a troca de material genético devido à proximidade das células bacterianas. Desta forma, a vida em comunidade facilita a transferência horizontal de genes através de plasmídeos, os quais podem codificar resistência para múltiplos agentes antimicrobianos (DAVEY e O'TOOLE, 2000; POZO e PATEL, 2007, MADSEN *et al.*, 2012).

d) Fuga da defesa imunológica humana: Devido à presença do EPS, o sistema imune encontra dificuldade de reconhecimento dos biofilmes, protegendo as células do interior do biofilme contra a ação de anticorpos, radicais livres e outros compostos reativos produzidos por fagócitos recrutados para o combate de infecções (FUX *et al.*, 2003; BRYERS, 2008).

Embora os fatores acima citados contribuam para o entendimento da baixa suscetibilidade das células na forma de biofilmes, isoladamente eles não suportam a ideia de que bactérias em biofilme sejam mais resistentes aos antimicrobianos do que bactérias planctônicas. Tanaka e colaboradores (1999) mostraram que a ação

bactericida de fluorquinolonas em biofilmes de *Pseudomonas aeruginosa* ocorreu independente da taxa de crescimento bacteriano. Walters e colaboradores (2003) compararam a penetração de antimicrobianos, com a limitação de oxigênio e com os efeitos da atividade metabólica na tolerância de biofilmes de *P. aeruginosa* ao ciprofloxacino e à tobramicina. Os resultados sugerem que a limitação de oxigênio e a baixa atividade metabólica no interior dos biofilmes, mais do que a penetração do antimicrobiano, sejam os responsáveis pela tolerância a esses fármacos. Assim, nota-se que a resistência dos biofilmes é multifatorial, e que estes mecanismos têm se desenvolvido como uma resposta de estresse bacteriano, onde a vida em biofilmes permite que as células respondam às diferentes alterações ambientais (MAH, 2012).

As infecções nosocomiais são a quarta causa de morte nos Estados Unidos, sendo responsáveis por 2-4 milhões de casos anualmente, elevando em mais de U$ 5 bilhões o custo médico adicional por ano (WENZEL, 2007). Considerando o Brasil, de acordo com o estudo do programa “SCOPE Surveillance and control of pathogens of epidemiological importance”, a taxa de mortalidade associada às infecções nosocomiais sanguíneas, de 2007 a 2010, atingiu 40% (MARRA *et al*., 2011); semelhantemente, no Rio Grande do Sul a taxa de mortalidade associada a infecções em Unidades de Terapia Intensiva (UTIs) foi de 45%, em 2003 (LISBOA *et al*., 2007). Sabe-se que 60-70% das infecções hospitalares estão associadas ao uso de dispositivo biomédico implantado (WENZEL, 2007) e estima-se que mais de 5 milhões destes dispositivos sejam usados por ano apenas nos Estados Unidos (BRYERS, 2008).

A inserção de dispositivos implantáveis tem se tornado indispensável em quase todas as áreas da medicina, particularmente em UTIs. Com o aumento da expectativa de vida humana, maior é a necessidade de substituição e reparo de funções biológicas e, portanto, é estimado um aumento no número de pessoas hospitalizadas e que irão receber implantes biomédicos. A indústria de biomateriais movimenta atualmente cerca de U$ 28 bilhões por ano e encontra-se em rápida expansão, com taxa de crescimento anual de 15% (HOLZAPFEL *et al.*, 2012). Independentemente da sofisticação do implante, todos os dispositivos médicos ou tecidos construídos por engenharia estão suscetíveis ao risco de colonização microbiana e infecção (BRYERS,

2008; TRETER e MACEDO, 2011; BUSSCHER *et al.*, 2012). O risco de infecção está associado ao déficit imunológico local na interface implante-hospedeiro, levando a uma reduzida habilidade de eliminar microrganismos próximos ao biomaterial, o que aumenta a propensão de infecção associada ao implante (ROCHFORD *et al*., 2012). A Tabela 1 mostra a magnitude das infecções associadas a implantes nos Estados Unidos, enquanto a Tabela 2 apresenta este panorama no Brasil.

Tabela 1: A magnitude do problema de infecções associadas a implantes médicos (adaptado de DAROUICHE, 2001).

Dispositivo	Número de inserções por ano	Taxa de infecção na primeira inserção (%)	Mortalidade atribuída[a]
Cateteres urinários	> 30 milhões	10-30	Baixa
Cateteres venosos central	5 milhões	3-8	Moderada
Dispositivos de fixação de fratura (placas e pinos)	2 milhões	5-10	Baixa
Implantes dentários	1 milhão	5-10	Baixa
Próteses articulares	600 mil	1-3	Baixa
Enxertos vasculares	450 mil	1-5	Moderada
Marca-passos cardíacos	300 mil	1-7	Moderada
Implantes mamários	130 mil	1-2	Baixa
Válvulas cardíacas mecânicas	85 mil	1-3	Alta
Dispositivos de assistência cardíaca	700	25-50	Alta

[a] Escala semiquantitativa: Baixa (< 5%); Moderada (5-25%); Alta (>25%).

As verdadeiras taxas de infecções podem ser maiores do que as apresentadas acima pois (i) a taxa de infecção de dispositivos re-implantados aumenta em diversas vezes, (ii) os antimicrobianos geralmente são administrados previamente à coleta para a realização de exame cultural, podendo gerar resultados falso negativos, e (iii) esses dados encontram-se defasados em no mínimo 12 anos.

Tabela 2: Infecções associadas a dispositivos médicos em UTIs de 3 hospitais brasileiros, no período de abril de 2003 a fevereiro de 2006 (adaptada de SALOMAO *et al*., 2008).

Dispositivo	Infecção associada	Taxa de infecção (%)	Mortalidade atribuída (%)
Ventilador mecânico	Pneumonia	13,2	34,5
Cateter venoso central	Infecção sanguínea	8,3	47,1
Cateter urinário	Infecção do trato urinário	8,2	30,0

Os esforços feitos para manter a esterilidade e assepsia, visando minimizar as possibilidades de contaminação durante a implantação de dispositivos médicos e para evitar infecções nos pacientes, através de protocolos adequados de profilaxia provaram ser eficazes, mas incapazes de controlar completamente a ocorrência de infecções (MONTANARO *et al.*, 2007). A dificuldade de tratamento devido ao efeito da resistência bacteriana em infecções associadas a biofilmes possui consequências diretas (maior tempo de internação, custo de antibioticoterapia e medicamentos complementares, custos com médicos e procedimentos diagnósticos) e indiretas (absenteísmo e desemprego) no desfecho clínico e na qualidade de vida do paciente. Frequentemente, o principal manejo nas infecções em dispositivos médicos é a sua remoção, no entanto, este procedimento pode estar associado com o aumento de morbidade e mortalidade, prolongando a hospitalização e elevando os custos para o sistema de saúde.

Estima-se que o gasto relacionado com o tratamento destas infecções seja maior do que o gasto envolvido com a retirada e troca do dispositivo médico. Em muitos casos, quando o dispositivo infectado não pode ser removido, os pacientes enfrentam uma supressiva terapia antimicrobiana para a prevenção de infecções sistêmicas recorrentes (SCHINABECK e GHANNOUM, 2005; FALAGAS *et al.*, 2009; ROHDE *et al.*, 2010). O custo para tratar infecções associadas a implantes biomédicos está estimado em torno de 5 a 7 vezes o custo da inserção original (BANDYK e ESSES, 1994). No caso de um cateter venoso central (CVC), a sua remoção e substituição pode ser tão elevada quanto US$ 14 mil, por incidência (THOMAS *et al.*, 2005) e, o custo anual de pacientes com infecções sanguíneas asssociadas a CVC varia de US$ 296 milhões a US$ 2.3 bilhões (VON EIFF *et al.*, 2005). Da mesma maneira, com base em cálculos para internações, uma incidência de sepse custa entre US$ 22 mil e US$ 70 mil, os custos de pneumonia variam de US$ 12 mil a U$ 22 mil e quando associada à ventilação US$ 41 mil, por paciente (THOMAS *et al.*, 2005).

II - 2.1. Bactérias de importância médica formadoras de biofilme

Biofilmes podem envolver apenas uma ou diferentes espécies microbianas. No caso de uma infecção multiespécie, algumas delas apenas desempenham o papel de favorecer a virulência e a organização estrutural do biofilme, o que protege e permite a sobrevivência das demais espécies envolvidas, que por sua vez participam ativamente da infecção (BURMOLLE *et al.*, 2010). Os patógenos formadores de biofilme mais comumente encontrados em infecções humanas relacionadas a dispositivos biomédicos estão listados na Tabela 3.

Tabela 3: Microrganismos formadores de biofilme comumente encontrados em infecções humanas e de implantes médicos (adaptada de GEORGOPAPADAKOU, 2005 e THOMAS *et al.*, 2005).

Infecção/sítio	Microrganismo(s) causador(es)
Cáries dentárias	*Streptococcus mutans*
Cateteres venosos central	*Sthaphylococcus* coagulase negativos (SCoN); *Sthaphylococcus aureus*; *Enterococcus faecalis*; *Pseudomonas aeruginosa*; *Klebsiella pneumoniae*; *Candida* spp
Dispositivos intrauterinos	*Sthaphylococcus epidermidis*; *Enterococcus* spp; *Streptocccus* β - hemolítico; Lactobacilos
Endocardite (válvulas nativas)	*Streptococcus* grupo viridans
Endocardite (válvulas mecânicas)	*S. epidermidis*, *S. aureus*, *Streptococcus* spp.; *Enterococcus* spp.; *P. aeruginosa; Candida* spp; *Aspergillus* spp
Esôfago (em aidéticos)	*Candida* spp
Enxertos vasculares	SCoN; *S. aureus*; *P. aeruginosa*; outros Gram- negativos
Lentes de contato e intraoculares	*P. aeruginosa*; *Serratia marcescens* e cocos Gram-positivos
Osteomielite	*S. aureus*
Otite média	*S. aureus*
Peritonite (cateteres de diálise peritoneal)	*S. aureus*; *Candida* spp; *P. aeruginosa*; outros Gram- negativos
Prostatites	*Escherichia coli*; *Clamydia trachomatis*; *Mycoplasma*
Próteses ortopédicas	*S. aureus* e *S. epidermidis*
Trato respiratório	*Streptococcus pneumoniae* e bacilos Gram-negativos
Trato respiratório (em fibrose cística)	*P. aeruginosa* e *Burkholderia cepacia*
Trato urinário (cateteres urinários)	*E. coli*; *Proteus mirabilis*; SCoN; *E.faecalis*; *Candida* spp.; *K. pneumoniae*; outros Gram-negativos
Tubos endotraqueais	Gram-negativos entéricos; *Staphylococcus* spp.; *Streptococcus* spp.; *Enterococcus* spp.;
Vagina	*Candida* spp

Dentre estes, cabe destacar: (a) *Staphylococcus* spp.– o principal causador de infecções associadas a implantes biomédicos, com crescente importância na medicina moderna; (b) *Pseudomonas aeruginosa* - microrganismo formador de biofilme, capaz de causar infecções crônicas progressivas em pacientes com fibrose cística; e (c) algumas enterobactérias, como *Escherichia coli*; *Klebsiella pneumoniae*; *Serratia marcescens* e *Enterobacter cloacae* – as quais demonstram grande potencial de epidemia devido a alta resistência aos antimicrobianos, incluindo carbapenêmicos.

II - 2.1.1. *Staphylococcus* spp.

Staphylococcus spp. são cocos Gram-positivos arranjados em aglomerado, reconhecidos como uma importante causa de infecções humanas nosocomiais e adquiridas na comunidade (WANG *et al.*, 2007; OTTO, 2008). Atualmente, o gênero *Staphylococcus* compreende 39 espécies, as quais podem ser divididas em dois grandes grupos: o coagulase positivo, no qual o *S. aureus* é a espécie exclusiva patógena em humanos, capaz de produzir a enzima responsável pela coagulação da fibrina no sangue, e o coagulase negativo, no qual se enquadram as demais espécies, comumente referidas como *Staphylococcus* coagulase negativos (SCoN) (BECKER e VON EIFF, 2011).

Dentre as espécies de estafilococos, *S. aureus* é considerado o patógeno humano oportunista mais importante. Aproximadamente 10 a 35% das pessoas saudáveis são consideradas carreadoras persistentes de *S. aureus* (BECKER e VON EIFF, 2011), servindo como reservatórios de microrganismos e importantes fontes de infecções invasivas. *Staphylococcus aureus* é a espécie mais importante clinicamente, capaz de causar uma grande variedade de doenças em humanos e em animais (BECKER e VON EIFF, 2011) e possui um arsenal intrínseco de determinantes de virulência, que contribuem para a sua patogenicidade. Dentre os fatores de virulência, incluem-se diferentes tipos de toxinas (citotoxinas, toxinas esfoliativas, enterotoxinas e a toxina 1 da síndrome do choque tóxico) e enzimas (coagulase, hialuronidase, fibrinolisina, lipases, nucleases) que facilitam a destruição tecidual e, um número de

componentes estruturais (cápsula, camada limosa, proteína A e adesinas), que impedem a ação de fagócitos e anticorpos e medeiam a adesão tecidual e colonização (MURRAY *et al.*, 2009a).

Por outro lado, os SCoN constituem a maior parte das bactérias pertencentes à microbiota normal humana, sendo importantes colonizadores da pele e membrana mucosa, e são importantes na manutenção da microbiota saudável competindo com microrganismos potencialmente prejudiciais, em particular, *S. aureus* (OTTO, 2012a). Embora desde a década de 50 já houvesse relatos de SCoN causando infecções, estes microrganismos foram considerados não patogênicos e seu isolamento no laboratório clínico era atribuído à contaminação pela microbiota cutânea normal. Somente nos anos 80 a comunidade científica começou a elucidar melhor a patogenicidade destes microrganismos (PFALLER e HERWALDT, 1988). Na verdade, a conversão dos SCoN de microrganismos simbiontes para patógeno humano é reflexo direto do aumento do uso de implantes médicos para a substituição intermitente ou permanente de órgãos e para o manejo de funções vitais em UTIs (O'GARA e HUMPHREYS, 2001; OTTO, 2009), sendo reconhecidos, atualmente, como principais patógenos associados a infecção de implantes (UÇKAY *et al*., 2009; OTTO, 2012b).

Um SCoN com importantes implicações na saúde é o *Staphylococcus epidermidis*. Em contraste com *S. aureus*, o qual possui um vasto arsenal de toxinas desenvolvido para causar infecção no hospedeiro humano, praticamente todos os fatores de virulência de *S. epidermidis* parecem ter funções originais no estilo de vida da bactéria comensal (OTTO, 2012a). Dentre estes, *S. epidermidis* é capaz de expressar determinantes que provocam sua persistência, como as moléculas promotoras da evasão ao sistema imune e as que medeiam a formação de biofilmes, como as PSMs (“phenolsoluble modulins”) e o exopolissacarídeo PIA (“polysaccharide intercellular adhesin”), fato que desperta crescente interesse clínico (VUONG e OTTO, 2002; OTTO, 2009; 2012a). *Staphylococcus epidermidis* apresenta uma substancial adaptação em nível de genoma para o crescimento na forma de biofilmes, incluindo a regulação negativa (“down-regulation”) de processos celulares básicos como biossíntese de ácidos nucléicos, de proteínas e de parede celular, fato

que associa a formação de biofilmes à sua patogenicidade (YAO *et al.*, 2005). Além disso, *S. epidermidis* estão sendo reconhecidos como importantes reservatórios de genes que promovem a colonização e a virulência de *S. aureus* resistente à meticilina (MRSA), como recentemente apresentado no trabalho de Otto (2013).

Em estudo do "SENTRY Antimicrobial Surveillance Program", envolvendo o Brasil e a América Latina, no período de 1997 a 2001, foi detectado uma prevalência de 21,3% de *S. aureus* e 13,9 % de SCoN em hemoculturas (SADER *et al.*, 2003). No período de 2005 a 2008, outro estudo realizado pelo SENTRY relatou aumento das taxas de resistência em SCoN para a maioria dos antimicrobianos utilizados nos hospitais brasileiros. Neste estudo, os índices reportados de resistência à meticilina foram de 78,7% em SCoN e 31,0% em *S. aureus*, sendo que entre os SCoN também foi encontrado valores elevados de resistência à eritromicina (70%), ao sulfametoxazol-trimetoprima (50%) e ao levofloxacino (45%) (GALES *et al.*, 2009). A mortalidade atribuída a infecções no sistema circulatório causadas por *S. epidermidis* varia de 10 a 34%, com aumento dos custos e acréscimo de 7 a 19 dias na duração da internação (OTTO, 2009; ROHDE *et al*., 2010). Devido à resistência à meticilina (CAMERON *et al.*, 2011) e aos outros antimicrobianos, 80% dos cateteres infectados com *Staphylococcus* spp. estão sendo tratados com vancomicina (RAAD *et al.*, 2007a). A resistência à vancomicina já foi descrita desde 2003 (CHANG *et al.*, 2003), mas felizmente o isolamento de cepas resistentes ainda é raro. Por outro lado, a prevalência de isolados com suscetibilidade intermediária à vancomicina aumenta progressivamente (BAE *et al.*, 2009) e a formação de biofilmes, o mecanismo não específico mais importante de resistência, diminui a atividade deste e de outros antimicrobianos de forma significativa (WEIGEL *et al.*, 2007; RAAD *et al.*, 2007b; ANTUNES *et al.*, 2011).

II - 2.1.2. *Pseudomonas aeruginosa*

Pseudomonas compreende um grande e complexo gênero formado por bacilos Gram-negativos não fermentadores de glicose e não fastidiosos. Estes bacilos são

ubíquos no ambiente, podendo se adaptar a uma variedade de nichos devido à sua habilidade de crescer em temperaturas abaixo de 25 °C até 42 °C e de utilizar diferentes moléculas orgânicas como fonte de carbono (HENRY e SPEERT, 2011).

Dentre estes, *P. aeruginosa* é um patógeno oportunista associado a uma vasta gama de infecções em humanos. Não há dúvidas com relação à versatilidade de *P. aeruginosa* como patógeno. Algumas infecções são mais encontradas na comunidade como foliculites, artrite séptica e otite externa; enquanto infecções mais sérias geralmente são encontradas no ambiente hospitalar e incluem bacteremia, pneumonia, endocardite, osteomielite, infecções no trato urinário e peritonite. Os indivíduos imunocomprometidos, incluindo pacientes com severas queimaduras, com câncer, com o vírus da imunodeficiência humana e portadores de fibrose cística (FC) constituem o grupo de risco com infecções envolvendo *P. aeruginosa* (HENRY e SPEERT, 2011; KERR e SNELLING, 2009). A morbidade e mortalidade associadas à FC, a doença autossômica recessiva mais comum entre os caucasianos (frequência de 1: 2500 nascidos vivos) (RATJEN e DORING, 2003), são causadas pela colonização crônica dos pulmões por microrganismos. Embora várias espécies microbianas possam colonizar com sucesso os pulmões de pacientes com FC, infecções por *P. aeruginosa* contribuem mais significativamente para a doença.

Pseudomonas aeruginosa expressa uma variedade de fatores de virulência, incluindo componentes estruturais (flagelo, pili, lipopolissacarídeo e o alginato), toxinas (exotoxina A, exoenzimas S e T), enzimas (proteases, elastases, fosfolipases), os pigmentos piocianina e pioverdina e o sistema de secreção tipo III, mecanismo pelo qual a bactéria injeta seus fatores de virulência na célula hospedeira. A maioria dos especialistas acredita que para *P. aeruginosa* causar doença, vários destes fatores devem estar expressos conjuntamente, promovendo o dano tecidual e a fuga ao sistema imune do hospedeiro (MURRAY *et al.*, 2009b). Aliado a estes, a capacidade de *P. aeruginosa* em formar biofilmes é uma importante habilidade que permite a sua persistência em diversos nichos (KERR e SNELLING, 2009). Quando cresce na forma de biofilme, *P. aeruginosa* pode secretar uma série de exopolissacarídeos, destacando-se o alginato, um polímero de ácido manurônico e ácido glicurônico, que caracteriza

seu fenótipo mucoide (RAMSEY e WOZNIAK, 2005). A conversão das microcolônias de *P. aeruginosa* do fenótipo não mucoide para o fenótipo mucoide marca a transição para um estado de persistência, marcado pela resistência aos antimicrobianos, ineficácia do sistema imune e acelerado declínio da função pulmonar, tornando a infecção por *P. aeruginosa* mucoide o principal patógeno da FC (PEDERSEN *et al.*, 1992; GOVAN e DERETIC, 1996; LYCZAK *et al.*, 2002).

Pseudomonas aeruginosa apresenta os quatro principais mecanismos de resistência: (i) a expressão de β-lactamase AmpC cromossomal induzida, a qual rende resistência à ampicilina, amoxacilina, amoxacilina-clavulanato, cefotaxima e ceftriaxona; (ii) os diversos sistemas de bombas de efluxo, capazes de expulsar o agente antimicrobiano da célula; (iii) a diminuição da permeabilidade de membrana externa, dificultando a entrada de antimicrobianos na célula, e (iv) a alteração do sítio de ação de alguns antimicrobianos (HENRY e SPEERT, 2011). Vários antimicrobianos podem ser utilizados no tratamento de infecções por *P. aeruginosa*, incluindo as penicilinas de amplo espectro (piperacilina e ticarcilina), certas cefalosporinas de largo espectro (ceftazidima e cefepima), carbapenêmicos (imipenem e meropenem), monobactâmicos (aztreonam), fluorquinolonas (ciprofloxacino e levofloxacino), aminoglicosídeos (gentamicina, tobramicina e amicacina) e colistina (HENRY e SPEERT, 2011). Infelizmente, resistência a todos antimicrobianos pode ser desenvolvida, uma vez que *P. aeruginosa* possui a habilidade de desenvolver resistência por mutações em diferentes *loci*, seja por aquisição horizontal de genes de resistência carreado por plasmídeos, por transposons ou por integrons (HENRY e SPEERT, 2011).

Mundialmente a resistência aos antimicrobianos, incluindo a resistência multifármacos (resistência a três ou mais classes de antimicrobianos) entre *P. aeruginosa* é generalizada e crescente. Conforme um estudo realizado em um período de dez anos (1993 a 2002) nos Estados Unidos, a resistência a multifármacos, incluindo ceftazidima, ciprofloxacino, tobramicina e imipenem aumentou de 4% no início do estudo para 14% em 2002 (OBRITSCH *et al.*, 2004), fato que demonstra a limitação nas terapias, a necessidade por novos fármacos e a importância do teste de

suscetibilidade para guiar o tratamento. Nas UTIs brasileiras, *P. aeruginosa* resistente a multifármacos é a principal causa de infecções nosocomiais, sendo a pneumonia a principal destas (ROSSI, 2011).

II - 2.1.3. Enterobacteriaceae

A família Enterobacteriaceae é a maior e mais heterogênea coleção de bacilos Gram-negativos de importância médica. Mais de 40 gêneros e centenas de espécies e subespécies já foram descritos. Os membros desta família possuem necessidades nutricionais simples, são fermentadores de glicose, e a ausência de atividade da enzima citocromo oxidase é uma característica importante para distingui-los dos demais bacilos Gram-negativos (MURRAY *et al.*, 2009c). As bactérias da família Enterobacteriaceae, também chamadas de enterobactérias, são microrganismos ubiquitários e fazem parte da microbiota normal humana. Apesar da complexidade desta família, relativamente poucas espécies são responsáveis pela maioria das infecções em humanos (Quadro 1). As enterobactérias podem causar uma variedade de doenças, incluindo 30 a 35% de todas as bacteremias, mais de 70% das infecções de trato urinário e infecções intestinais (MURRAY *et al.*, 2009c).

Quadro 1: Enterobacteriaceae de importância médica (adaptada de MURRAY *et al.*, 2009c).

Microrganismos
Citrobacter freundii; *C. Koseri*
Enterobacter aerogenes; *E. cloacae*
Escherichia coli
Klebsiella pneumoniae; *K. Oxytoca*
Morganella morganii
Proteus mirabilis
Salmonella enterica
Serratia marcescens
Shigella sonnei; *S. flexneri*
Yersinia pestis; *Y. enterocolitica*; *Y. Pseudotuberculosis*

Numerosos fatores de virulência foram identificados nos membros da família Enterobacteriaceae, sendo comuns a todos os microrganismos a cápsula e a variação de fase antigênica (alterando o antígeno capsular K e flagelar H) que conferem proteção frente à fagocitose e à morte mediada por anticorpos; as proteínas (enterobactina e aerobactina) capazes de quelar o ferro do hospedeiro e permitir o crescimento bacteriano; e, as endotoxinas, que podem causar choque e morte (MURRAY *et al.*, 2009c). Adicionalmente, algumas enterobactérias, como *K. pneumoniae* (DI MARTINO *et al.*, 2003), *S. marcescens* (RICE *et al.*, 2005) e *E. cloacae* (THOMPSON *et al.*, 2006) têm demonstrado a capacidade de formar biofilmes. Tão rapidamente quanto novos antimicrobianos são introduzidos no mercado, as enterobactérias podem desenvolver mecanismos de resistência aos mesmos. Os principais mecanismos encontrados neste grupo de bactérias são cromossômico e plasmidial-induzíveis através de β-lactamase do tipo AmpC e resistência plasmidial com produção de β-lactamase de espectro estendido (ESBL), que confere resistência a todas as penicilinas e cefalosporinas, incluindo os monobactâmicos (ROSSI e ANDREAZZI, 2005).

Conforme Rossi (2011), em hospitais brasileiros, a frequência de microrganismos produtores de ESBL isolados de hemocultura (54% das *K. pneumoniae* e 32.4% das *E. coli*) têm sido maior do que em hospitais americanos e europeus e, a resistência às fluoroquinolonas também foi alta nestes isolados (81% e 41%, respectivamente). Dentre algumas razões para explicar esta disparidade pode-se citar: (i) o atraso na entrega de laudos do perfil de susceptibilidade microbiana aos antimicrobianos em alguns hospitais brasileiros, contribuindo para uma alta pressão seletiva devido ao grande uso de agentes antimicrobianos de amplo espectro; (ii) a venda de antimicrobianos sem prescrição médica até o ano passado, acarretando na automedicação e colaborando para o surgimento de cepas resistentes. De acordo com o "Study for Monitoring Antimicrobial Resistance Trends" (2004), o qual analisou amostras de 28 países, 10% das *E. coli*, 17% das espécies de *Klebsiella* e 22% das espécies de *Enterobacter* eram isolados produtores de ESBL (ROSSI *et al.*, 2006). Yang e Zhang (2008) demonstraram em *K. pneumoniae* isoladas de escarro e urina, a

associação entre a produção de ESBL e a formação de biofilmes, e em *K. pneumoniae* isoladas de infecções do trato respiratório e urinário, a associação entre a produção de ESBL, formação de biofilmes e a presença do dispositivo médico.

Os carbapenêmicos são, muitas vezes, uma das poucas opções de tratamento disponíveis em infecções graves causadas por bactérias Gram-negativas resistentes a multifármacos. Entretanto, em 2001, o primeiro isolado produtor de carbapenemase, enzima conhecida como *Klebsiella pneumoniae* carbapenemase (KPC), foi descrito por Yigit e colaboradores. Esta enzima é caracterizada por conferir resistência a todos os fármacos β-lactâmicos, motivo pelo qual a sua disseminação mundial entre família Enterobacteriaceae é de extrema preocupação (QUALE, 2008; WALSH, 2010; NORDMANN e CORNAGLIA, 2012). KPCs foram identificadas em praticamente todos os membros da família Enterobacteriaceae (BRATU *et al*., 2005;. HIRSCH e TAM, 2010; GOREN *et al*. 2010; SOULI *et al*., 2010), no entanto, a prevalência de isolados produtores de KPC no Brasil (MONTEIRO *et al*., 2009) e, especificamente, no Rio Grande do Sul ainda é baixa (ZAVASCKI *et al*., 2009). Em 2011, WON e colaboradores (2011) observaram uma extensa transmissão de enterobactérias produtoras de KPC entre hospitais e lares de idosos, alertando a alta prevalência destas infecções em pacientes que fazem uso de dispositivos biomédicos, como cateter venoso central (67.5%), ventilação mecânica (30%) e que realizam hemodiálise (50%).

II - 3. Estratégias de combate aos biofilmes

A abordagem multidisciplinar para o tratamento e controle de biofilmes tem resultado da valorização crescente do papel que eles desempenham na medicina moderna. As estratégias para o combate de biofilmes podem, basicamente, ser divididas em dois segmentos: (a) a inibição da formação de biofilmes e (b) a erradicação ou tratamento de biofilmes já formados, cujos principais alvos para intervenção são ilustrados na Figura 6. Considerando o modo como pode ser alcançada a inibição da formação de biofilmes, foco do presente trabalho, duas grandes classificações podem ser feitas: através da via da inibição do crescimento bacteriano,

pelo uso de compostos bactericidas ou bacteriostáticos ou, através do bloqueio da adesão bacteriana e, consequentemente, da formação de biofilme por uma via que não envolve a morte bacteriana - característica marcante de um novo conceito de terapia: as terapias antivirulência. As terapias antivirulência exploram novos mecanismos de ação de compostos, visando dificultar o rápido desenvolvimento de resistência bacteriana. Ainda, por não afetar o crescimento de bactérias e manter as células em estado planctônico, o desligamento da expressão de virulência e atenuação do patógeno deve tornar os microrganismos mais suscetíveis aos antimicrobianos tradicionalmente utilizados e ao sistema imunológico (CLATWORTHY *et al.*, 2007; ESCAICH, 2010; RASKO e SPERANDIO, 2010). Assim, estas novas terapêuticas podem ser consideradas alternativas e/ou complementares à antibioticoterapia tradicional, com base em novos mecanismos de ação em diferentes alvos.

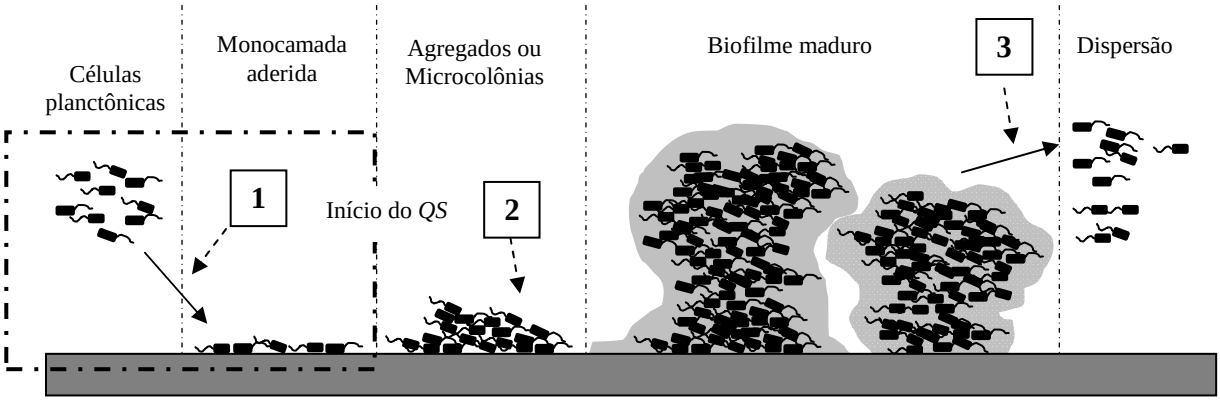

Figura 6: Principais alvos para combate aos biofilmes microbianos: inibição da adesão bacteriana via bloqueio da adesão bacteriana à superfície (etapa 1) ou o rompimento da comunicação celular bacteriana - *Quorum sensing* (etapa 2) e, a erradicação ou tratamento de biofilmes já formados (etapa 3) (adaptada de MACEDO e ABRAHAM, 2009).

(a). Inibição da formação de biofilmes: O desenvolvimento de novas estratégias para prevenir a formação de biofilme, principalmente em biomateriais, tem recebido bastante atenção. A inibição da formação de biofilmes pode ser obtida através (a1) do bloqueio da adesão celular bacteriana a uma superfície ou por meio (a2) do rompimento da comunicação celular bacteriana (*Quorum sensing*), conforme ilustrado na etapa 1 e 2 da Figura 6, respectivamente.

(**a1). Bloqueio da adesão celular bacteriana à superfície** (Figura 6, etapa 1). Considerando superfícies abióticas, o uso profilático de antibióticos e biocidas

(incluindo revestimentos de dispositivos médicos, imersão do dispositivo em soluções antibióticas, irrigação do sítio cirúrgico com antibióticos, e terapia de bloqueio com antibiótico, a última geralmente utilizada em cateteres) podem reduzir a incidência de adesão e infecções associadas a biofilmes em implantes médicos. Embora o uso da profilaxia antimicrobiana seja controverso devido à curta duração da sua eficácia e ao seu potencial para aumentar a resistência antimicrobiana, esta prática é cada vez mais comum em grupos de pacientes de alto risco (FUX *et al.*, 2003; LYNCH e ROBERTSON, 2008). Ainda em relação a substratos abióticos, o desenvolvimento de novas superfícies com características físicas de antiaderência, (evitando interações físico-químicas que medeiam a adesão primária ao substrato), bem como o recobrimento de superfícies com os mais diversos compostos, incluindo antibióticos e moléculas inibidoras do sistema *QS*, representam uma outra alternativa na prevenção da formação de biofilmes, que está descrito no item I - 3.2. Modificação de superfícies.

Para a inibição da adesão celular bacteriana a um substrato biótico, importantes exemplo são os compostos pilicidas e os compostos quelantes de ferro. Os pilicidas, como por exemplo as 2-piridonas biciclícas, são capazes de inibir a biossíntese de pili, uma das estruturas bacterianas responsáveis pela adesão de certas bactérias às células humanas. Desta maneira, estes compostos são capazes de evitar a adesão de *E. coli* às células da bexiga (PINKNER *et al.*, 2006; BERG *et al.*, 2008) e do *Vibrio cholerae* às células intestinais (HUNG *et al.*, 2005). Com relação aos compostos quelantes de ferro, Singh e colaboradores (2002) demostraram que a lactoferrina, uma proteína encontrada na maioria dos fluidos corporais, é capaz de quelar ferro, um elemento crítico para a adesão bacteriana a superfícies. A lactoferrina em concentrações subinibitórias foi capaz de inibir a adesão bacteriana e a formação de biofilme de *P. aeruginosa* sem afetar o crescimento bacteriano, através da privação de ferro. Em concentrações mais altas, a lactoferrina é conhecida por inibir o crescimento bacteriano, rompendo a membrana bacteriana através da ligação aos lipopolissacarídeos (ELLISON, 1994). Devido à eficácia comprovada da lactoferrina contra patógenos capazes de formar biofilme, patentes que descrevem o seu uso em tratamentos de lesões superficiais de pele foram registradas (AMMONS, 2010). Estudos similares foram desenvolvidos com outros quelantes de ferro, como ácido

2,2’-dipiridil dietilenotriaminopentacético (DTPA), ácido etilenodiaminotetracético (EDTA), etilenodiamina-N,N’- ácido diacético (EDDA) e mesilato de deferoxamina (O’MAY *et al.*, 2009).

Ao se considerar ambas as superfícies, abióticas e bióticas, um novo alvo para impedir a adesão bacteriana encontra-se em estudo. Trata-se da adesina sortase, uma enzima de membrana que catalisa reações de transpeptidação ligando covalentemente proteínas ao peptidioglicano, além de estarem envolvidas na montagem do pili (MARRAFFINI *et al.*, 2006). Bactérias mutantes em sortase A (gene *str*A), apresentam uma deficiência de proteínas de superfície e dificuldade em aderir a superfícies de hidroxiapatita na presença de saliva. Outras moléculas, como o metanotiosulfonato e o ácido p-hidroxi-mercuribenzóico estão sendo reconhecidas como inibidores de sortases. Sendo um fator de virulência universal de bactérias Gram-positivas, estas enzimas poderão servir como um bom alvo para fármacos antiadesão, com amplas aplicações clínicas (CHEN e WEN, 2011).

(a2). Rompimento da comunicação celular bacteriana (*Quorum sensing*) (Figura 6, etapa 2). Para estabelecer uma infecção e produzir uma doença, bactérias patogênicas devem desenvolver diferentes mecanismos de virulência para colonizar, disseminar e se adaptar aos vários microambientes impostos. Na fase posterior à formação de agregados e no início da maturação de formação do biofilme, as bactérias usam sistemas de comunicação célula-célula para regular a expressão dos genes envolvidos na formação do biofilme.

A inibição da formação de biofilmes, após a etapa da adesão primária, pode ser obtida através da interferência nesta sinalização intercelular bacteriana. O uso de moléculas inibidoras do sistema *QS* (QSI), as quais competem com o receptor das moléculas sinalizadoras, ou de enzimas conhecidas como *Quorum Quenching* (QQ), as quais degradam as moléculas de sinalização, podem bloquear a comunicação celular bacteriana (MARTIN *et al.* 2008). Desta maneira, a produção de EPS, dentre outras atividades, é inibida, o que dificulta a manutenção da estrutura tridimensional dos biofilmes (adesão irreversível) (MACEDO e ABRAHAM, 2009; LAZAR, 2011)

(Figura 6, etapa 2). Acredita-se que as moléculas moduladoras do *QS* sejam capazes de tornar os microrganismos mais suscetíveis aos antimicrobianos e ao sistema imune. Desta maneira, combinando esses interferentes do sistema QS com antimicrobianos tradicionais pode-se aumentar a efetividade dos fármacos correntemente utilizados, facilitando o controle de infecções bacterianas relacionadas a biofilmes. Dentre um grande número de patentes (ROMERO *et al.*, 2012) e compostos QSI relatados na literatura, as furanonas halogenadas e o peptídeo inibidor do RNA Ⅲ (RIP) parecem ser os compostos mais investigados, inibindo a formação de biofilme de *P. aeruginosa* e *Staphylococcus* spp. (MUSK e HERGENROTHER, 2006; CHEN e WEN, 2011). No entanto, alguns estudos estão demonstrando que a formação de biofilmes de *P. aeruginosa* e de *S. aureus* pode ocorrer independente da modulação do sistema *QS* (SCHABER *et al.*, 2007, COELHO *et al.*, 2008), o que corrobora com a hipótese de que os mecanismos envolvidos na formação dos biofilmes são complexos e provavelmente multifatoriais.

(b). Erradicação ou tratamento de biofilmes já formados (Figura 6, etapa 3). Atualmente, o tratamento de biofilmes, quando possível, ocorre basicamente através do uso de antimicrobianos e da substituição de dispositivos médicos. Esta prática, porém, acarreta onerosos custos tanto ao paciente quanto ao sistema de saúde, conforme descrito na seção anterior.

A formação de biofilme não é um processo irreversível e os microrganismos por si só são capazes de dissolver um biofilme em condições desfavoráveis, como mudanças de pH e privação nutricional. Para isto, eles devem coordenar o seu comportamento para se converter ao estado planctônico. Assim, o conhecimento e elucidação destas moléculas, as quais estimulam o mecanismo natural de dispersão de biofilmes, auxilia a busca de compostos para a erradicação de biofilmes (MACEDO e ABRAHAM, 2009; OTTO, 2012b). Ainda que incipiente, o recente progresso na descoberta dos mecanismos implicados na conversão de células sésseis em células planctônicas, no caso de *S. aureus* e de *S. epidermidis*, indicam o envolvimento do colapso ou a solubilização da matriz do biofilme (BOLES e HORSWILL, 2011).

Para erradicar biofilmes já formados, encontra-se em fase de estudo principalmente enzimas e algumas outras moléculas capazes de desintegrar a matriz (EPS) que engloba as células bacterianas. A intenção não é necessariamente inibir o crescimento bacteriano, mas sim perfurar a estrutura do biofilme (através de ruptura enzimática), sendo útil em combinação com um agente antimicrobiano para o tratamento de infecções associadas a biofilmes. A diversificada constituição química da matriz do biofilme, que inclui material protéico, DNA extracelular e polissacarídeos, torna o EPS suscetível à degradação por uma série de enzimas exogenamente adicionadas (como proteinase K, tripsina e DNAse I) (BOLES e HORSWILL, 2011). Neste sentido, o uso enzimas do tipo alginato liase, capazes de degradar o polímero alginato, provocou importante erradicação de biofilme de *P. aeruginosa* (ALKAWASH *et al.*, 2006); a adição de ânions polivalentes (poliaspartato) e/ou DNAse, também ocasionaram importante erradicação de biofilme de *P. aeruginosa* e de *S. aureus* (MANN *et al.*, 2009; TOLKER-NIELSEN e HOIBY, 2009). A dispersina B, uma N-acetilglucosaminidase produzida por *Actinobacillus actinomycetemcomitans* (KAPLAN *et al.*, 2003) é um outro exemplo. Esta enzima é capaz de inibir a formação de biofilme e de promover a erradicação de biofilmes já formados em muitas cepas de *S. epidermidis* e *S. aureus* que possuem poli-*N*-acetilglicosamina (PNAG) como um componente dominante da sua matriz (KAPLAN *et al.*, 2004). Ainda, a lisostafina, uma glicina endopeptidase produzida por *Staphylococcus simulans*, foi eficaz em erradicar biofilme de *S. aureus* em modelo de infecção de cateter em camundongos (KOKAI-KUN *et al.*, 2009). Esta enzima degrada a ponte pentaglicina da parede celular estafilocócica, indicando que o material da parede celular possui um papel subestimado na matriz do biofilme.

Após duas décadas de incansável esforço, recentes patentes (PAN e REN, 2009; AMMONS, 2010, CARVALHO, 2012; ROMERO *et al*., 2012) e mais de 250 mil publicações (CHEN e WEN, 2011) ainda estamos à espera para o lançamento do primeiro produto antibiofilme. Neste ponto, os esforços coletivos no campo da antiadesão, interrupção do sistema *QS* e erradicação de biofilmes - os três grandes alvos no combate aos biofilmes - geram expectativa no fornecimento de novos

fármacos, embora o conjunto de diferentes alvos pareça ser a maneira mais apropriada para combater os biofilmes.

II - 3.1. Plantas como fonte de compostos antibiofilmes e antibacterianos

Encontrar poderes de cura nas plantas é uma ideia muito antiga. Hipócrates (no final do século V aC) já havia mencionado cerca de 300 a 400 plantas medicinais. As plantas têm sido utilizadas como recurso terapêutico em virtualmente todas as culturas. Em regiões de baixo desenvolvimento econômico ou em zonas rurais, a falta de acesso aos medicamentos industrializados determina o tratamento das doenças com base no uso de plantas medicinais (LOPEZ *et al.*, 2001; MOTHANA e LINDEQUIST, 2005; TADEG *et al.*, 2005). Nos países industrializados, desde o advento de antibióticos na década de 1950, o uso de derivados de plantas como antimicrobianos diminuiu. Em contrapartida, nestes locais, a medicina alternativa ou complementar - medicina que não desempenha um papel principal em um sistema de saúde, como fitoterapia, acupuntura, homeopatia e quiropraxia - vem apresentando um aumento significativo. Nos Estados Unidos, o uso de apenas 1 das 16 terapias alternativas aumentou de 33,8%, em 1990, para 42%, em 1997. Na Bélgica, em 1998, quase 40% da população já havia feito uso de medicinas complementares e 25% dos médicos utilizavam esses recursos (ZHANG, 2000).

O interesse em pesquisas que envolvem atividade antimicrobiana dentre a comunidade científica dedicada a investigar as propriedades das plantas medicinais tem aumentado exponencialmente (RÍOS e RECIO, 2005). Quando o número de publicações sobre a atividade antimicrobiana de plantas medicinais é analisado no banco de dados científicos "*Web of Science*" (tópico: antimicrobial activity AND medicinal plants), encontra-se 7, 135 e 1874 artigos publicados durante os períodos de 1945 a 1990, 1991 a 2000 e 2001 a 2012, respectivamente (pesquisa feita no dia 11 de dezembro de 2012). No período de 2005 a 2010, um total de 19 fármacos com base em produtos naturais foram aprovados para comercialização em todo o mundo, dentre os quais 7 são classificadas como produtos naturais, 10 como produtos naturais semi-

sintéticos e 2 como fármacos derivados de produtos naturais. Dentre os 19 fármacos aprovados, 5 são antibacterianos: doripenem, tigeciclina, retapamulina, telavancina e o aztreonam monobactâmico (MISHRA e TIWARI, 2011).

O grande número de compostos derivados de produtos naturais em vários estágios de desenvolvimento clínico indica que o uso destes como modelo ainda é uma fonte viável para novos fármacos (MISHRA e TIWARI, 2011; NEWMAN e CRAGG, 2012). Além disso, quando comparado com bibliotecas de produtos sintéticos, os produtos naturais apresentam perspectivas de descobrimento de maior número de compostos e com estruturas muito mais complexas (HENKEL *et al.*, 1999).

As plantas produzem diversos metabólitos secundários e a escolha das mesmas como fonte de compostos antimicrobianos é apropriada dada a razão ecológica de que elas produzem naturalmente moléculas que atuam como defesa química contra microrganismos. Compostos antimicrobianos provenientes de plantas são extensamente conhecidos. Dentre as principais classes de antimicrobianos encontram-se o grande grupo dos polifenóis, os terpenoides, as lectinas e polipeptídeos e os poliacetilenos (COWAN, 1999). Exemplos da literatura se destinam a ser representativos, mas não exaustivos do assunto (Tabela 4), uma vez que diversos exemplos e algumas outras classes de compostos com atividade antimicrobiana provenientes de plantas poderiam ser citados (GIBBONS, 2004; MAHADY, 2005; MAHADY *et al.*, 2008, PERUMAL SAMY e GOPALAKRISHNAKONE, 2010).

Mais recentemente, a atividade antibiofilme de compostos provenientes de plantas começou a ser considerada. Wittschier e colaboradores (2007) destacaram a importância de grandes moléculas (como taninos e carboidratos de alta massa molecular) provenientes de plantas como importantes antagonistas de interações adesivas.

Com relação à inibição de formação de biofilme, um dos mais conhecidos e talvez mais estudados compostos de ação antiaderência e inibidores de biofilme são as proantocianidinas (taninos condensados) purificadas do cranberry (*Vaccinium macrocarpon*), com ação principalmente contra *E. coli* (FOO *et al*., 2000;

EYDELNANT e TUFENKI, 2008), *Streptococcus mutans* (KOO *et al*., 2010) e *Candida albicans* (FELDMAN *et al*., 2012). Janecki e Kolodziej (2010) mostraram que proantocianidinas do *Pelargonium sidoides* são capazes de inibir a adesão bacteriana de *Streptococcus* spp. a células de laringe humanas "in vitro". O tanino 1,2,3,4,6-penta-O-galoil-β-D-glicopiranose (purificado da *Eustigma oblongifolium*) apresenta atividade antiformação de biofilme de *S. aureus* através da privação de ferro (LIN *et al.*, 2011, 2012) e a proantocianidina A2 (isolada de *Aesculus hippocastanum*) previne a transição de células planctônicas de *S. aureus* e *S. epidermidis* para sésseis sem exercer efeito sobre o crescimento bacteriano (ARTINI *et al*., 2012). Vandeputte e colaboradores (2010) identificaram uma catequina das cascas de *Combretum albiflorum* que reduz a produção de fatores de virulência de *P. aeruginosa* via inibição do *QS*. Semelhantemente, Taganna e colaboradores (2011) demonstraram que a fração rica em taninos de *Terminalia catappa* é capaz de inibir o sistema bacteriano *QS* e prevenir a formação de biofilmes de *P. aeruginosa*. Dois derivados diterpenoides, a dimetilfruticulina A e a fruticulina A, isolados de *Salvia corrugata*, demostraram a habilidade de inibir a formação de biofilme de *S. aureus*, *S. epidermidis* e *Entercoccus faecalis* em concentrações subinibitórias, sendo que a interação dos compostos com estes microrganismos parece ser bastante complexa e envolver a inibição da síntese de EPS, atividade quelante e ainda mudanças na hidrofobicidade de superfície das bactérias (SCHITO *et al.*, 2011). O diterpeno casbano (isolado de *Croton nepetaefolius*) também apresenta atividade antiformação de biofilme sem interferir no crescimento de diversas espécies de microrganismos (CARNEIRO *et al.*, 2010).

Alguns poucos estudos de rastreamento também são relatados na literatura na busca de moléculas moduladoras do sistema bacteriano *QS*. Estes trabalhos envolvem extratos de diversas plantas e, geralmente, utilizam como modelo o biosensor *Chromobacterium violaceum* CV026 e CV12472 e *P. aeruginosa* PA01 -os quais são utilizados para identificar inibidores do *QS* de bactérias Gram-negativas - e, a quantificação de δ-hemolisina, para avaliar a modulação em *S. aureus* (MUSTHAFA

et al., 2010; SONG *et al.*, 2010; ZAHIN *et al.*, 2010; KOH e THAM, 2011; QUAVE *et al.*, 2011).

Com relação à erradicação de biofilmes, a literatura descreve o efeito antimicrobiano principalmente de polifenóis. Estes estudos geralmente enfocam microrganismos de importância odontológica. Sampaio e colaboradores (2009), mostraram que extratos ricos em polifenóis de frutos de *Caesalpiniea ferrea* apresentam atividade antibiofilme em um modelo multiespécies, envolvendo *Streptococcus* spp., *Candida albicans* e *Lactobacillus casei*. Prabhakar e colaboradores (2010) demostraram que polifenóis de trifala (mistura de ervas ayurvédicas) e chá verde apresentam atividade antibacteriana contra biofilme de *E. faecalis* formados em dentes durante 3 e 6 semanas. Ainda, proantocianidinas parecem ser as responsáveis pela atividade antiformação de biofilme (interferindo na adesão) e de erradicação de biofilmes de *Streptococcus mutans* em modelos de hidroxiapatita e em dentes humanos (DAGLIA *et al.*, 2010). Recentemente, Coenye e colaboradores (2012) mostraram que extratos de *Epimedium brevicornum* e *Polygonum cuspidatum*, assim como os seus compostos ativos (icariina e resveratrol, respectivamente) erradicam biofilmes de *Propionibacterium acnes* em concentrações subinibitórias, indicando que a morte bacteriana não é o único modo de ação envolvido nesta atividade.

As diversas atividades descritas mostram que os metabólitos secundários de plantas possuem, além da bem reconhecida propriedade antimicrobiana, um alto potencial como agentes bloqueadores da formação de biofilmes microbianos e capazes de agir sobre células que encontram-se na forma de biofilmes. A utilidade de produtos naturais como fontes de novas estruturas, mas não necessariamente a entidade química final, permanece grande sendo que a área de compostos antiinfecciosos é dependente de produtos naturais e das suas estruturas (NEWMAN e CRAGG, 2012).

Tabela 4: Principais classes de compostos antimicrobianos de plantas, exemplos e mecanismos de ação sugeridos (adaptado de COWAN, 1999 e PERUMAL SAMY e GOPALAKRISHNAKONE, 2010).

Classes	Subclasses	Exemplos	Mecanismos
Polifenóis	Fenóis simples	Catecol	Privação de substrato
		Epicatequina	Rompimento de membrana
	Ácidos fenólicos	Ácido cinâmico	
	Quinonas	Hipericina	Ligação a adesinas, complexação com parede celular e inativação enzimática
	Flavonoides	Crisina	Ligação a adesinas, complexação com parede celular e inativação enzimática
	Flavonas	Abissinona	Inativação enzimática, inativação da transcriptase reversa HIV
	Taninos	Elagitaninos	Ligação a proteínas e a adesinas, inativação enzimática, privação de substrato, complexação com parede celular e com íons metálicos, rompimento de membrana
	Cumarinas	Warfarina	Interação com DNA eucariótico (atividade antiviral)
Terpenoides, óleos essenciais		Capsaicina e Ácido imbérbico	Rompimento de membrana
Alcaloides		Berberina e Piperina	Intercalante da parede celular e/ou DNA
Lectinas e polipeptídeos		Aglutinina específica de manose	Formação de canais iônicos na membrana microbiana, inibição competitiva da adesão de proteínas microbianas aos receptores polissacarídeos do hospedeiro, bloqueio da fusão ou adsorção viral
		WjAMP-1	
		Fabatina	Formação de pontes dissulfeto
Poliacetilenos		8S -Heptadeca 2(*Z*),9(*Z*)dieno - 4,6-diino-1,8-diol	Não descrito

II - 3.1.1. Etnofarmacologia e o bioma Caatinga

Existem diferentes abordagens a fim de facilitar a selação de plantas para a busca de metabólitos secundários bioativos (CORDELL *et al.*, 1996; FABRICANT e FARNSWORTH, 2001): (a) seleção randômica das plantas, onde todas as plantas dentro de uma área geográfica são coletadas e avaliadas por um amplo espectro de atividades biológicas (geralmente utilizando técnicas de “high throughput screening”); (b) seleção das plantas a partir de estudos taxonômicos e quimiotaxonômicos, a qual se baseia na premissa de que plantas relacionadas herdam a capacidade genética para produzir metabólitos secundários semelhantes; (c) seleção das plantas a partir de relatos etnofarmacológicos.

A etnofarmacologia é uma abordagem diversificada para a descoberta de compostos, que envolve a observação, a descrição e a investigação experimental de plantas medicinais e suas atividades biológicas. Congrega conhecimentos de botânica, química, bioquímica, farmacologia, e muitas outras disciplinas como, antropologia, arqueologia, história, e linguística, que contribuem para a descoberta de produtos naturais bioativos. Segundo Elisabetsky (1999), a definição mais aceita de etnofarmacologia é a exploração científica interdisciplinar dos agentes biologicamente ativos, tradicionalmente empregados ou observados pelo homem. Como estratégia para a investigação de plantas medicinais, a etnofarmacologia combina as informações adquiridas junto a comunidades locais que fazem uso da flora medicinal com estudos químicos e farmacológicos em laboratórios de pesquisa.

A etnofarmacologia permite a formulação de hipóteses quanto a(s) atividades farmacológicas e quanto à(s) substância(s) ativa(s) responsável(eis) pelas ações terapêuticas relatadas pelas populações usuárias (ELISABETSKY, 1999), utilizando basicamente duas abordagens complementares (GRAZ *et al.*, 2010; REYES-GARCÍA, 2010):

(a) a validação da eficácia da medicina tradicional para as pessoas que as utilizam, contribuindo para (i) assegurar o registro e a preservação dos conhecimentos tradicionais, conservando o patrimônio cultural em nível local, bem como para (ii)

estimular o desenvolvimento de políticas públicas sobre a proteção da espécie contra os danos ambientais.

(b) a contribuição das informações obtidas a partir da medicina popular associada à evidência científica, sendo um valioso atalho para guiar os estudos de bioprospecção e aumentar o sucesso na descoberta de novos fitofármacos.

Um estudo etnofarmacológico necessita a interação multidisciplinar, envolvendo, os seguintes passos (ELISABETSKY, 1999):

1 - Coleta das plantas e análise dos dados farmacológicos;

2 - Identificação botânica, e depósito de material-testemunho em herbário;

3 - Análise química preliminar para detectar as classes de compostos presentes na parte da planta utilizada medicinalmente (farmacógeno);

4 - Estudo farmacológico preliminar dos extratos vegetais em modelos experimentais relacionados às ações farmacológicas sugeridas pela análise das informações populares;

5 - Fracionamento químico, onde as frações de interesse são detalhadas pelo uso de técnicas analíticas;

6 - Estudo farmacológico abrangente e toxicologia pré-clínica de frações padronizadas ou compostos purificados;

7 - Elucidação das estruturas das substâncias ativas isoladas e/ou obtenção de derivados semi-sintéticos.

Fabricant e Farnsworth (2001) mostram que os fármacos provenientes de plantas de maior utilidade na medicina foram descobertos pelo acompanhamento de usos etnofarmacológicos, incluindo o sedativo escopolamina, o antitumoral teniposídeo, os diuréticos e broncodilatores teobromina e teofilina, dentre outros. A Tabela 5 correlaciona alguns estudos etnofarmacológicos e atividade antimicrobiana.

Tabela 5: Algumas plantas utilizadas na medicina tradicional e suas classes de compostos antimicrobianos (adaptado de PERUMAL SAMY e GOPALAKRISHNAKONE, 2010).

Nomes científicos	Partes usadas/ Solventes	Classes	Compostos	Medicina tradicional e Mecanismos
Baccharis grisebachii Hieron (Asteraceae)	Exudato da resina	Diterpenos, derivados de ácido *p*-cumárico, flavonas	Ácido 3- prenil- *p*-cumárico Ácido 3,5- diprenil- *p*-cumárico	Utilizado na medicina tradicional argentina. Mostrou atividade frente a fungos dermatófitos e bactérias.
Origanum virens L. (Lamiaceae)	Partes aéreas	Óleo essencial	Carvacrol (68.1%), γ-terpineno (9.9%), e *p*-cimeno (4.5%)	Utilizado na medicina tradicional portuguesa. Atividade antifúngica contra espécies de *Candida*. Efeito primariamente devido a lesões na membrana.
Osmitopsis asteriscoides L. (Asteraceae)	Partes aéreas	Óleo essencial	1,8-Cineol, cânfora	Atividade antimicrobiana frente a *Candida* spp, atividade bacteriostática para *S. aureus* e *P. aeruginosa*. Possivelmente apresente efeito sinérgico com o uso tradicional para dores pulmonares na África do Sul.
Santiria trimera (Oliv.) Aubrev. (Burseraceae)	Cascas	Óleo essencial monoterpeno	β-Pineno (20%) e α-pineno (80%)	Utilizado na medicina tradicional de São Tomé e Príncipe na África. Planta amplamente usada pelos curandeiros, atividade antimicrobiana principalmente contra *Proteus vulgaris* e *Cryptococcus neoformans*.
Zanthoxylum chalybeum Engl. (Rutaceae)	Sementes	Alcalóide	Skimmianina	Utilizado na medicina tradicional da Uganda, na África. Atividade antibacteriana. Mecanismo de ação não estabelecido.
Zingiber officinale Rosc (Gingiberaceae)	Rizoma	Polifenóis	6-,8-,10-Gingerois	Utilizado na medicina tradicional chinesa. Inibição do crescimento de *Helycobacter pylori* “in vitro”.

A diversidade da flora não está distribuída uniformemente em todo o planeta, sendo que aproximadamente 70% das espécies do mundo ocorrem apenas em 12 países: Austrália, Brasil, China, Colômbia, Equador, Índia, Indonésia, Madagascar, México, Peru e Zaire (BASSO *et al.*, 2005). Neste contexto, o Brasil possui uma posição de destaque, contando uma grande biodiversidade (Floresta Amazônica, Mata Atlântica, Floresta de Araucárias, Cerrado, Caatinga e Pampa) e um rico conhecimento tradicional acumulado pelas populações locais que possuem acesso direto à natureza e aos produtos desta biodiversidade (BASSO *et al.*, 2005; ALBUQUERQUE *et al.*, 2007). A extensão do país, particularmente na direção Norte-Sul, cobre uma grande variedade de climas, tipos de solo e altitudes, fornecendo um conjunto único de pressões seletivas para a adaptação da vida vegetal nesses diversos cenários. Estima-se que apenas 5-15% das plantas superiores têm sido sistematicamente investigadas quanto a presença de compostos bioativos, desta forma a biodiversidade permanece amplamente inexplorada (PIETERS e VLIETINCK, 2005).

A região da Caatinga ocupa uma área de 800.000 km^2, cobrindo quase 60% do território nordestino do Brasil e uma pequena parte da região sudeste do estado de Minas Gerais (ARAÚJO *et al.*, 2007). A Caatinga é o único bioma exclusivamente brasileiro, caracterizado por savana semi-árida, que apresenta temperaturas elevadas (média anual de 27,5 °C), com baixa e irregular pluviosidade (média anual de 250–500 mm) (BASSO *et al.*, 2005; COUTINHO, 2006; ALVES *et al.*, 2011). Os solos são de diferentes origens e, via de regra, são quimicamente férteis, bem drenados, e oxigenados. A estação seca dura sete meses ou mais, sendo que o inverno é a estação chuvosa, em que as temperaturas não são tão altas (BASSO *et al.*, 2005). Assim, a paisagem da região é dominada por um mosaico de formas fisionômicas, como a arbórea, a arbustiva e a espinhosa, todas adaptadas à seca (COUTINHO, 2006). No Nordeste do Brasil vivem diferentes comunidades étnicas, as quais praticam a agricultura de subsistência, pecuária e colheita de produtos florestais (ALBUQUERQUE *et al.*, 2007; ARAÚJO *et al.*, 2007).

Várias publicações descrevem que a rica flora desta região possui diversas propostas medicinais para as comunidades rurais (AGRA *et al.*, 2007a, 2007b, 2008; ALBUQUERQUE *et al.*, 2007; CARTAXO *et al.*, 2010), entretanto, muitas espécies ainda não foram submetidas a estudos científicos para confirmar sua eficácia no tratamento de uma determinada doença (CARTAXO *et al.*, 2010). A investigação das plantas conhecidas pelo uso etnomedicinal na região Nordeste brasileira, realizada por Agra e colaboradores (2008), revelou um total de 650 espécies e 407 gêneros pertencentes a 111 famílias. Destas espécies, cerca de 126 são exóticas e cultivadas na região, correspondendo a cerca de 20% do total. Com relação à região da Caatinga, 389 espécies de plantas são utilizadas pela população local com fins medicinais (ALBUQUERQUE *et al.*, 2007).

Diante da infinidade de espécies que apresentam indicações etnofarmacológicas e que possuem potencial químico e farmacológico para várias propostas medicinais, fica evidente que estudos científicos para a comprovar a efetividade são necessários. Assim, as espécies da Caatinga, sujeitas a um ambiente de extrema seca o qual poderia conduzir a uma produção diferenciada de metabólitos secundários, associadas ao conhecimento tradicional da comunidade local, representam uma valiosa fonte para prospecção de produtos naturais bioativos.

II – 3.1.2. Fracionamento bioguiado de extratos aquosos: o estudo de taninos

Após a coleta e identificação do material vegetal, o passo seguinte é de extrema importância: a escolha de solvente para a extração. Frequentemente são utilizados solventes orgânicos polares ou moderadamente polares. Extratos aquosos geralmente são evitados por conta de sua complexidade, entretanto, na medicina tradicional a preparação dos remédios é realizada através da extração com água ou ainda com cachada (SAMUELSSON *et al.*, 1985). É preciso, portanto, considerar que se estas plantas contêm compostos farmacologicamente ativos e se estes são extraíveis pela água, o uso de outros solventes pode prejudicar ou interferir na detectação da atividade

farmacológica relatada pelo uso popular. Neste contexto, merece destaque a purificação de compostos ativos através do fracionamento bioguiado, onde após a triagem primária com bioensaios, os extratos que demonstram uma expressiva e reprodutível atividade podem ser submetidos ao fracionamento. Neste procedimento, o extrato fracionado e cada fração que é produzida durante este processo são avaliados em ensaio biológico, sendo que somente as frações ativas serão fracionadas novamente até a etapa de purificação de um composto biologicamente ativo (SAMUELSSON *et al.*, 1985; CORDELL *et al.*, 1996; HOSTETTMANN, 1998; PIETERS e VLIETINCK, 2005) (Figura 7). O isolamento dos constituintes farmacologicamente ativos de plantas, quando possível, é um longo processo. Por esta razão o rastreamento fitoquímico, gera informações sobre as classes estruturais dos compostos presentes em extratos ou nas primeiras fases de fracionamento, sendo importantes para direcionar os passos subsequentes de purificação.

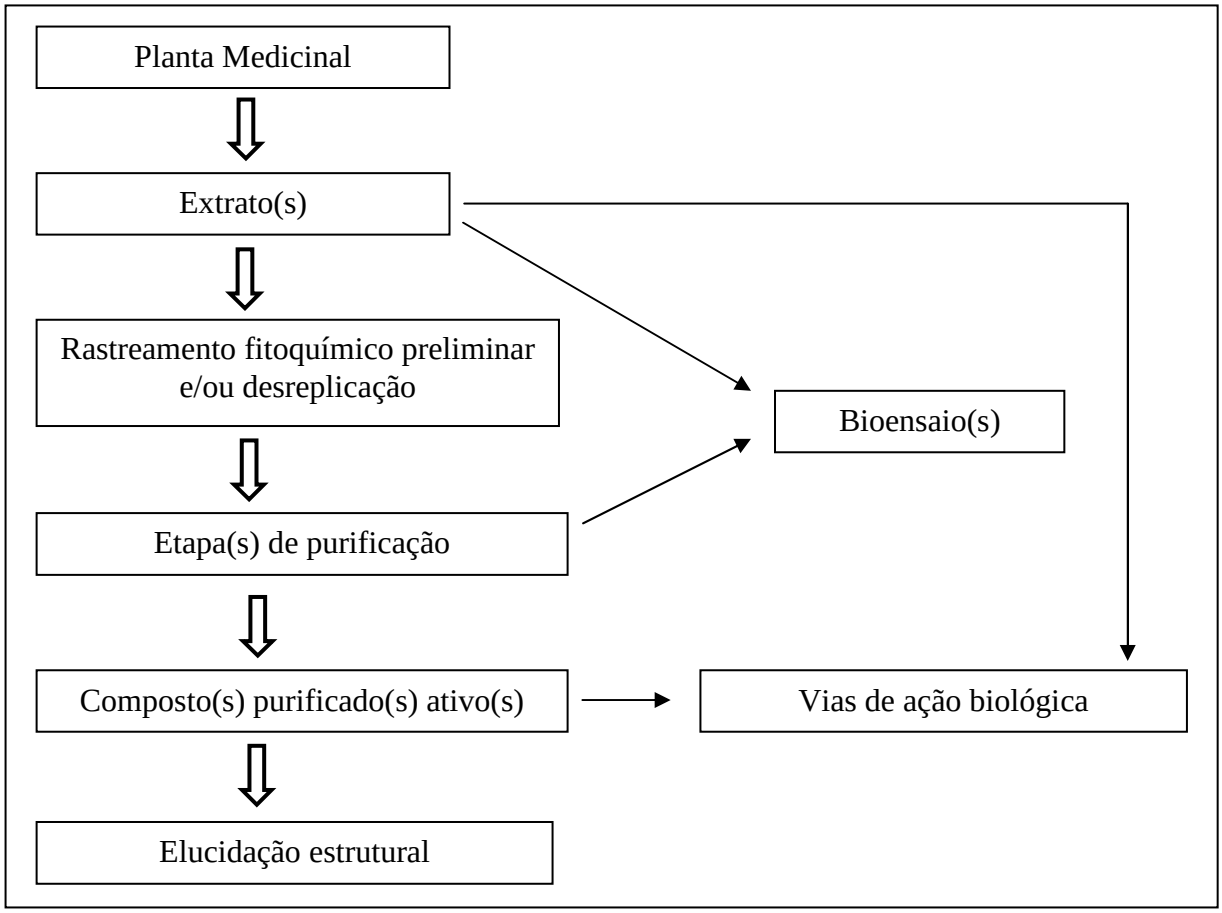

Figura 7: Etapas no processo de purificação química guiado por atividade biológica (adaptado de PIETERS e VLIETINCK, 2005).

Uma vez determinada a extração do material vegetal com água, a presença de saponinas, flavonoides, lectinas e taninos como prováveis classes de compostos bioativos deve ser considerada. Taninos são compostos fenólicos solúveis em água com massa molecular entre 500 e 4000 Daltons. Tradicionalmente, são classificados segundo sua estrutura química em dois grupos: taninos hidrolisáveis e taninos condensados (SANTOS e MELLO, 1999). Os hidrolisáveis são caracterizados por um grupo poliol central, geralmente β - D – glicose, cujas funções hidroxilas são parcial ou totalmente esterificadas com o ácido gálico (no caso de galotaninos) ou com ácido hexa-hidroxidifênico (no caso de elagitaninos) (Figura 8A e B), formando estruturas condensadas de unidades monoméricas que se interconectam. Já os taninos condensados, também conhecidos como proantocianidinas, são estruturalmente mais complexos que os hidrolisáveis, constituindo-se de oligômeros e polímeros formados pela policondensação de duas ou mais unidades de flavan-3-ol (referidas como catequinas) ou flavan-3,4-diol (pertencente à classe das leucoantocianidinas) ou, ainda, a mistura de ambos (CHUNG *et al*, 1998) (Figura 8C e D).

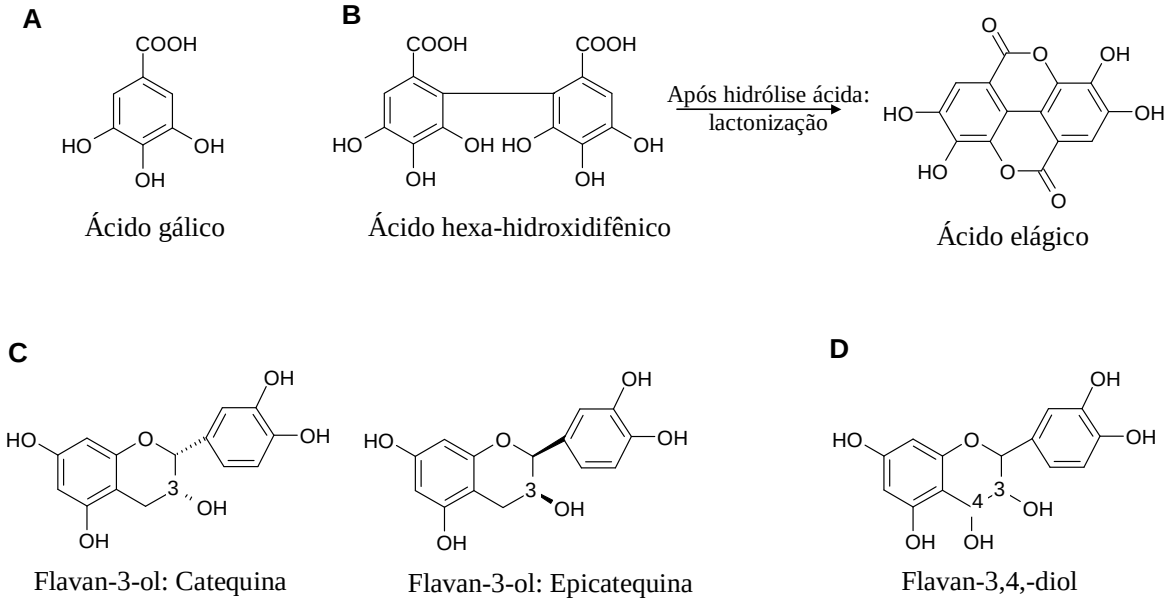

Figura 8: Precursores de taninos hidrolisáveis: ácido gálico (A) e ácido hexa-hidroxidifênico que produz após degradação em meio ácido o ácido elágico (B). Precursores de taninos condensados derivados de flavan-3-ol: catequina e epicatequina (C) e a estrutura básica de flavan-3,4-diol (D) (Adaptado de CHUNG *et al*, 1998).

Uma das principais características destes polifenóis, estruturalmente ricos no número de hidroxilas, é a capacidade de formar complexos insolúveis em água com alcaloides, proteínas e outras macromoléculas (SANTOS e MELLO, 1999), fato que lhes confere diversas propriedades. Os taninos são tradicionalmente utilizados no curtimento do couro, sendo esta uma importante aplicação comercial, assim como para o desenvolvimento do sabor e cor do vinho tinto durante o processo de envelhecimento. Além disso, os taninos são utilizados para a fabricação de adesivos para madeira (PIZZI, 1977; KIM, 2009) e também como resinas de purificação de água (BELTRAN-HEREDIA e SANCHEZ-MARTIN, 2009).

A adstringência de muitos frutos é também conferida pela propriedade dos taninos de precipitar as glicoproteínas salivares. O papel biológico de taninos como uma estratégia de defesa nas plantas é bastante investigado (MONTEIRO *et al.*, 2005). Estudos demonstram que taninos diminuem a taxa de predação de herbívoros por serem impalatáveis e por apresentarem potencial antinutricional devido à adstringência, afastando os predadores naturais (HEIL *et al.*, 2002). Taninos também apresentam diversos modos de defesa para combater bactérias, fungos e leveduras, dentre eles: a inibição de enzimas microbianas extracelulares, a privação de substratos e íons necessários para o crescimento microbiano e a ação em membranas microbianas (SCALBERT, 1991). Outras ações fisiológicas e farmacológicas, como antiviral, antiparasitária, antioxidante, anticarcinogênica, bem como carcinogênica estão relatadas na literatura (HASLAM, 1996; CHUNG *et al.*, 1998).

Muitas espécies produtoras de taninos são usadas na medicina popular para diferentes finalidades, destacando as propriedades antimicrobianas, as quais são bem documentadas. De forma que os taninos eram reconhecidos como resíduos ineficazes dos produtos fotossintéticos e respiratórios, poucos estudos têm avaliado o potencial medicinal de tais compostos (MONTEIRO *et al.*, 2005). Os métodos mais indicados para a determinação de taninos são os de precipitação de proteínas, enquanto que os ensaios colorimétricos são amplamente utilizados para estimar a quantidade de taninos específicos (MONTEIRO *et al.*, 2005). Os ensaios colorimétricos não fornecem dados

quantitativos acurados, principalmente devido ao baixo entendimento em relação às estruturas dos taninos, à dificuldade de seleção de um padrão que represente fielmente a amostra e devido ao fato de que não exista um método ideal para caracterizá-los. Entretanto, os mesmos podem ser utilizados para fins de propostas comparativas (MUELLER-HARVEY, 2001; SCHOFIELD *et al.*, 2001; HÜMMER e SCHREIER, 2008). No caso de polifenóis de alta massa molecular, embora a separação e a elucidação estrutural sejam bastante difíceis, devido à grande diversidade dos taninos e a sua capacidade de complexação com diversos compostos, alguns métodos estão sendo aplicados com este propósito. Destacam-se o uso de técnicas cromatográficas por exclusão de tamanho, por adsorção (fase estacionária normal) e por contra-corrente como efetivas para a purificação destes compostos de acordo com o grau de polimerização e, o uso de espectrometria de massas para caracterizar a distribuição de massa molecular de constituintes oligoméricos (OKUDA *et al*., 1989; YANAGIDA *et al*., 2003).

II - 3.2. Modificação de superfícies

Polímeros são amplamente utilizados como biomateriais em próteses, cateteres, nos sistemas de “drug delivery” e na engenharia de tecidos (LANGER e TIRRELL, 2004; LUTOLF e HUBBELL, 2005). Entretanto, como relatado no item “I - 2. Importância clínica e impacto econômico das infecções associadas a biofilmes”, materiais que são utilizados como dispostivos biomédicos são facilmente colonizados por microrganismos após a sua implantação.

O tipo de material utilizado na fabricação dos dispositivos biomédicos pode diferenciar em relação à propensão de adesão bacteriana (ROCHFORD *et al*., 2012). De acordo com Darouiche (2001), o cloreto de polivinila (PVC) favorece mais a adesão bacteriana do que o teflon; o polietileno (PE) mais que o poliuretano (PU); o látex mais que o silicone; o silicone mais que o politetrafluoretileno (PTFE); e o aço inoxidável mais que o titânio. Além disso, superfícies irregulares e texturizadas

favorecem mais a adesão bacteriana do que superfícies regulares e lisas. As respostas biológicas a materais poliméricos dependem basicamente da química e estrutura das superfícies envolvidas, e a utilização de materiais implantáveis tem progredido rapidamente, de modo que a modificação de superfície de materiais se torna de extremo interesse (VON EIFF *et al.*, 2005; PAVITHRA e DOBLE, 2008; FERNEBRO, 2011).

A modificação de superfícies, de maneira geral, permite: (a) modificar a superfície externa de um material para dificultar a adesão e colonização microbiana e/ou facilitar a biocompatibilidade material-tecido, mantendo inalteradas as propriedades mecânicas, o volume e as funcionalidades desejáveis do material (BALAZS *et al.*, 2004; ZHANG *et al.*, 2006; CHENG *et al.*, 2008; HAUSER *et al.*, 2009; KADOR e SUBRAMANIAN, 2011; BAZAKA *et al*., 2012) e, (b) introduzir sítios na superfície do material para a imobilização de moléculas ativas (GODDARD e HOTCHKISS, 2007). A Figura 9 destaca, de forma simples, algumas técnicas existentes para realizar a modificação de superfícies de materiais.

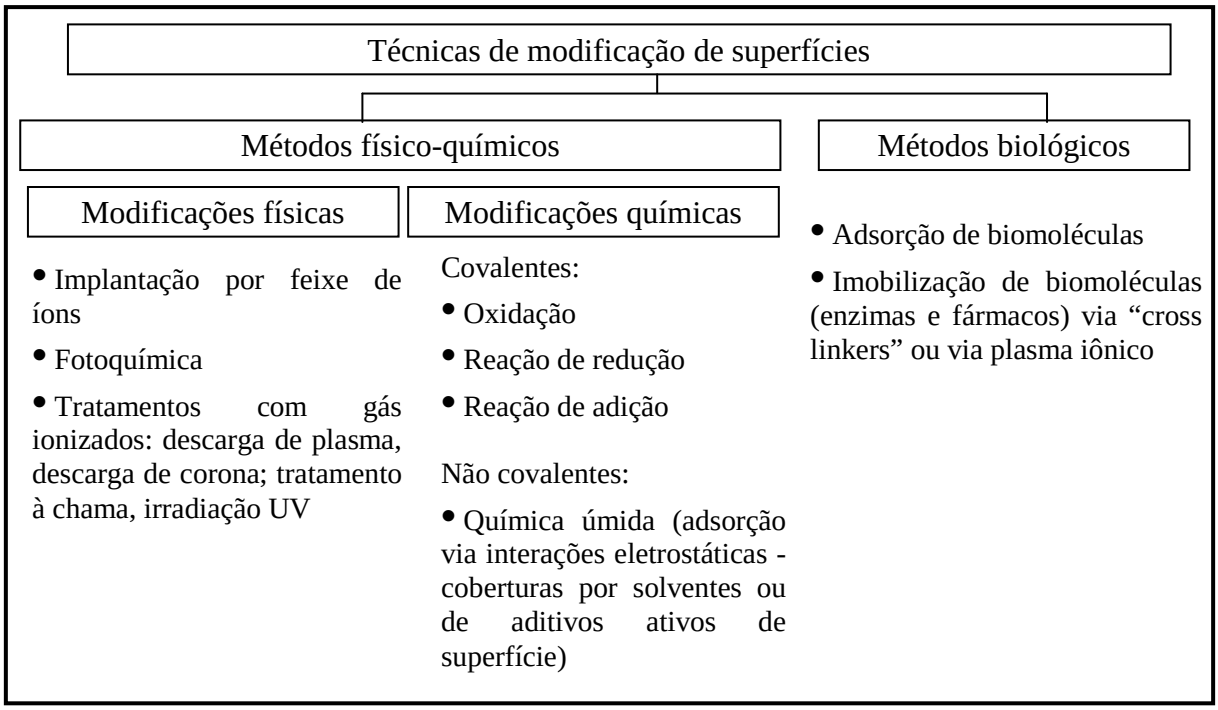

Figura 9: Algumas técnicas de modificação de superfície de materiais (adaptada de GODDARD e HOTCHKISS, 2007 e PAVITHRA e DOBLE, 2008)

II - 3.2.1. Modificação via plasma iônico

Plasma é um estado altamente energético da matéria, no qual um gás é ionizado a partículas carregadas, elétrons e moléculas neutras. No processo de modificação de superfícies à plasma, uma descarga de plasma é gerada através da evacuação da câmara e do preenchimento da mesma com um gás à baixa pressão. O gás é então energizado, através de fontes de energia (radiofrequência, corrente direta ou por microondas) e a superfície em contato com o plasma é bombardeada pelas espécies energéticas geradas. A natureza das interações entre as espécies reativas e a superfície determinarão o tipo e o grau de modificação química e física (Figura 10 e 11) (CHEN e CHANG, 2002; DENES e MANOLACHE, 2004; BAZAKA *et al.*, 2011).

No tratamento à plasma, diversos gases podem ser utilizados, como ar, nitrogênio, argônio, oxigênio, óxido nitroso, hélio, tetrafluormetano, vapor de água, dióxido de carbono, metano e amônia (BAZAKA *et al.*, 2011). A técnica é considerada altamente versátil, uma vez que a exposição a cada um destes gases pode levar à introdução de diferentes funcionalidades químicas, as quais são altamente dependentes da composição química do material e do gás utilizado. Por exemplo, oxidações, nitretações, hidrólises ou aminações à plasma aumentam a energia de superfície e a hidrofilicidade do material, mudando desta maneira a sua interação com o ambiente. Radicais livres que também são formados podem levar a processos de funcionalização, remoção e reticulação (Figura 10) (BAZAKA *et al.*, 2011). Dentre as técnicas físicas de modificação de superfícies, o plasma iônico apresenta algumas vantagens, devido ao custo relativamente baixo da técnica, baixo impacto ambiental (não gera resíduos químicos), e a possibilidade de modificar a superfície de diferentes materiais, como polímeros, metais e cerâmica e ao mesmo tempo esterilizá-los (RAINER *et al.*, 2010). O plasma iônico é efetivo à temperatua ambiente sem causar dano a materiais termosensíveis. Ele pode ser aplicado a materiais com as mais diversas formas, o que pode ser um problema para outras técnicas de modificação de superfície, e a reação ocorre em profundidades como centenas de angstroms a micrômetros, não alterando as propriedades de volume do material (LOH, 1999).

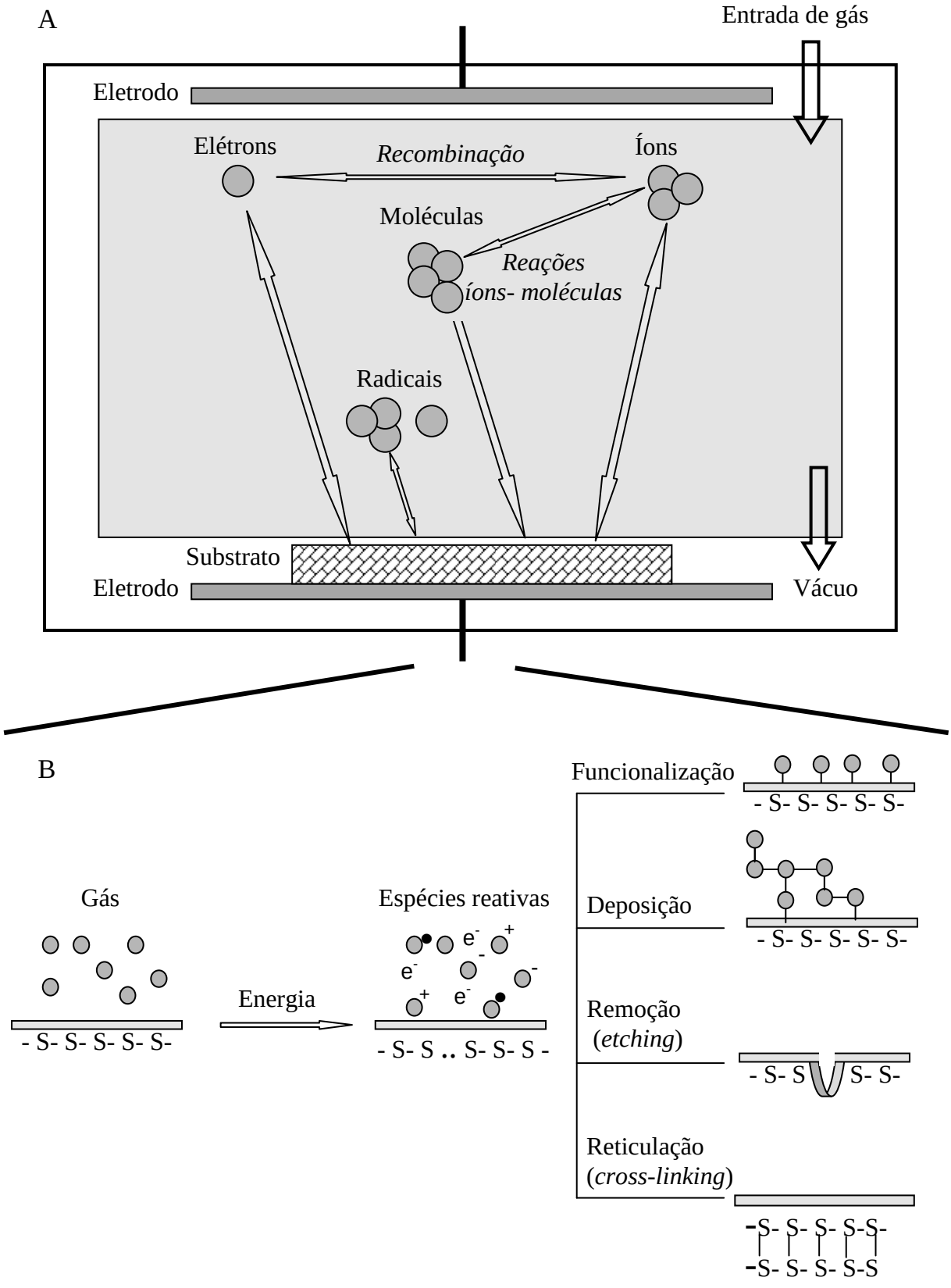

Figura 10: A. Esquema de uma câmara de plasma. B. Os processos de modificação de superfície que podem ser alcançados utilizando a técnica de plasma (adaptado de DENES e MANOLACHE, 2004 e BAZAKA *et al.*, 2011).

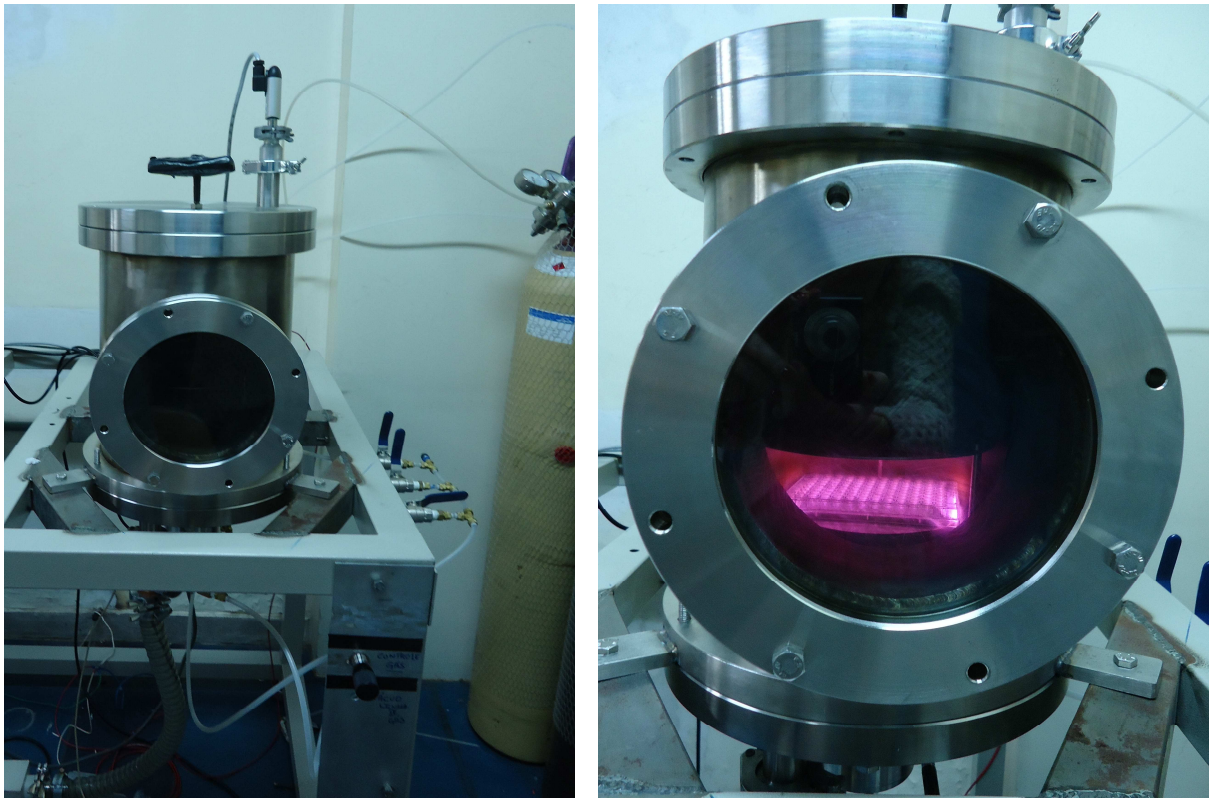

Figura 11: À esquerda: câmara de plasma do Laboratório de Superfícies e Interfaces Sólidas - Instituto de Física da Universidade Federal do Rio Grande do Sul. À direita, o processo de plasma de nitrogênio e hidrogênio sob uma placa de poliestireno.

A realização de todo o potencial do plasma a um material específico depende, entretanto, da aplicação ideal dos parâmetros de processamento (tipo de gás e tempo de exposição, fluxo, pressão de operação, distância da superfície à fonte de plasma, dentre outros). Diversos trabalhos vêm demonstrando o grande potencial das técnicas de plasma em evitar a adesão bacteriana e previnir a formação de biofilme através da criação de superfícies antiaderentes, incluindo materiais, como: o PVC com o uso de plasma de oxigênio (ZHANG *et al.*, 2006); o silicone e o titânio com plasma de argônio (EVERAERT *et al.*, 1998; AMOROSO *et al.*, 2006); o PE com plasma de prata e mistura de prata com nitrogênio (ZHANG *et al.*, 2008); o politereftalato de etileno (PET) com o uso de plasma de hélio e mistura de hélio e oxigênio (KATSIKOGIANNI *et al.*, 2008). Em adição, Joaquin e colaboradores (2009) mostraram que biofilmes podem ser erradicados por meio de descarga de plasma de hélio, sustentando ainda mais o potencial do plasma como método de esterilização. Além de o plasma ter uma atrativa aplicação como ferramenta única na modificação de

superfícies para fins biomédicos, conforme os exemplos citados acima, as superfícies modificadas por plasma também podem servir como uma plataforma para futuros processos de modificação (como o ancoramento de biomoléculas e outras estruturas funcionais), criando propriedades no material para aplicações específicas (BALAZS *et al.*, 2004; GODDARD e HOTCHKISS, 2007).

II – 3.2.2. Recobrimento de superfícies com compostos bioativos

O recobrimento de superfícies com os mais diversos compostos bioativos passou a ser fortemente investigado para prevenir da formação de biofilmes em biomaterais. Este recobrimento pode ocorrer, basicamente, por adsorção ou por ligação covalente do composto com a superfície. A tabela 6 exemplifica materiais e coberturas com funções anti-infectivas, destacando o estado atual em que se encontram as avaliações biológicas ou clínicas.

Conforme a tabela 6, os principais biomateriais com cobertura atualmente disponíveis no mercado são aqueles com revestimentos a base de antissépticos ou antibacterianos. Entretanto, um dos principais desafios é obter “in vivo” a efetividade encontrada nos experimentos “in vitro”, principalmente em períodos de uso mais prolongados. Um estudo de metanálise sobre cateteres urinários mostrou que o uso de cateteres cobertos por liga de prata, por minociclina/rifampicina ou nitrofurazona, diminui o risco de infecção do trato urinário a curto prazo (menos de uma semana) em adultos hospitalizados, entretanto, após este período, a redução não foi significativa (SCHUMM e LAM, 2008). Da mesma maneira, endopróteses uretrais expansíveis (“stents”) cobertas com triclosan não apresentaram efetividade para reduzir infecções urinárias em longos períodos de uso (120 dias) (CADIEUX *et al.*, 2009). Da mesma forma, clinicamente, os cateteres revestidos com clorexidina ou sulfadiazina de prata não mostraram vantagens na prevenção de sepse em relação aos cateteres não-revestidos (PEMBERTON *et al.*, 1996). Uma desvantagem adicional dos cateteres com clorexidina são os relatos de choque anafilático (STEPHENS *et al.*, 2001).

Embora Balazs e colaboradores (2004) tenham demonstrado que a incorporação de íons prata na superfície de tubos endotraqueais seja extremamente efetiva na redução da colonização bacteriana, cateteres urinários revestidos com prata não apresentaram diferença significativa nas taxas de infecção em relação a cateteres não modificados, em estudo incluindo 1.300 pacientes (SCHIERHOLZ *et al.*, 1999). Evidências experimentais sugerem que a diminuída atividade antimicrobiana pode ser devida à adsorção de íons de prata à albumina. Já os compostos de amônio quaternário, alguns têm se mostrado tóxicos para as células humanas (NAGAMUNE *et al.*, 2000).

Por outro lado, resultados promissores têm sido demonstrados em estudos de impregnação do antisséptico gendine (violeta genciana e clorexidina) a cateteres urinários e a tubos endotraqueais (CHAIBAN *et al.*, 2005; HACHEM *et al.*, 2009; RAAD *et al.*, 2011). Considerando o cateter venoso central, um estudo de metanálise indicou que a cobertura de cateteres por antibacterianos, principalmente minociclina/rifampicina, reduzem as infecções sanguíneas relacionadas ao cateter (HOCKENHULL *et al.*, 2009). Da mesma forma, a imobilização de antibióticos em implantes ortopédicos, principalmente em titânio, previne a colonização bacteriana, mas a eficácia a longo prazo ainda não foi estabelecida (HICKOK e SHAPIRO, 2012). Recentemente, a imobilização de peptídeos antimicrobianos (AMPs), isolados de animais, plantas, bactérias, fungos e vírus, em diversos biomateriais têm sido investigada. Estes peptídeos oferecem vantagens atraentes: eles exibem propriedades bactericidas e fungicidas em concentrações muito baixas, sendo menos propensos a promover resistência bacteriana. Além disso, exibem a alta estabilidade, sendo resistentes a alterações de temperatura e pH, quando encontram-se imobilizados (COSTA *et al.*, 2011).

Entretanto, o uso da profilaxia antibiótica é controverso devido a curta duração da sua eficácia, uma vez que ocorre a liberação do mesmo na circulação, e devido ao potencial para aumentar a resistência aos antimicrobianos (VON EIFF *et al.*, 2005). Isto resulta na demanda por coberturas alternativas, como moléculas que controlem a adesão bacteriana por vias que não envolvam a inibição do crescimento bacteriano.

Tabela 6: Exemplos de materiais e coberturas com funções anti-infectivas, "status" atual e exemplos de aplicação clínica (adaptada de BUSSCHER *et al*., 2012).

Funcionalidade	Base química	"Status" atual	Aplicação clínica
Antiadesiva	Revestimento de polímero hidrofílico	Aplicado clinicamente	Lentes de contato, tubos endotraqueais, cateteres urinários
	Revestimento de polímeros em escova	Experimentos "in vitro" e em animais	Não especificado
Integradora de tecidos	Peptídeos promotores de adesão celular	"In vitro"	Enxerto vascular
	Revestimento de hidroxiapatita	Aplicado clinicamente	Implantes ortopédicos e dentários
	Revestimento de filmes finos de titânio	"In vitro"	Implantes dentários
Matadores de contato	Imobilização de compostos de amônio quaternário	"In vitro"	Não especificado
	Revestimento de selênio	"In vitro"	Lentes de contato
	Revestimento de prata	Aplicado clinicamente	Cateteres urinários
Liberadores de antimicrobianos	Acrilatos liberadores de antibióticos	Aplicado clinicamente	Próteses ortopédicas articulares
	Liberadores de carbonato de prata e diacetato de clorexidina	Aplicado clinicamente	Malhas cirúrgicas
	Revestimento de prata, clorexidina, rifampicina ou minociclina	Aplicado clinicamente	Cateteres vasculares
	Suturas liberadoras de triclosan	Aplicado clinicamente	Suturas
	Revestimento de polímero biodegradável e gentamicina	Aplicado clinicamente	Malhas cirúrgicas
Recobrimento multifuncional	Co-enxerto de polímero em escovas e antibiótico	"In vitro"	Possivelmente para implantes de titânio
	Co-enxerto de polímero em escovas e ligante celular	"In vitro"	Não especificado

Considerando produtos naturais, o uso e potencial aplicação comercial de biosurfactantes no campo da medicina têm aumentado durante a última década. Biosurfactantes incluem diversas estruturas químicas, tais como glicolipídeos, lipopeptídeos, complexos polissacarídeo-proteína, fosfolipídeos, ácidos graxos e lipídios neutros (RODRIGUES *et al.*, 2006a). A adsorção de biosurfactantes, produzidos por lactobacilos, em superfícies de silicone demonstra-se capaz de inibir a adesão de bactérias e fungos (RODRIGUES *et al.*, 2006b). Os estudos de Rodrigues e colaboradores (2006a e 2011) revisam com detalhes os diversos biosurfactantes com potencial para revestimento de superfíces e destaca que a utilização dos mesmos têm sido limitada pelo custo de produção relativamente alto e por haver escassas informações sobre a toxicidade em humanos.

Polissacarídeos (quitosana e quitosana/pectina) foram covalentemente imobilizados em PVC através de ativação da superfície com descarga de plasma. Estas superfícies funcionalizadas foram caracterizadas por diversas técnicas e apresentaram uma redução de 50% e 20% na adesão de *E. coli*, respectivamente (ASADINEZHAD *et al.*, 2010). O revestimento de superfícies com produtos vegetais bioativos também está sendo investigado, sendo que um dos principais desafios é a manutenção da atividade biológica de um composto, mesmo quando retido em uma superfície. Bazaka e colaboradores (2010) enfocam esta problemática, ao descrever a síntese e a caracterização de substratos revestidos por filmes finos de terpinen-4-ol, o principal constituinte antimicrobiano do óleo de *Melaleuca alternifolia*, para a prevenção do crescimento de *P. aeruginosa*. A técnica de plasma foi empregada para o ancoramento dos monômeros de terpinenol e a inibição do crescimento de *P. aeruginosa* foi atingida.

A enzima disperina B, capaz de inibir a formação e de promover a erradicação de biofilmes, está sendo estudada juntamente com o antisséptico triclosan em cateteres venosos centrais. Estes cateteres mostraram eficácia em evitar a colonização de *S. aureus* em modelo animal e uma atividade mais prolongada foi obtida quando comparado com cateteres revestidos por clorexidina/sulfadiazina de prata. O efeito

sinérgico de combinação da atividade antimicrobiana do triclosan e antibiofilme da dispersina B “in vitro”, pode ser devido ao fato de que a dispersina evita o recobrimento bacteriano pela matriz de EPS, tornando as células mais suscetíveis à ação antimicrobiana do triclosan (DAROUICHE *et al.*, 2009).

Inibidores do sistema *QS*, como as furanonas e o peptídeo inibidor do RNA III, também estão sendo explorados quanto ao recobrimento de superfícies. Hume e colaboradores (2004) descrevem a incorporação do 3-(10-bromoexil)-5-dibromometileno- 2(5 H)-furanona em estireno e a sua ligação covalente a cateteres previamente ativados com descarga de plasma. Resultados “in vivo” mostraram a inibição da colonização por *S. epidermidis* e sugerem que as furanonas sejam capazes de controlar a infecção por até 65 dias no modelo animal. Cirioni e colaboradores (2007) mostraram que “stents” ureterais revestidos com o peptídeo inibidor do RNAIII, implantados em bexigas de ratos, apresentaram a formação de biofilme de *S. aureus* suprimida. O “stent” revestido foi especialmente eficaz quando combinado com teicoplanina, aumentando a eficácia do antimicrobiano na prevenção de infecções ureterais estafilocócicas associadas ao “stent”.

Apesar das interações entre superfícies (bióticas, abióticas, com ou sem filme condicionante), e diferentes tipos de células (bactérias, fungos, células humanas) serem muito complexas e de difícil entendimento frente a tantas variáveis, a importante aplicação prática destes estudos vem impulsionando os contínuos esforços neste campo multidisciplinar de pesquisa.

III – OBJETIVOS

III - 1. OBJETIVO GERAL

O presente trabalho tem como principal proposta a busca por estratégias alternativas para o combate da adesão bacteriana e formação de biofilme.

III - 1. 1. OBJETIVOS ESPECÍFICOS

- Rastrear plantas da Caatinga que possuem indicação etnofarmacológica para atividade antiformação de biofilme e antibacteriana contra as bactérias modelo *Staphylococcus epidermidis* ATCC 35984 e *Pseudomonas aeruginosa* ATCC 27853;

- Realizar o fracionamento bioguiado do(s) extrato(s) bioativo(s) com atividade promissora visando à purificação de compostos ativos;

- Estudar as potenciais vias de ação de extratos ou compostos purificados envolvidos na não-adesão de bactérias às superfícies modelo;

- Modificar superfícies, utilizando como modelo o poliestireno, através do recobrimento com frações ou compostos purificados obtidos dos extratos de plantas da Caatinga e, testá-las quanto à adesão bacteriana;

- Avaliar a possível citotoxicidade de extratos e compostos ativos bem como a compatibilidade de superfícies modificadas com os compostos purificados;

- Modificar superfícies, utilizando como modelo o poliestireno, através da técnica de descarga de plasma iônico, com diversos gases e testá-las frente a diferentes bactérias de importância médica, como *Staphylococcus epidermidis*, *Staphylococcus aureus* meticilina resistente e enterobactérias (*Klebsiella pneumoniae*, *Enterobacter cloacae e Serratia marcescens*) produtoras de carbapenemase

- Estudar os potenciais mecanismos envolvidos na não-adesão de bactérias nas superfícies modificadas por plasma.

IV – CAPÍTULOS

IV - CAPÍTULO 1 - Potential of medicinal plants from the Brazilian semi-arid region (Caatinga) against *Staphylococcus epidermidis* planktonic and biofilm lifestyles

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Potential of medicinal plants from the Brazilian semi-arid region (Caatinga) against *Staphylococcus epidermidis* planktonic and biofilm lifestyles

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ABSTRACT

Ethnopharmacological relevance: Medicinal plants from the Caatinga, a Brazilian xeric shrubland, are used in folk medicine to treat infections. These ethnopharmacological data can contribute to obtaining new antimicrobial/antibiofilm extracts and natural product prototypes for the development of new drugs. The aim of this study was to investigate the antibiofilm and antibacterial activities of 45 aqueous extracts from 24 Caatinga plant species.
Materials and methods: The effect of aqueous extracts on planktonic cells and on biofilm formation by *Staphylococcus epidermidis* was studied by the OD_{600} absorbance and by the crystal violet assay, respectively. Scanning electron microscopy (SEM) was used to generate comparative images of extract-treated and untreated biofilms. Chromatographic analyses were performed to characterize the active extracts.
Results: The *in vitro* screening, at 0.4 mg/mL and 4.0 mg/mL, showed 20 plants effective in preventing biofilm formation and 13 plants able to inhibit planktonic bacterial growth. SEM images demonstrated distinct profiles of bacterial adhesion, matrix production and cell morphology according to different treatments and surfaces. The phytochemical analysis of the selected active extracts indicates the polyphenols, coumarins, steroids and terpenes as possible active compounds.
Conclusion: This study describes the first antibiofilm and antibacterial screening of Caatinga plants against *S. epidermidis*. The evaluation presented in this study confirms several ethnopharmacological reports and can be utilized to identify new antibiofilm and antibacterial products against *S. epidermidis* from traditional Brazilian medicine.

1. Introduction

The Caatinga, a xeric shrub-dominated biome of northeastern Brazil, supports a high, yet poorly studied, diversity of plant resources. Caatinga is a matrix of thorn shrublands and seasonally dry forests, with pockets of montane rain forests and savannah. It covers most of the states of Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia and the northeastern part of Minas Gerais in Jequitinhonha Valley, occupying an area of approximately 735,000 km^2 (Basso et al., 2005). The annual mean temperature in the region is 27.5 °C (Alves et al., 2011), the humidity is low, and the average annual rainfall is 250–500 mm (Basso et al., 2005). With respect to soils, there is a predominance of luvisols, eutrophic inceptisols and vertisols, in addition to sparsely scattered rocky outcroppings (Ab'Saber, 1974). They are fertile, well drained, and oxygenated (Basso et al., 2005). The dry season lasts seven months or more and the winter is the rainy season, in which temperatures are not as high. These characteristics making the Caatinga a peculiar kind of vegetation adapted to the prevailing local ecological conditions.

A review of the literature from the northeast of Brazil reveals that traditional medicinal practices have been used to treat a variety of illnesses including skin and gastrointestinal disorders, tuberculosis and urinary tract infections (Agra et al., 2007a,b). However, some of them still have not been subjected to scientific study to confirm their effectiveness for a given disease (Cartaxo et al., 2010). Taking into account current concerns over bacterial multi-resistance, the

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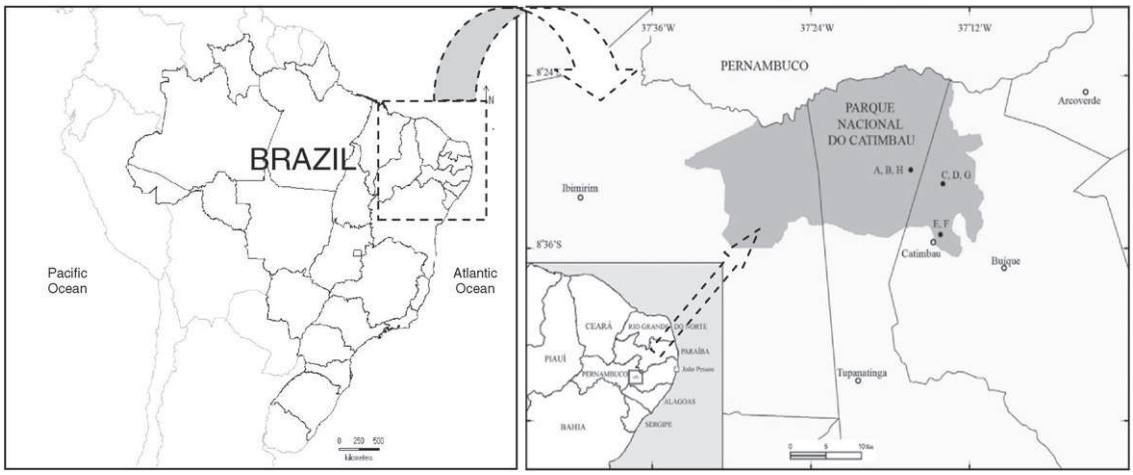

Fig. 1. Geographical localization of PARNA do Catimbau (Buíque, Tupanatinga and Ibimirim municipalities), Pernambuco state, Brazil.

screening of potential new antibiofilm and antibacterial agents as therapeutic alternatives is an important issue.

Staphylococcus epidermidis has attracted substantial interest in recent years because it has become the most important cause of nosocomial infections (Vuong and Otto, 2002). Its pathogenicity is mainly due to the ability to form biofilms (an important virulence factor), a process in which planktonic bacteria adhere to a surface and initiate the development of sessile microcolonies surrounded by an extracellular matrix, existing as a bacterial community (Otto, 2009). Biofilm-associated microorganisms have been shown to be related to more than 65% of all medical infections, including endocarditis, otitis, prostatitis, periodontitis, conjunctivitis, vaginitis, and infections related to cystic fibrosis. In addition, biofilms are important colonizers of a wide variety of medical devices, such as catheters and prostheses (Donlan and Costerton, 2002). The costs linked to vascular catheter-related blood-stream infections caused by *S. epidermidis* amount to an estimated US$2 billion annually in the United States (Otto, 2009). In a biofilm, the bacterium is protected against attacks from both, the immune system and antibacterial treatment, making *S. epidermidis* infections difficult to eradicate (Vuong and Otto, 2002; Davies, 2003; Antunes et al., 2011). The inhibition of virulence targets could bring new antibacterial molecules with radically new mechanism of action and represent an innovative therapeutic concept (Escaich, 2008).

Therefore, we screened 45 aqueous extracts from 24 Caatinga plants, used in local traditional medicine, for the potential to inhibit the biofilm formation and the planktonic bacterial growth of *S. epidermidis*.

2. Materials and methods

2.1. Plant material: source, sampling and identification

Based on ethnobotanical information (Agra et al., 2007a,b and local community information) the plants were collected from several habitats at Parque Nacional do Catimbau (PARNA do Catimbau), Pernambuco, Brazil (Fig. 1), between July and August 2009. Voucher specimens were identified at the herbarium of the Instituto Agronômico de Pernambuco (IPA), where a voucher of each species was deposited (Table 1).

The aerial parts of the plants (leaf, fruit, inflorescence, branch and cephalium – the flowering adult phase of a cactus), stem bark and roots were dried in an incubator at 45 °C, for 2–3 days. The dried material was ground into powder using a grinder followed by a blender (Waring Laboratory, USA).

2.2. Extracts

The powdered dried material was mixed for 15 min with water [1:9; (w:v)] and kept in the dark at room temperature (22 °C) for 24 h. After this period the mixture was centrifuged for 10 min, at 10,000 rpm, and the supernatant was collected; the water was then evaporated at 40 °C, during 96–120 h. A 1 mg/mL and 10 mg/mL aqueous solution were filtered through a 0.2 μm pore membrane and stored at −20 °C.

2.3. Phytochemical analysis

A preliminary phytochemical analysis to detect the major components of the extracts was carried out using thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC).

The different extracts were developed by TLC plates (Kieselgel 60 F254 0.2 mm, Merck, Germany) using dichloromethane:methanol (8:2) as eluent and visualized under UV light (254 and 365 nm, Handheld UV Lamp Model 9403E, BioAmerica Inc., USA). Polyphenol compounds were detected with aluminum chloride 1% and ferric chloride 2%, potassium hydroxide 5% was used for coumarins, Dragendorff's reagent for alkaloids, anisaldehyde/sulfuric acid for steroids and terpenes, ninhydrin for amines and aminoacids, and iodine vapor as an universal reagent (Wagner and Bladt, 1996).

The HPLC analyses were carried out on a Shimadzu LC-20AT coupled to a Shimadzu SPD M20A diode array detector, and a reversed-phase column Shim-pack VP-ODS (250 mm × 6 mm I.D., Shimadzu) was used. Gradient elution was performed with solution A, acetonitrile:water (5:95 [v:v]), and solution B comprising 100% of acetonitrile, delivered at a flow rate of 0.6 mL/min as follows: initially 5% of solution B increasing up to 100% for 60 min and finally 100% of B for 15 min. The injection volume was 20 μL from a solution at 2.5 mg/mL.

2.4. Surfaces, bacterial strain and culture conditions

Bacterial adhesion is determined both by the type of microorganism and by the properties of the surface of the material involved (Pavithra and Doble, 2008). Two surfaces were studied: sterile 96-well polystyrene flat-bottom microtiter plates (Costar 3599) purchased from Corning Inc. (USA) as a model of a hydrophobic material, and glass as a model of a hydrophilic material. *Staphylococcus epidermidis* ATCC 35984 was grown in Mueller Hinton agar (Oxoid Ltd., England) overnight, at 37 °C, and a bacterial suspension

Table 1
List of plant species from the Brazilian Caatinga for tested against *S. epidermidis*.

Family	Scientific name	Popular name	Voucher	Usage forms, preparation and therapeutic indication
Anacardiaceae	*Myracrodruon urundeuva* Alemão	Aroeira, aroeira-do-sertão	IPA 84059	Drink or wash the affected site. A decoction or maceration of a handful of stem bark in a liter of water. Used in cases of ovarian inflammation and in ulcerative external afflictions This species has many other medicinal indications (Agra et al., 2007b).
Apocynaceae	*Allamanda blanchetii* A.DC.	Quatro-patacas-roxa, leiteiro	IPA 84112	Drink. One teaspoon of the latex in a cup of water. Latex is used as a laxative, emetic, cathartic and vermifuge. It is described as poisonous (Agra et al., 2007a,b).
Burseraceae	*Commiphora leptophloeos* (Mart.) J.B.Gillett	Imburana, amburana, imburana de cambão	IPA 84037	Drink or wash/bathe the affected site. A decoction of a handful of roots in a liter of water is prepared with sugar as syrup. Used in the treatment of influenza, coughs, bronchitis, to treat urinary and liver diseases. Also, external use against vaginal ulcers and others (Agra et al., 2007a).
Cactaceae	*Melocactus zehntneri* (Britton & Rose) Luetzelb.	Coroa-de-frade	IPA 85028	Drink. Stem pulp is used mashed with sugar to treat bronchitis, coughs and physical debility (Agra et al., 2007a,b).
Combretaceae	*Buchenavia tetraphylla* (Aubl.) R.A. Howard	Caicaró	IPA 84104	Drink. An infusion of stem bark or leaves is used as a digestive after meals (Agra et al., 2007b).
Euphorbiaceae	*Jatropha mutabilis* (Pohl) Baill.	Pinhão bravo, pinhão manso	IPA 84054	Eat and drink. The oil extracted from seeds is ingested as a veterinary vermifuge and the latex is drunk to treat snake bites. A spoonful is used once only (Agra et al., 2007b).
Fabaceae Cercideae	*Bauhinia acuruana* Moric.	Mororó, pata de vaca	IPA 84042	Drink. An infusion or decoction of a handful of leaves in a liter of water it is drunk during the meals until the symptoms disappear. Used as tonic, depurative and against diabetes (Agra et al., 2007a,b).
Fabaceae "Caesalpinioideae"	*Chamaecrista cytisoides* (DC. ex Collad.) H.S. Irwin & Barneby	Vassourinha	IPA 84103	Drink. According to a local community the whole plant is used as a laxative and purgative.
Fabaceae "Caesalpinioideae"	*Chamaecrista desvauxii* (Collad.) Killip	Vassourinha	IPA 84064	Drink. According to the local community the whole plant is used as a laxative and purgative.
Fabaceae "Caesalpinioideae"	*Libidibia ferrea* (Mart. ex Tul.) L.P. Queiroz *var. ferrea*	Pau ferro, jucá	IPA 84035	Drink. The stem bark in "cachaça" (bottled) is used against anemia, diarrhea and dysentery (Agra et al., 2007b).
Fabaceae "Caesalpinioideae"	*Pakinsonia aculeata* L.	Turco, tangerim	IPA 84113	Drink. The seeds are roasted, powdered, prepared as coffee and drunk as tea until the symptoms disappear; used to treat fevers and malaria. Prepared as an infusion or decoction of a handful in a liter of water and drunk as antiepileptic, febrifuge, and to treat snake bites (Agra et al., 2007a,b).
Fabaceae "Caesalpinioideae"	*Senna macranthera* (Collad.) H.S. Irwin & Barneby *var. macranthera*	Pau fava, fedegoso	IPA 84045	Drink. According to the local community a decoction of a spoonful of the fruit in a cup of water is used against influenza and colds four times a day.
Fabaceae "Caesalpinioideae"	*Senna splendida* (Vogel.) H.S. Irwin & Barneby	Feijão-brabo	IPA 84040	Drink. According to the local community the fruits are roasted, powdered and prepared as coffee to be used against anemia. A cup is drunk after meals until the symptoms disappear.
Fabaceae Mimosoideae	*Anadenanthera colubrina* (Vell.) Brenan *var. colubrina*	Angico	IPA 84039	Drink. A maceration of a handful of stem bark in a liter of wine or "cachaça" is used against coughs, whooping cough and bronchitis (Agra et al., 2007a,b).
Fabaceae Mimosoideae	*Piptadenia viridiflora* (Kunth) Benth.	Jacurutu, espinheiro-preto	IPA 84036	Drink. The stem bark is used in a decoction against asthma, intestinal spasms and toothaches (Agra et al., 2007b).
Fabaceae Mimosoideae	*Pityrocarpa moniliformis* (Benth.) Luckow & R.W. Jobson	Canzenzo, angico de bezerro, quipembe	IPA 84048	In communication with local people was informed that the stem bark and root are used as healing.
Fabaceae Faboideae	*Dioclea grandiflora* Mart. ex Benth.	Mucunã, parreira-brava	IPA 84057	Drink. A decoction of a handful of roots in a liter of water is used against prostate inflammation. It is drunk in place of water until the symptoms disappear (Agra et al., 2007a,b).
Fabaceae Faboideae	*Myroxylon peruiferum* L.f.	Bálsamo	IPA 84110	Topic. In communication with local community was informed that the stem-bark is used like as anti-inflammatory.
Malpighiaceae	*Stigmaphyllon paralias* A. Juss.	Amarelinho	IPA 84041	Drink. According to the local community the leaf is used as a decoction against fevers and diarrhea, syphilis and kidney diseases.
Malvaceae	*Sida galheirensis* Ulbr.	Malva-veludo, malva-branca, malva, malvão	IPA 84078	Drink or wash the affected site. According to the local community an infusion of a spoonful of stem bark and root in a cup of water are used against acne, coughs and leucorrhea. Also used for skin afflictions (Agra et al., 2007b).
Myrtaceae	*Eugenia brejoensis* Mazine	Cutia	IPA 84033	Drink. According to the local community a decoction or infusion of the leaves is drunk against diarrhea and dysentery. (Endemic plant).
Ochnaceae	*Ouratea blanchetiana* Engl.	Batiputá	IPA 84044	Topical. The oil extracted by heat from the fruits is used against earache. It is dropped into the ears until the pain disappears (Agra et al., 2007b).
Polygalaceae	*Polygala boliviensis* A.W.Benn.	Arrozinho	IPA 84066	Drink or eat and topic. An infusion or decoction of a handful of leaves in water is used as diuretic, emetic, expectorant and against blenorrheas. The roots are eaten and placed above the affected area to treat snake bites (Agra et al., 2007a,b).
Polygalaceae	*Polygala violacea* Aubl.	Erva-iodeque	IPA 84051	Drink or eat and topical. An infusion or decoction of a handful of roots in water is used as a diuretic, emetic, expectorant and against blenorrheas. The roots are eaten and placed over the affected area to treat snake bites. (Agra et al., 2007b).

in 0.9% NaCl, corresponding to 1 McFarland scale (3×10^8 CFU/mL), was used in the assays.

2.5. Antibiofilm formation assay

A protocol adapted from Antunes et al. (2010), employing crystal violet in 96-well microtiter plates and glass tubes was used. In the case of microtiter plates, 80 μL of the bacterial suspension, 80 μL of the aqueous extract (concentration of 0.4 mg/mL or 4.0 mg/mL in the wells) and 40 μL of tryptone soya broth (TSB) (Oxoid Ltd., England) were added. In the case of glass tubes, 800 μL of the bacterial suspension, 800 μL of the aqueous extract (concentration of 0.4 mg/mL or 4.0 mg/mL in the tubes) and 400 μL of TSB were added. In both experiments, following the incubation period (37 °C for 24 h) the content of the wells/tubes was removed and the wells/tubes were washed three times with sterile saline. The remaining attached bacteria were heat-fixed at 60 °C for 1 h. The adherent biofilm layer formed was stained with 0.4% crystal violet for 15 min at room temperature. The stain bound to the cells was solubilized with 99.5% DMSO (Sigma–Aldrich Co., USA) and absorbance was measured at 570 nm (Spectramax M2e Multimode Microplate Reader, Molecular Devices, USA). The biofilm formation control was considered to represent 100% of biofilm formation, and the extracts were replaced by 80 μL and 800 μL of water in 96-well microtiter plates and glass tubes, respectively. Values higher than 100% represent a stimulation of biofilm formation in comparison to the control.

2.6. Scanning electron microscopy

Biofilms of *S. epidermidis* ATCC 35984 were grown in 96-well microtiter plates, as described in Section 2.5, with a piece of Permanox™ slide (Nalge Nunc International, USA) or a glass coverslip in each well. After 24 h of incubation at 37 °C, the samples were fixed in 2.5% glutaraldehyde for 2.5 h, washed with 100 mM cacodylate buffer pH 7.2, treated for 2 h with 2% osmium tetroxide and dehydrated in increasing concentrations of acetone. The Permanox™ slides and glass coverslips were dried by the CO_2

Table 2
Biological activity of 45 aqueous extracts of Caatinga plants against biofilm formation and growth of *S. epidermidis* ATCC 35984.

Species	Part used	*S. epidermidis*			
		0.4 mg/mL		4.0 mg/mL	
		Biofilm formation (%)	Bacterial growth (%)	Biofilm formation (%)	Bacterial growth (%)
Allamanda blanchetii	Branches	110.9 ± 0.1	100.7 ± 4.6	183.2 ± 1.7*	33.0 ± 31.7*
Allamanda blanchetii	Leaves	93.1 ± 6.2	108.1 ± 7.0	74.2 ± 7.6	170.6 ± 12.5*
Anadenanthera colubrina var *cebil*	Fruits	118.0 ± 14.1	91.9 ± 5.4	101.0 ± 6.8	131.2 ± 13.8*
Anadenanthera colubrina var *cebil*	Leaves	167.1 ± 1.1	93.8 ± 14.4	119.4 ± 2.6*	257.6 ± 4.7*
Anadenanthera colubrina var *cebil*	Branches	113.2 ± 12.3	102.2 ± 4.3	130.8 ± 5.6*	207.5 ± 0.6*
Anadenanthera colubrina var *cebil*	Stem bark	136.8 ± 10.2	95.2 ± 7.5	11.6 ± 1.7*	63.9 ± 5.8*
Bauhinia acuruana	Branches	99.0 ± 7.2	83.1 ± 9.6	18.3 ± 3.7*	184.0 ± 8.0*
Bauhinia acuruana	Fruits	121.4 ± 12.2	81.6 ± 7.2	22.2 ± 5.0*	135.4 ± 3.1*
Bauhinia acuruana	Leaves	128.0 ± 14.5	74.1 ± 1.6*	144.5 ± 3.9*	343.1 ± 7.0*
Buchenavia tetraphylla	Leaves	179.4 ± 6.5*	136.0 ± 5.9*	177.4 ± 9.4*	268.5 ± 19.5*
Chamaecrista desvauxii	Leaves	103.8 ± 4.9	91.4 ± 10.5	126.2 ± 12.9*	153.6 ± 12.4*
Chamaecrista desvauxii	Fruits	68.3 ± 2.7*	154.7 ± 12.5*	12.6 ± 1.3*	201.3 ± 9.9*
Chamaecrista cytisoides	Branches	108.4 ± 17.5	113.0 ± 6.9	131.1 ± 9.0*	122.5 ± 6.6*
Commiphora leptophloeos	Branches	153.8 ± 9.6	97.2 ± 8.3	174.1 ± 0.8*	144.6 ± 3.1*
Commiphora leptophloeos	Stem bark	32.7 ± 8.5*	93.3 ± 5.1	15.3 ± 1.3*	0 ± 4.5*
Dioclea grandiflora	Leaves	166.1 ± 6.5	109.9 ± 13.9	172.4 ± 12.0*	178.4 ± 6.6*
Dioclea grandiflora	Branches	110.9 ± 4.5	92.7 ± 4.6	177.8 ± 18.0*	99.2 ± 4.4
Dioclea grandiflora	Fruits	86.5 ± 5.1	107.6 ± 5.4	68.5 ± 1.9*	127.5 ± 11.9*
Eugenia brejoensis	Leaves	140.3 ± 23.1	91.2 ± 2.4	76.0 ± 6.9	131.8 ± 1.2*
Jatropha mutabilis	Roots	110.3 ± 17.5	87.6 ± 4.3*	227.3 ± 7.8*	130.6 ± 8.5*
Jatropha mutabilis	Branches	102.0 ± 7.2	82.3 ± 7.1*	201.7 ± 7.4*	177.4 ± 16.9*
Libidibia ferrea var ferrea	Leaves	106.0 ± 13.5	113.6 ± 14.5	122.0 ± 11.0*	160.5 ± 1.1*
Libidibia ferrea var ferrea	Fruits	120.7 ± 12.2	133.0 ± 3.4	30.0 ± 1.2*	178.9 ± 0.5*
Melocactus zehntneri	Roots	66.5 ± 6.8*	122.4 ± 3.1	86.2 ± 9.9	139.0 ± 5.5*
Melocactus zehntneri	Cephalium	68.8 ± 7.8*	100.8 ± 5.0	154.4 ± 5.3*	206.0 ± 7.7*
Myracrodruoun urundeuva	Branches	81.5 ± 14.5	82.6 ± 11.9	159.1 ± 7.9*	158.1 ± 12.2*
Myracrodruoun urundeuva	Leaves	71.4 ± 5.8*	90.3 ± 5.1	171.5 ± 8.4*	161.4 ± 20.3*
Myracrodruoun urundeuva	Stem bark	141.2 ± 7.2*	99.7 ± 1.6	16.1 ± 0.4*	62.7 ± 3.4*
Myroxylon peruiferum	Leaves	71.6 ± 2.4*	146.1 ± 4.1*	53.6 ± 6.7*	195.4 ± 6.1*
Ouratea blanchetiana	Branches	195.5 ± 10.2	117.2 ± 9.8	122.2 ± 2.7*	239.9 ± 8.6*
Ouratea blanchetiana	Leaves	172.8 ± 14.2	130.4 ± 11.2	50.0 ± 2.5*	273.1 ± 9.8*
Parkinsonia aculeata	Leaves	90.2 ± 11.4	102.9 ± 12.9	47.2 ± 12.0*	85.2 ± 2.7*
Piptadenia viridiflora	Branches	91.4 ± 17.0	107.3 ± 9.4	117.2 ± 26.0	102.4 ± 1.4
Piptadenia viridiflora	Fruits	85.3 ± 11.6	98.6 ± 10.2	61.3 ± 9.2*	218.5 ± 17.7*
Pityrocarpa moniliformis	Leaves	108.7 ± 3.8	126.1 ± 11.2	23.0 ± 0.7*	266.7 ± 6.2*
Polygala boliviensis	Inflorescences	105.9 ± 5.0	101.1 ± 4.9	112.2 ± 7.6	244.3 ± 1.7*
Polygala boliviensis	Leaves	111.1 ± 8.7	92.7 ± 9.5	93.0 ± 10.1	134.3 ± 5.8*
Polygala boliviensis	Branches	75.7 ± 12.4	83.1 ± 10.1*	96.5 ± 6.3	154.6 ± 12.8*
Polygala violacea	Leaves	93.8 ± 2.1	113.9 ± 2.7*	86.2 ± 4.7*	166.5 ± 8.2*
Polygala violacea	Roots	82.8 ± 10.2	90.5 ± 9.0	52.6 ± 7.4*	138.1 ± 4.1*
Senna macranthera	Fruits	43.3 ± 3.1*	152.8 ± 8.7	103.4 ± 13.8	88.9 ± 10.6
Senna splendida	Branches	98.0 ± 0.7	104.8 ± 0.5	58.8 ± 2.3*	164.5 ± 1.3*
Sida galheirensis	Branches	88.2 ± 14.0	126.7 ± 10.6	110.0 ± 24.8	112.0 ± 3.6
Sida galheirensis	Leaves	84.8 ± 8.0	105.8 ± 7.8	95.0 ± 8.3	107.3 ± 8.6
Stigmaphyllon paralias	Leaves	138.6 ± 10.9*	113.5 ± 4.3	197.5 ± 7.7*	319.4 ± 6.3*

Results represent mean ± standard deviation of 3 experiments.
* Represents significant difference in relation to control ($p < 0.05$).

critical point technique (CPD 030 Balzers, Liechtenstein), fixed on aluminum stubs, covered with gold film and examined in a JEOL JSM-6060 scanning electron microscope.

2.7. Bacterial growth assay

The planktonic bacterial growth was evaluated by the difference between the OD_{600} absorbance measured at the end and at the beginning of the incubation time in polystyrene 96-well microtiter plates. As a control for bacterial growth the extracts were replaced by 80 μL of water, with this being considered to represent 100% of planktonic bacterial growth. Values higher than 100% represent a stimulation of bacterial growth in comparison to the control. The minimum inhibitory concentration (MIC) to kill 100% of bacterial cells was determined and 50 μL of a serial dilution was spread on to Mueller Hinton agar plates. After incubation (37 °C, 24 h), the number of colony-forming units was counted to determine the bacteriostatic or bactericidal effect of the extract. Rifampicin 8 μg/mL (Sigma–Aldrich Co., USA) was used as a control for the inhibition of bacterial growth.

2.8. Statistical analysis

All microbiological experiments were carried out at least in triplicate and data are presented as percentage mean ± S.D. Differences between groups were evaluated by Student's *t*-test and a $p \leq 0.05$ was considered significant.

3. Results

3.1. Screening of bioactive Caatinga plant aqueous extracts

Table 2 shows the screening of 45 aqueous extracts against *S. epidermidis* on polystyrene.

Considering the extracts at 0.4 mg/mL, stem bark of *Commiphora leptophloeos* and fruits of *Senna macranthera* allowed a biofilm formation of 32.7% and 43.3%, respectively, although the screening did not reveal any significant antibacterial activity at this concentration. At 4.0 mg/mL, the lowest rates of biofilm formation were obtained using: stem bark of *Anadenanthera colubrina* (11.6%), *Commiphora leptophloeos* (15.3%) and *Myracrodruoun urundeuva* (16.1%); fruits of *Bauhinia acuruana* (22.2%), *Chamaecrista desvauxii* (12.6%) and *Libidibia ferrea* (30.0%); leaves of *Parkinsonia aculeata* (47.2%) and *Pityrocarpa moniliformis* (23.0%), and branches of *Bauhinia acuruana* (18.3%). At this concentration, two extracts presented marked inhibition of planktonic bacterial growth since, branches of *Allamanda blanchetii* and stem bark of *Commiphora leptophloeos* permitted just 33.0% and none of bacterial growth, respectively (Table 2).

We chose four active extracts for further investigation (branches of *Bauhinia acuruana* – BBA11; stem bark of *Commiphora leptophloeos* – SBCL33; fruits of *Bauhinia acuruana* – FBA35 and leaves of *Pityrocarpa moniliformis* – LPM45), which were obtained in higher amounts during preparation and represent the different parts of a plant (branches, stem bark, fruits and leaves). The extracts BBA11, FBA35 and LPM45 inhibited biofilm formation by a mechanism that did not involve bacterial death, but instead they stimulated cell growth (Table 2). At 0.4 mg/mL, these extracts did not affect biofilm formation or bacterial growth (Table 2). However, the SBCL33 extract did kill *S. epidermidis* at the highest concentration, although at 0.4 mg/mL the discrete biofilm formation (32.7%) was not associated with bacterial death (Table 2).

To compare the various extracts at both concentrations, they were classified into six categories according to their effect against *S. epidermidis* (Table 3).

Table 3
Percentage effect of Caatinga plant extracts against *S. epidermidis*.

	0.4 mg/mL		4 mg/mL	
	Biofilm (%)	Growth (%)	Biofilm (%)	Growth (%)
Stimulation	46.7	35.5	48.8	82.2
No effect	11.1	24.4	4.4	4.4
Up to 25% inhibition	26.7	37.8	11.1	4.4
From 26 to 50% inhibition	11.1	2.2	15.5	4.4
From 51 to 75% inhibition	4.4	0	4.4	2.2
From 76 to 100% inhibition	0	0	15.5	2.2

3.2. Evaluation of S. epidermidis biofilm on glass

The four active extracts selected after screening were also tested for their capacity to inhibit the formation of a biofilm on a glass surface. The results were similar to those obtained using polystyrene plates (Section 3.1 and Table 2), but showed a discrete decrease in antibiofilm activity (Table 4).

Table 4
Biological activities of four Caatinga aqueous extracts against *S. epidermidis* ATCC 35984.

	Biofilm inhibition					Growth inhibition	
	0.4 mg/mL	4.0 mg/mL				0.4 mg/mL	4.0 mg/mL
	Polystyrene	Polystyrene	SEM Permanox™	Glass	SEM glass	Polystyrene	
Branches of *Bauhinia acuruana*	–	81.7 ± 3.7%*	Small clusters/single cells; DC; MO	69.0 ± 11.9%*	Small clusters/single cells; DC; MO	16.9 ± 9.6%	–
Fruits of *Bauhinia acuruana*	–	77.8 ± 5.0%*	Small clusters/single cells; DC; MO	68.5 ± 7.4%*	Small clusters/single cells; MO	18.4 ± 7.2%	–
Leaves of *Pityrocarpa moniliformis*	–	77.0 ± 0.7%*	Small clusters	71.0 ± 0.6%*	Small clusters/single cells; MO	–	–
Stem bark of *Commiphora leptophloeos*	67.3 ± 8.5%	84.7 ± 1.3%*	Small clusters; DC	82.8 ± 5.6%*	Small clusters/single cells	6.7 ± 5.1%	100 ± 4.5%*

Results represent mean ± standard deviation of 3 experiments. DC represents deformation of the cells and MO represents matrix overproduction.
* Represents significant difference in relation to control ($p < 0.05$).

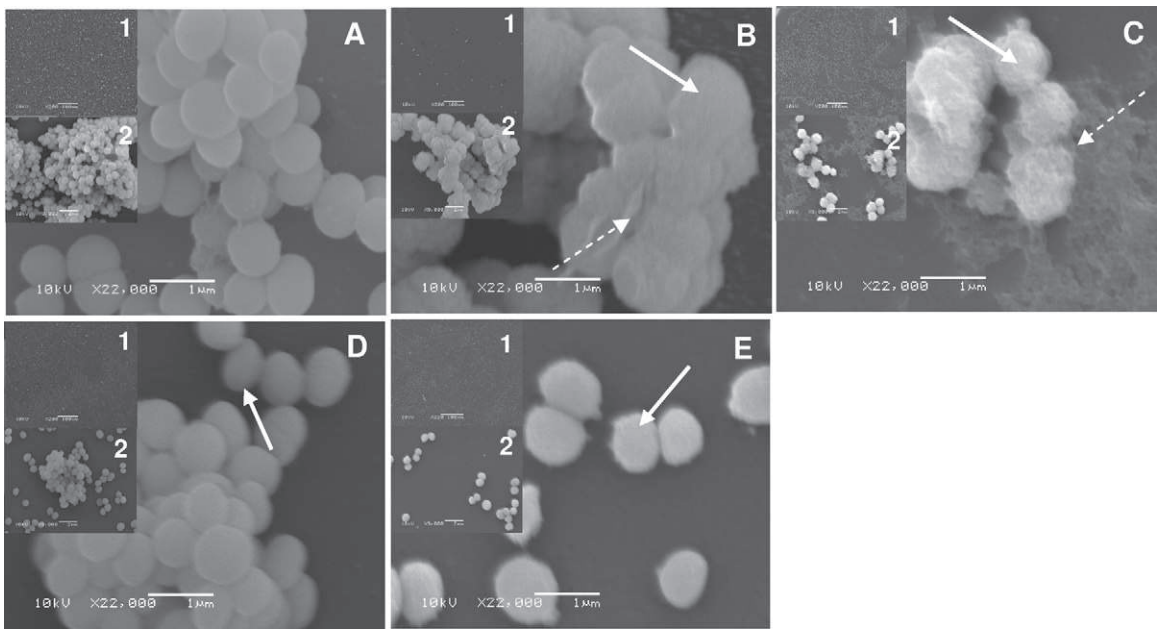

Fig. 2. Scanning electron microscopy images of the biofilms upon Permanox™. Untreated biofilms (control for biofilm formation) (A); Extract-treated biofilms: BBA11 (B); FBA35 (C); LPM45 (D); and SBCL33 (E). Scale bars: 22,000× magnification (in the images: insert 1 200× magnification and insert 2 9000× magnification). Solid arrows: cell deformation. Dotted arrows: matrix overproduction.

3.3. Scanning electron microscopy: biofilm visualization

The inhibition of *S. epidermidis* biofilm formation by the selected active extracts was observed by SEM using Permanox™, in order to mimic polystyrene, and glass coverslips. Images showed a dense and uniform staphylococcal biofilm covered the Permanox™ and glass surfaces in untreated biofilms (control for biofilm formation) (Figs. 2 and 3, panel A). By contrast, treated biofilms displayed a significant reduction in the number of adherent bacteria and in the size of aggregates, which were reduced to small clusters or even single cells (Figs. 2 and 3, panels B–E, respectively) and confirmed the results obtained by crystal violet assay.

Extract of BBA11 almost completely prevented bacterial adherence on both surfaces (Figs. 2 and 3, panel B), whereas LPM45 and SBCL33 extracts were similarly effective on glass coverslips only (Fig. 2, panels D and E, respectively). Regarding the FBA35 extract, the bacteria were equally able to form a biofilm on both surfaces, although to a lesser extent than the positive control (Figs. 2 and 3, panels C and A, respectively). Interestingly, the images indicated that some extract-treated biofilms presented a structural deformation in the bacterial cells and/or an overproduction of the exopolymeric matrix, the latter probably as a way of protecting themselves against the aggression of the extract (Figs. 2 and 3 and Table 4).

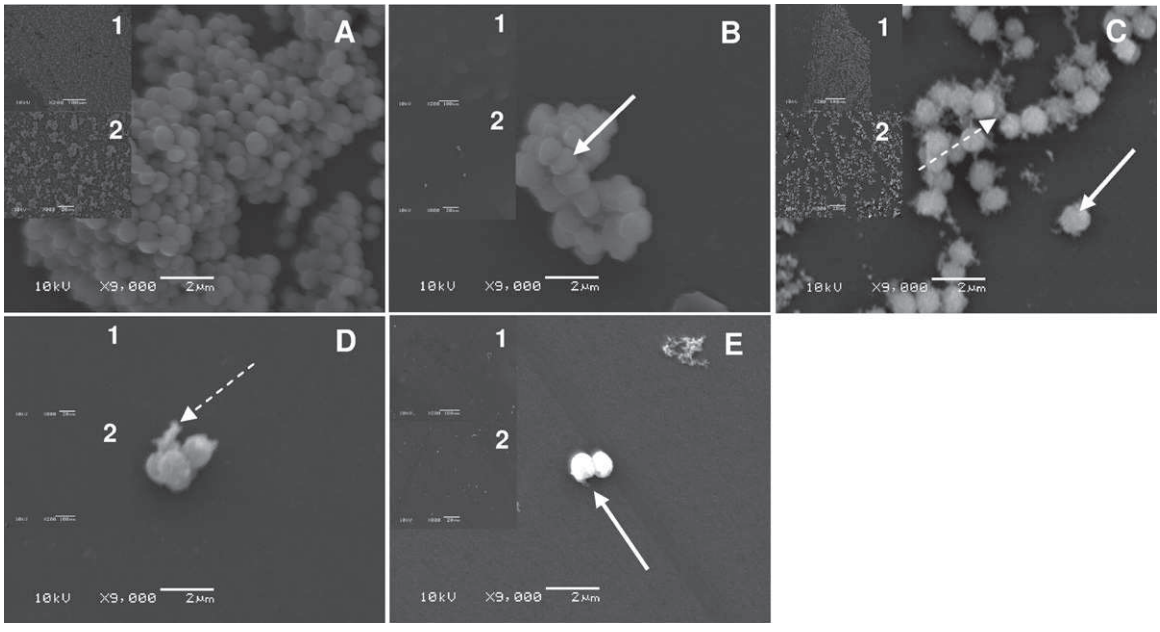

Fig. 3. Scanning electron microscopy images of the biofilms upon glass coverslips. Untreated biofilms (control for biofilm formation) (A); extract-treated biofilms: BBA11 (B); FBA35 (C); LPM45 (D); and SBCL33 (E). Scale bars: 9000× magnification (in the images: insert 1 200× magnification and insert 2 800× magnification). Solid arrows: cell deformation. Dotted arrows: matrix overproduction.

3.4. Antibacterial activity of Commiphora leptophloeos stem bark

Extract of SBCL33, at 4.0 mg/mL, caused 100% bacterial death according to OD_{600} measurements. The serial dilution (from 4.0 to 0.4 mg/mL) showed that the MIC was 1.0 mg/mL and the counting of the colony-forming units confirmed the bactericidal effect.

3.5. Phytochemical analysis

A preliminary qualitative phytochemical screening was carried out with the four extracts chosen. The TLC analysis revealed the absence of alkaloids and amines/aminoacids in the extracts and indicated the presence of polyphenols, coumarins, steroids and terpenes.

Although each sample was obtained from different species (3 plants) and from distinct plant structures (4 parts), the HPLC analysis presented similar chromatographic profiles among these four extracts. Each extract was monitored at 210, 254, 273 and 365 nm and compared by overlay (Fig. 4A–D). In addition, an overlay of the chromatograms from the differents extracts was obtained at 210 nm (Fig. 4E).

4. Discussion

Ethnopharmacology rescues the historical use of plants by people who have acquired great knowledge through family tradition. The information that is held and that can be accumulated about the use of plants in folk health care systems around the world is of inestimable importance. For example, the ethnobotanical and medicinal knowledge of the native people who live in or near tropical forests is recognized as valuable to bioprospecting (Quave et al., 2008). Likewise, ethnopharmacological data from residents of the Caatinga as well as from the literature pointed to the species collected and investigated in this study (Table 1). In addition, the

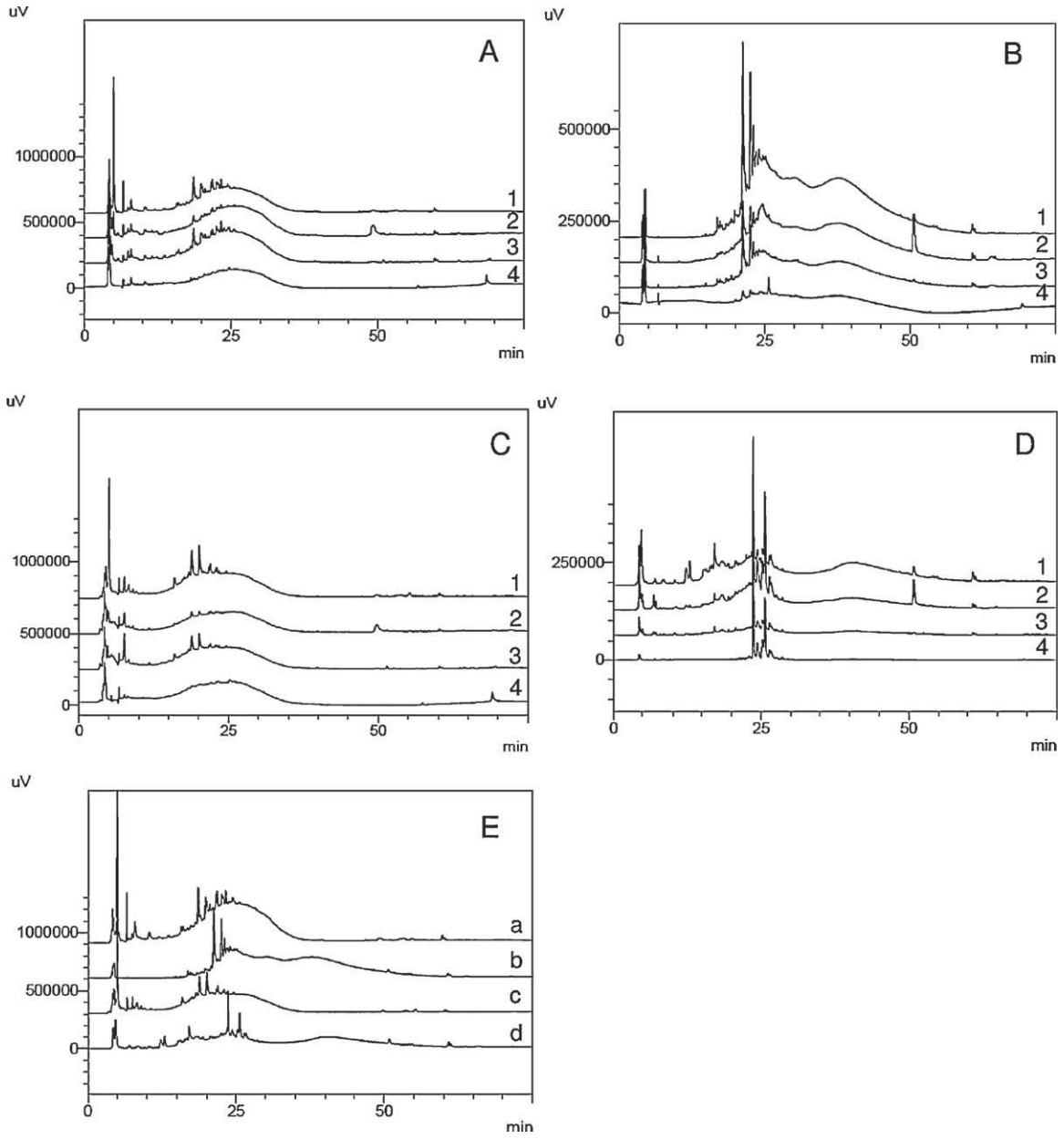

Fig. 4. HPLC-DAD chromatograms of the aqueous extracts. Extract BBA11 (A); SBCL33 (B); FBA35 (C); and LPM45 (D). Chromatograms 1, 2, 3, and 4 represent wavelengths of 210, 254, 273 and 365 nm, respectively and (E) represents an overlay of BBA11 (a); SBCL33 (b); FBA35 (c); and LPM45 (d) at 210 nm.

preparation of the aqueous extracts aimed to reproduce the form of use in traditional medicine and thereby enable us to confirm or refute their effectiveness, considering antibacterial/antibiofilm properties.

Among the 24 collected plants, 17 (70.8%) of them presented antimicrobial potential because they have been used in someway as antiseptics, anti-inflammatory or healing agents by folk medicine (Table 1). When tested *in vitro* against *S. epidermidis*, the most important cause of nosocomial infections, our results identified 13 plants (54.2% of the collection) with antibacterial properties, corresponding to 10 plants (58.6%) of those with antimicrobial potential. However, considering antibiofilm activity, 20 plants (83.3% of the collection), were active, corresponding to 15 plants (88.2%) used in folk medicine with antimicrobial potential. *Chamaecrista cystisoides* (branches) and *Stigmaphyllon paralias* (leaves) were the unique plants that not presented antibacterial neither antibiofilm activity, the last one was indicated by Caatinga community against diarrheas, syphilis and renal disorders. Interestingly, we found that 78% of the extracts that presented a high ability to inhibit *S. epidermidis* biofilm on polystyrene (Section 3.1), without killing the bacteria, came from plants that belong to the Fabaceae family (Table 2).

Of the four active extracts chosen for further investigation (BBA11, SBCL33, FBA35 and LPM45), one presented antibacterial activity (SBCL33), demonstrating a bactericidal effect against *Staphylococcus epidermidis* with a MIC of 1.0 mg/mL. Consequently, the biofilm structure was poorly formed when examined at 4.0 mg/mL on polystyrene and glass (more than 80% of antibiofilm activity) (Table 4). However, at 0.4 mg/mL, SBCL33 permitted a biofilm formation of 32.7% without antibacterial activity (Table 2). Additionally, the other three extracts selected for a more detailed study (BBA11, FBA35 and LPM45) demonstrated marked antibiofilm activity on polystyrene and glass surfaces without causing bacterial death (Table 4). This observation points out the wide potential of *Bauhinia acuruana* as a source of antivirulence compounds, since both branches (BBA11) and fruits (FBA35) prevented *S. epidermidis* biofilm formation on the two surfaces.

The notable activity demonstrated by the extracts was visualized by SEM, a valuable tool to improve understanding of the qualitative and quantitative impact of the samples upon the bacteria. Based on SEM images all four selected extracts affected the bacterial adhesion on Permanox™ and glass, two surfaces with distinct characteristics of hydrophobicity (Figs. 2 and 3 and Table 4). We found that the cells which adhered to surfaces presented an overproduction of extracellular matrix (BBA11 on Permanox™; FBA35 on both surfaces and LPM45 on glass), suggesting that molecules involved in the activity of the extracts did not exert their effect through extracellular matrix inhibition. Also, we observed bacterial cells with deformed morphology (BBA11, SBCL33 and FBA35 on both surfaces, and LPM45 on Permanox™), which could indeed be related to the extracts mechanism of action (Figs. 2 and 3 and Table 4).

Impairment of bacterial adhesion and biofilm formation by a pathway that does not involve bacterial death is a remarkable characteristic of a new concept in antivirulence therapies. Importantly, it explores new mechanisms of action that may difficult the rapid development of bacterial resistance. Moreover, in this alternative approach, which does not affect bacterial growth and maintains the cells in a planktonic state, the switching off of virulence expression and attenuation of the pathogen make microorganisms more susceptible to other antimicrobials and to the immune system (Clatworthy et al., 2007; Martin et al., 2008; Macedo and Abraham, 2009).

In this context, natural products are an important source of bioactive molecules and the medicinal plants used in popular medicine could facilitate the search for new agents. The results of TLC phytochemical screening of the four extracts chosen enabled us to exclude alkaloids and amines/aminoacids from the compounds potentially responsible for the bioactivity. In contrast, polyphenols, coumarins, steroids and terpenes do appear to be among the compounds involved with the extracts effects. HPLC-DAD analyses were carried out to characterize the four extracts selected and the results demonstrated similar profiles of compounds among them, especially at 210 nm where the highest number of compounds was detected (Fig. 4).

Polyphenols have received some attention recently regarding their antimicrobial effect upon microorganisms in biofilms, including a small number of studies involving *S. epidermidis* (Ferrazzano et al., 2009; Prabhakar et al., 2010; Schito et al., 2010). Sampaio et al. (2009) showed that polyphenol-rich extracts of fruits from *Caesalpiniea ferrea* had antibiofilm activity in a multispecies biofilm model involving *Streptococcus* sp., *Candida albicans* and *Lactobacillus casei*. This agrees with our results for fruits of the synonym *Libidibia ferrea*, which inhibited the formation of a biofilm by *S. epidermidis* by 70.0% (Table 2). Considering coumarins, we found only a few studies involving biofilm inhibition (Girennavar et al., 2008; Praud-Tabaries et al., 2009). However, antimicrobial activity of coumarins, terpenes (excluding volatile oils) and steroids against bacteria and fungi has been more widely described (Ojala et al., 2000; Sparg et al., 2004; Zhang et al., 2008; Popova et al., 2009; Smyth et al., 2009).

The antivirulence therapy is one of the most promise alternative to combat pathogenic microorganisms. The study presented herein show the Caatinga plants potential as a new and valuable source of prototype compounds. Our efforts now are concerned with the isolation and elucidation of active molecules from the four selected extracts.

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IV - CAPÍTULO 2 - Brazilian Caatinga medicinal plants: antibiofilm and antibacterial activities against *Pseudomonas aeruginosa*

Manuscrito submetido para o periódico **Revista Brasileira de Plantas Medicinais**

Brazilian Caatinga medicinal plants: antibiofilm and antibacterial activities against *Pseudomonas aeruginosa*

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ABSTRACT

The Caatinga biome covers a vast area in northeastern Brazil and presents a high level of biodiversity. It is known that at least 389 plant species are used by semi-arid local communities for medical purposes. Considering ethnopharmacological reports, this study aims to screen 24 species from Caatinga regarding the ability to prevent biofilm formation and to inhibit growth of *Pseudomonas* ae*ruginosa* - a major opportunistic human pathogen and an important causative agent of morbidity and mortality. The effects of aqueous extracts, at 0.4 and 4.0 mg/mL, on biofilm formation and on growth of *P. aeruginosa* ATCC 27853 were studied using the crystal violet assay and the OD_{600} absorbance, respectively. The more active extracts were analyzed by thin-layer chromatography and high performance liquid chromatography. The investigation pointed three species with potential application for the control of *P. aeruginosa*: *Anadenanthera colubrina*, *Myracrodruoun urundeuva* and *Commiphora leptophloeos*. The qualitative phytochemical analyses indicate similiarities among the samples as well as the presence of compounds with high molecular weight. This is an unprecedented evaluation about the potential of semi-arid Caatinga plants to prevent the formation of the *P. aeruginosa* biofilm.

Keyword: biofilm, *Anadenanthera colubrina*, *Myracrodruoun urundeuva*, *Commiphora leptophloeos*, Traditional Brazilian medicine.

RESUMO

O bioma Caatinga abrange uma vasta área no nordeste do Brasil e apresenta uma expressiva biodiversidade. Sabe-se que pelo menos 389 espécies de plantas são utilizadas por comunidades locais para fins medicinais. Considerando relatos etnofarmacológicos, este estudo tem por objetivo rastrear 24 espécies de plantas da Caatinga quanto à capacidade de impedir a formação de biofilme e de inibir o crescimento de *Pseudomonas aeruginosa* - importante patógeno oportunista humano e agente causador de morbidade e mortalidade. Os efeitos dos extratos aquosos, a 0,4 e 4,0 mg /mL, sobre a formação de biofilme e o crescimento de *P. aeruginosa* ATCC 27853 foram avaliados através do ensaio de cristal violeta e da densidade óptica a 600 nm, respectivamente. Os extratos mais ativos foram analisados por cromatografia em camada delgada e cromatografia líquida de alta eficiência. A investigação indicou três espécies com potencial aplicação para o controle de *P. aeruginosa*: *Anadenanthera colubrina*, *Myracrodruoun urundeuva* e *Commiphora leptophloeos*. As análises qualitativas fitoquímicas indicam similiaridades entre as amostras, bem como a presença de compostos com elevada massa molecular. Este estudo trata-se de uma avaliação sem precedentes sobre o potencial de plantas da Caatinga semi-árida para evitar a formação de biofilme por *P. aeruginosa*.

Palavras-chave: biofilme, *Anadenanthera colubrina*, *Myracrodruoun urundeuva* e *Commiphora leptophloeos*, medicina tradicional brasileira.

INTRODUCTORY REMARKS

Folk medicine has a great contribution to human health care long before modern medicine began. Nowadays, the use of medicinal plants as a source of remedies remains common to the medical traditions of many cultures and there is a strong evidence of integration between traditional and modern methods of health care (Zhang, 2000). Understanding the practices of the past, maintained by the traditional knowledge passed generation by generation, provides insight into and may lead to improvements in actual pharmaceutical practice (Vandebroek et al., 2011, Medeiros & Albuquerque, 2012). Therefore, there is an urgent call to collect information on and to identify the best health practices all over the world, and use them cost-effectively to enhance health care delivery (Ortega, 2006). The Caatinga, a typical semi-arid vegetation, represents the fourth largest area covered by a single vegetation form in Brazil, account for about 60% of the northeast territory (Sampaio et al., 2002). Several publications describe the rich flora in this region as having many medicinal purposes and great phytochemical potential (Agra et al., 2007; Albuquerque et al., 2007a; 2007b; 2012). In addition to being widely known and used by local communities, many medicinal species in the Caatinga are sold as herbal products (Albuquerque et al., 2007c; Cartaxo et al., 2010; Monteiro et al., 2011).

Pseudomonas aeruginosa is a major opportunistic human pathogen and an important causative agent of morbidity and mortality. This bacterium is able to express all known mechanisms of antimicrobial resistance, such as the low outer membrane permeability, the expression of broad multidrug efflux systems and the horizontal gene transfer (Strateva & Yordanov, 2009). In addition, *P. aeruginosa* has emerged as a pathogen adept at adhering to surfaces and forming biofilms, a bacterial lifestyle which cells are significantly more resistant to antimicrobials agents than as planktonic cells, causing chronic and very difficult to eradicate infections (Donlan & Costerton, 2002). In this context, we investigated 24 species of Caatinga plants (45 aqueous extracts) for their ability to inhibit growth and to prevent biofilm formation by *P. aeruginosa*. Also, we performed preliminary phytochemical analysis of the most active extracts.

MATERIALS AND METHODS

Plant material

The plants were collected at Parque Nacional do Catimbau (PARNA do Catimbau), Pernambuco State, Brazil, in July and August 2009. The species were chose according to the published ethnopharmacological data and using information from the local community. They were identified by the MSc. Alexandre Gomes da Silva from the Universidade Federal de Pernambuco, Brazil. A voucher specimen is deposited in the herbarium at Instituto Agronômico de Pernambuco (IPA), Brazil (Table 1).

Extraction

Aqueous extracts were prepared by static maceration of the powdered dried material with sterilized water [1:9; (w:v)] at 22 °C during 24 h (Trentin et al., 2011). Stock solutions (1.0 and 10 mg/mL) were prepared by solubilization of the dried extract in water and sterilized by filtration (0.22 µm).

Preliminary phytochemical analysis

The extracts were analyzed by TLC (Kieselgel 60 GF_{254}, 0.2 mm, Merck, Germany) using ethyl acetate:water:acetic acid:formic acid (9:2.3:1:1) as eluent. The plates were visualized under UV light (254 and 365 nm) and using standard procedures: polyphenol compounds were detected with aluminum chloride 1% and ferric chloride 2%, potassium hydroxide 5% was used for coumarins, Dragendorff's reagent for alkaloids, anisaldehyde/sulfuric acid for steroids and terpenes, ninhydrin for amines and aminoacids, and iodine vapor as an universal reagent (Wagner & Blat, 1996). The HPLC analyses were carried out on a Shimadzu LC-20AT coupled to a diode array detector. A reversed-phase column Shim-pack VP-ODS (250 mm × 4.6 mm I.D., 5µm, Shimadzu) was used. The gradient elution was performed with solution A - acetonitrile: water (5:95, [v/v]), and solution B - comprising 100 % acetonitrile, at a flow rate of 0.6 mL/min. Solution B increasing up from 5% to 100 % in 60 min and, finally, 100 % of solution B was preserved for 15 min. The injection volume was 20 µL (2.5 mg/mL solution).

***Pseudomonas aeruginosa* culture**

P. aeruginosa ATCC 27853 was cultured overnight at 37 °C in Mueller Hinton agar (Oxoid Ltd., England). Colonies were suspended in 0.9% saline to obtain a bacterial suspension corresponding to 1 McFarland scale (3×10^8 CFU/mL).

Antibiofilm formation assay and bacterial growth assay

In 96-well polystyrene plates, 80 µL of the bacterial suspension, 80 µL of the aqueous extract (0.4 mg/mL or 4.0 mg/mL in the wells) and 40 µL of TSB (Oxoid Ltd., England) were added and incubated at 37 °C for 6 h. After, the content of the wells was removed and they were washed with saline. The remaining attached bacteria were fixed and stained with crystal violet. The stain was solubilized with DMSO (Sigma-Aldrich Co., USA) and the absorbance was measured at 570 nm (Spectramax M2e Multimode Microplate Reader, Molecular Devices, USA). Bacterial growth was evaluated measuring the difference between OD_{600} absorbance at initial time and after 6 h (incubation time). Gentamicin sulfate 8 µg/mL (Sigma-Aldrich Co., USA) was used as a control for the inhibition of bacterial growth. Since does not exist a commercially available non-biocidal compound possessing antibiofilm activity, we can not apply a positive control to antibiofilm activity. The extracts were replaced with sterile water to represent 100% of biofilm formation and bacterial growth (untreated control). Values higher than 100% represent a stimulation of biofilm formation or bacterial growth in comparison to the untreated control.

Experiments were carried out in triplicate. The results were expressed as mean (%) ± SD and analyzed using the Student's t-test ($p \leq 0.05$).

Table 1: Aqueous extracts of the Caatinga plants against biofilm formation and planktonic growth of *P. aeruginosa* ATCC 27853.

Family	Species	Voucher	Part used	*P. aeruginosa*			
				0.4 mg/mL		4.0 mg/mL	
				Biofilm formation (%)	Bacterial growth (%)	Biofilm formation (%)	Bacterial growth (%)
Apocynaceae	*Allamanda blanchetii* A.DC.	IPA 84112	Branches	116.5 ± 4.8*	95.3 ± 0.4	109.4 ± 8.0	123.5 ± 3.0*
	Allamanda blanchetii A.DC.		Leaves	100.2 ± 4.4	89.5 ± 0.4	91.8 ± 9.4	99.6 ± 1.4
Fabaceae Mimosoideae	*Anadenanthera colubrina* (Vell.) Brenan *var. colubrina*	IPA 84039	Fruits	97.6 ± 6.2	88.2 ± 6.3	95.3 ± 0.7	100.2 ± 1.0
	Anadenanthera colubrina (Vell.) Brenan *var. colubrina*		Leaves	127.0 ± 1.8*	86.4 ± 4.3*	95.5 ± 1.4	67.8 ± 0.7*
	Anadenanthera colubrina (Vell.) Brenan *var. colubrina*		Branches	132.1 ± 1.9	104.3 ± 3.1	130.7 ± 31.4	99.3 ± 0.5
	Anadenanthera colubrina (Vell.) Brenan *var. colubrina*		Stem bark	124.1 ± 0.2*	97.8 ± 5.8	10.6 ± 4.1*	0.1 ± 12.5*
Fabaceae Cercideae	*Bauhinia acuruana* Moric.	IPA 84042	Branches	93.1 ± 7.8	94.3 ± 0.1	98.5 ± 8.2	98.3 ± 6.4
	Bauhinia acuruana Moric.		Fruits	155.2 ± 12.1*	82.0 ± 5.0*	119.8 ± 6.0	114.2 ± 3.1*
	Bauhinia acuruana Moric.		Leaves	108.8 ± 1.4	95.1 ± 2.9	102.7 ± 3.6	78.7 ± 1.5*
Combretaceae	*Buchenavia tetraphylla* (Aubl.) R.A. Howard	IPA 84104	Leaves	140.2 ± 6.7*	73.9 ± 4.7*	103.7 ± 2.4	83.5 ± 4.1*
Fabaceae "Caesalpinioideae"	*Chamaecrista desvauxii* (Collad.) Killip	IPA 84064	Leaves	128.4 ± 8.8*	91.7 ± 3.4	96.2 ± 5.5	110.7 ± 2.0*
	Chamaecrista desvauxii (Collad.) Killip		Fruits	122.2 ± 1.7*	83.5 ± 4.2	113.9 ± 4.5	97.4 ± 1.1
	Chamaecrista cytisoides (DC. ex Collad.) H.S. Irwin & Barneby	IPA 84103	Branches	118.6 ± 3.2	103.6 ± 2.7	114.1 ± 17.4	92.8 ± 1.4

Table 1 (*Continued*)

Family	Species	Voucher	Part used	*P. aeruginosa*			
				0.4 mg/mL		4.0 mg/mL	
				Biofilm formation (%)	Bacterial growth (%)	Biofilm formation (%)	Bacterial growth (%)
Burseraceae	*Commiphora leptophloeos* (Mart.) J.B.Gillett	IPA 84037	Branches	95.8 ± 6.7	87.7 ± 0.2	103.3 ± 7.8	153.6 ± 6.7*
	Commiphora leptophloeos (Mart.) J.B.Gillett		Stem bark	75.6 ± 0.9*	65.1 ± 2.1*	44.0 ± 2.8*	0.0 ± 15.3*
Fabaceae Faboideae	*Dioclea grandiflora* Mart. ex Benth.	IPA 84057	Leaves	122.9 ± 6.9	82.4 ± 6.5	114.3 ± 3.6*	85.4 ± 1.6*
	Dioclea grandiflora Mart. ex Benth.		Branches	113.0 ± 1.7	100.0 ± 3.0	84.5 ± 1.8*	103.9 ± 3.0
	Dioclea grandiflora Mart. ex Benth.		Fruits	86.6 ± 3.9*	92.0 ± 1.4	85.9 ± 5.2*	112.2 ± 3.8
Myrtaceae	*Eugenia brejoensis* Mazine	IPA 84033	Leaves	107.9 ± 4.0	98.9 ± 1.2	104.1 ± 12.0	100.3 ± 2.8
Euphorbiaceae	*Jatropha mutabilis* (Pohl) Baill.	IPA 84054	Roots	124.6 ± 2.5*	87.6 ± 4.7	126.1 ± 5.2*	110.3 ± 1.7
	Jatropha mutabilis (Pohl) Baill.		Branches	116.5 ± 6.6	84.5 ± 5.2	130.0 ± 5.8	132.1 ± 2.2*
Fabaceae "Caesalpinioideae"	*Libidibia ferrea* (Mart. ex Tul.) L.P. Queiroz *var. ferrea*	IPA 84035	Leaves	104.7 ± 5.4	78.4 ± 0.6	163.3 ± 5.3*	84.5 ± 2.4*
	Libidibia ferrea (Mart. ex Tul.) L.P. Queiroz *var. ferrea*		Fruits	91.7 ± 0.6	78.3 ± 2.8	202.3 ± 10.4*	56.1 ± 0.7*
Cactaceae	*Melocactus zehntneri* (Britton & Rose) Luetzelb.	IPA 85028	Roots	98.6 ± 0.3	110.6 ± 5.8	103.7 ± 1.2	164.6 ± 0.9*
	Melocactus zehntneri (Britton & Rose) Luetzelb.		Cephalium	85.8 ± 2.4*	102.4 ± 5.8	122.0 ± 5.3	138.3 ± 1.7*
Anacardiaceae	*Myracrodruoun urundeuva* Alemão	IPA 84059	Branches	108.3 ± 4.8	83.0 ± 1.7*	100.4 ± 4.5	90.0 ± 1.2
	Myracrodruoun urundeuva Alemão		Leaves	115.5 ± 6.9	75.3 ± 1.0*	109.2 ± 5.9	97.6 ± 2.0
	Myracrodruoun urundeuva Alemão		Stem bark	147.3 ± 3.8*	81.9 ± 3.8*	20.7 ± 2.0*	0.1 ± 2.6*
Fabaceae Faboideae	*Myroxylon peruiferum* L.f.	IPA 84110	Leaves	100.2 ± 9.0	106.2 ± 4.0	128.0 ± 0.4*	111.5 ± 1.2*

Table 1 (*Continued*)

Family	Species	Voucher	Part used	*P. aeruginosa*			
				0.4 mg/mL		4.0 mg/mL	
				Biofilm formation (%)	Bacterial growth (%)	Biofilm formation (%)	Bacterial growth (%)
Ochnaceae	*Ouratea blanchetiana* Engl.	IPA 84044	Branches	118.2 ± 6.4	84.4 ± 3.9	119.6 ± 2.1	103.3 ± 2.8
	Ouratea blanchetiana Engl.		Leaves	150.7 ± 12.3*	82.5 ± 2.3	104.2 ± 11.7	104.5 ± 5.0
Fabaceae “Caesalpinioideae”	*Parkinsonia aculeate* L.	IPA 84113	Leaves	98.9 ± 0.3	92.4 ± 2.1	101.2 ± 4.4	128.4 ± 7.8*
Fabaceae Mimosoideae	*Piptadenia viridiflora* (Kunth) Benth.	IPA 84036	Branches	109.4 ± 5.6	121.8 ± 7.3	96.7 ± 2.0	118.8 ± 0.3*
	Piptadenia viridiflora (Kunth) Benth.		Fruits	71.6 ± 8.6*	92.5 ± 2.0	90.6 ± 2.0	97.9 ± 0.1
Fabaceae Mimosoideae	*Pityrocarpa moniliformis* (Benth.) Luckow & R.W. Jobson	IPA 84048	Leaves	205.0 ± 13.5*	76.0 ± 3.2*	32.3 ± 6.4*	69.2 ± 10.8*
	Polygala boliviensis A.W.Benn.		Inflorescences	95.8 ± 4.3	84.9 ± 3.0	119.2 ± 2.1	102.5 ± 0.9
	Polygala boliviensis A.W.Benn.	IPA 84066	Leaves	104.2 ± 0.4*	82.2 ± 0.7	91.5 ± 1.9*	88.6 ± 3.0*
Polygalaceae	*Polygala boliviensis* A.W.Benn.		Branches	106.3 ± 4.6	86.7 ± 6.1	115.4 ± 0.3	139.8 ± 9.0*
	Polygala violacea Aubl.	IPA 84051	Leaves	114.2 ± 0.4*	112.9 ± 4.6	112.4 ± 16.6	116.7 ± 1.0*
	Polygala violacea Aubl.		Roots	151.2 ± 13.2*	91.3 ± 5.8	96.1 ± 0.7	107.5 ± 1.6*
Fabaceae “Caesalpinioideae”	*Senna macranthera* (Collad.) H.S. Irwin & Barneby *var. macranthera*	IPA 84045	Fruits	93.8 ± 1.5	95.7 ± 1.9	89.9 ± 2.0*	118.8 ± 3.0*
	Senna splendida (Vogel.) H.S. Irwin & Barneby	IPA 84040	Branches	118.9 ± 2.7	103.7 ± 2.0	71.8 ± 1.6	71.7 ± 7.6*
Malvaceae	*Sida galheirensis* Ulbr.	IPA 84078	Branches	111.8 ± 4.9*	97.8 ± 2.5	75.7 ± 3.8	98.0 ± 1.4
	Sida galheirensis Ulbr.		Leaves	121.2 ± 0.6*	99.5 ± 3.7	69.9 ± 9.6*	104.7 ± 2.9
Malpighiaceae	*Stigmaphyllon paralias* A. Juss.	IPA 84041	Leaves	155.2 ± 1.4*	79.3 ± 0.9	126.3 ± 15.1	124.9 ± 9.4*

Results represent mean ± standard deviation of 3 experiments. * represents significant difference in relation to control ($p < 0.05$).

RESULTS AND DISCUSSION

Nosocomial infections having multidrug-resistant *P. aeruginosa* as ethiologic agent is frequently worldwide, accounting for about of 25% of isolated strains from two nationwide surveillance programmes in China (Xiao et al., 2012). Moreover, *P. aeruginosa* ranks first among all nosocomial pathogens related to pneumonia in intensive care in Brazil (Rossi, 2011) and in United States (Richards et al. 1999) – a commonly biofilm-associated infection.

Targeting bacterial virulence (like biofilm formation) is an alternative approach to antibacterial therapy that offers promising opportunities to inhibit pathogenesis without threatening bacterial existence, resulting in a reduced selection pressure for drug-resistant mutations (Cegelski et al., 2008). Plants have been used as medicine in all cultures and the interest in research involving antimicrobial activity and medicinal properties of plants has increased. We used aqueous extracts to reproduce the traditional medicine preparation, and thereby, it enables us to evaluate their effectiveness, considering *in vitro* antibacterial/antibiofilm properties against this important pathogen.

Pseudomonas aeruginosa was exposed to the 45 extracts, as showed in Table 1. At 0.4 mg/mL no extract showed remarkable activity, even considering values of at least 20% of inhibition on growth and on biofilm formation. At this concentration, the stem-bark of *Commiphora leptophloeos* was the most effective extract to inhibit the *P. aeruginosa* growth. Regarding extracts at 4.0 mg/mL, the lowest rates of biofilm formation, when compared to untreated control, were found using *Anadenanthera colubrina* (11%), *Myracrodruoun urundeuva* (21%) and *C. leptophloeos* (44%) stem barks. Table 2 summarizes the effect of the extracts against *P. aeruginosa*, classifying them according to the activity range. At 4.0 mg/mL, the results demonstrated that among all tested extracts, only 2 (4.4%) possess high ability to prevent biofilm formation and that 3 (6.7%) present high ability to inhibit *P. aeruginosa* growth (Table 2). The more active plants (*A. colubrina*, *C. leptophloeos* and *M. urundeuva*) are traditionally used in folk medicine as anti-inflammatory and antiseptic agents (Agra et

al., 2007). Regarding these 3 species (stem-bark), the formation of biofilm by *P. aeruginosa* seems to be avoided through antibacterial properties, since their extracts strongly affected bacterial growth (Table 1). However, our previous screening evaluating the same extracts against *Staphylococcus epidermidis* revealed a different scenario, since 15.5% and 2.2% of them presented high inhibition of biofilm formation and planktonic growth, respectively. In addition, only one extract showed *S. epidermidis* biofilm prevention associated to its bactericidal effect (Trentin et al., 2011).

TABLE 2: Correlation of the number of aqueous extracts (%) and their anti-*P. aeruginosa* activities.

	0.4 mg/mL		4.0 mg/mL	
	Biofilm (%)	Growth (%)	Biofilm (%)	Growth (%)
Stimulate	53.3	6.7	35.6	33.3
No effect	35.6	35.6	37.8	37.8
Up to 25% inhibition	8.9	53.3	13.3	13.3
From 26 to 50% inhibition	2.2	4.4	4.4	8.9
From 51 to 75% inhibition	-	-	4.4	-
From 76 to 100% inhibition	-	-	4.4	6.7

The qualitative phytochemical screening was carried out with the three most active extracts. The TLC-fingerprint indicated the presence of polyphenols, steroids, terpenes and amines/aminoacids (data not shown). The chromatograms obtained by HPLC showed a similar profile of the three extracts (Figure 1) in agreement with TLC analysis. They presented several absorption peaks in overlapping retention times even after several attempts of chromatogram optimization. It is indicative of compounds with high molecular weight. Siqueira et al. (2012) highlighted that the group of plant with antimicrobial potential showed a higher content of tannins compared to a control group. In addition, it is know the potential Anacardiaceae, Bursereaceae and Fabaceae to synthesize tannins, particularly by stem-bark tissues. On this way, the qualitative phytochemical analysis reveals the complexity of these aqueous stem-bark extracts and enables us to suggest the tannins as the possible bioactive secondary metabolites in these samples.

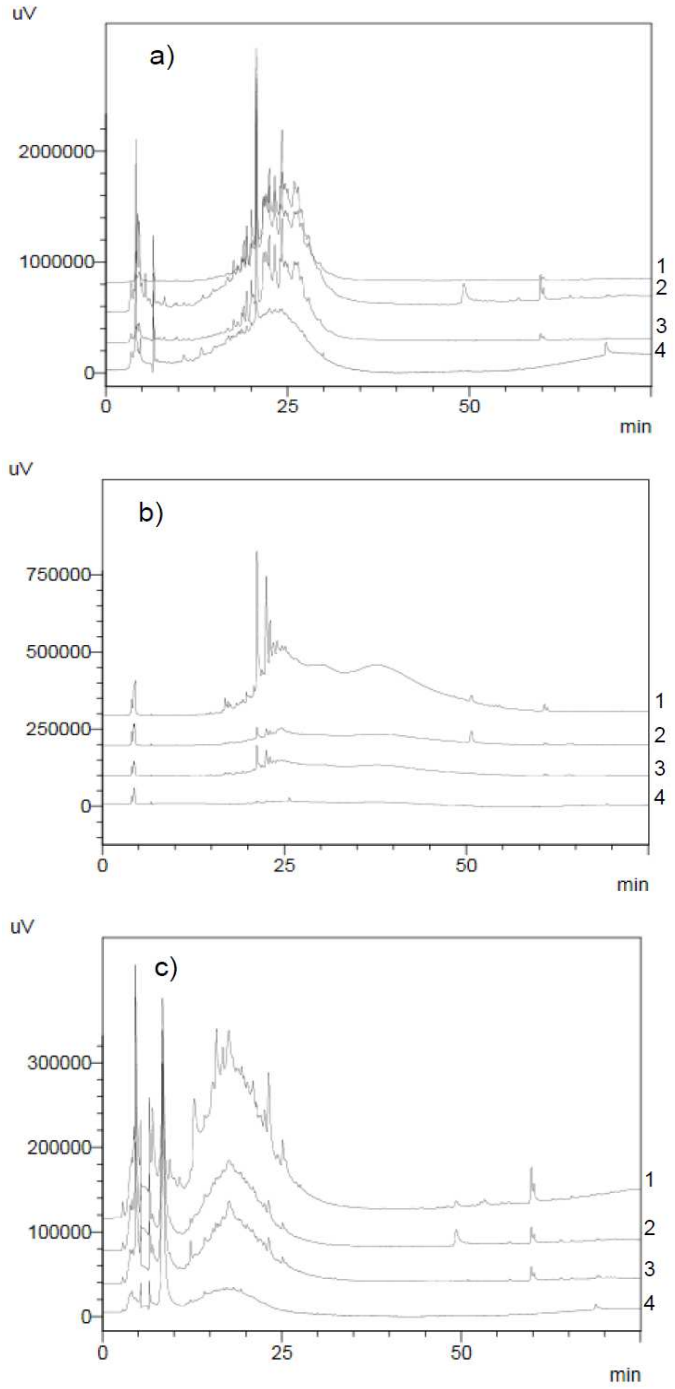

Figure 1: Qualitative HPLC-DAD chromatogram of the aqueous extracts: a) stem bark of *A. colubrina*; b) stem bark of *C. leptophloeos* and c) stem bark of *M. urundeuva*. Chromatograms 1, 2, 3, and 4 represent wavelengths of 210, 254, 273 and 365 nm, respectively.

Since just 6.7% of the assayed extracts demonstrated expressive activity against *P. aeruginosa*, the difficulty to control this pathogen with some Brazilian plant extracts is stressed. This is in agreement with many studies that investigate

antibacterial activity of medicinal plants extracts in several countries, such as Spain, Cuba, Colombia, Siberia, Africa, Arabia and India (Herrera et al., 1996; Martínez et al., 1996; Lopez et al., 2001; Kokoska et al., 2002; Koné et al., 2004; Mothana & Lindequist, 2005; Kumar et al, 2006). Given the great importance of biofilm formation in the infectious process, the association of screening of medicinal plants and the modulation of *P. aeruginosa* biofilm started to be considered (Ding et al., 2011). This is the first study that investigates the potential of Caatinga plants extracts to prevent the biofilm formation by *P. aeruginosa*. Antimicrobial resistance of non-fermenting bacteria, including *P. aeruginosa*, remains a challenge for clinical treatment, antibiotic selection and effectiveness. Indeed, *P. aeruginosa* often exhibits a multidrug-resistant or even pandrug-resistant phenotype, warranting its reputation as a 'superbug' and highlighting its clinical significance (Souli et al., 2008; Xiao et al., 2012).

Overall, this work point to the importance of 3 plant aqueous extracts (stem-bark of *A. colubrina, C. leptophloeos* and *M. urundeuva*) against *P. aeruginosa*. We might hypothesize that the low number of active extracts observed against *P. aeruginosa* could be related to the lower presence of Gram-negative bacteria in semi-arid soil of Northeastern Brazil, as already reported by Gorlach-Lira & Coutinho (2007). In addition, this bacterium is recognized as an important phytopathogen, since it can form a biofilm that confers resistance against root-secreted antibiotic by plants during root colonization (Walker et al., 2004). Considering that plant secondary metabolism is responsive and modulated by environmental conditions and by plant-pathogen interactions, our results are in accordance with previous studies. Our efforts now are concerned with the elucidation of active compounds from the stem-bark of *A. colubrina*, *C. leptophloeos* and *M. urundeuva*.

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IV - CAPÍTULO 3 - Tannins possessing bacteriostatic effect impair *Pseudomonas aeruginosa* adhesion and biofilm formation

Short title: Tannins prevent *Pseudomonas aeruginosa* biofilm

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Tannins possessing bacteriostatic effect impair *Pseudomonas aeruginosa* adhesion and biofilm formation

Short title: Tannins prevent *Pseudomonas aeruginosa* biofilm

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Abstract

Plants produce many compounds that are biologically active, either as part of their normal program of growth and development or in response to pathogen attack or stress. Traditionally, *Anadenanthera colubrina*, *Commiphora leptophloeos* and *Myracrodruon urundeuva* have been used by communities in the Brazilian Caatinga to treat several infectious diseases. The ability to impair bacterial adhesion represents an ideal strategy to combat bacterial pathogenesis, because of its importance in the early stages of the infectious process; thus, the search for anti-adherent compounds in plants is a very promising alternative. This study investigated the ability of stem-bark extracts from these three species to control the growth and prevent biofilm formation of *Pseudomonas aeruginosa*, an important opportunistic pathogen that adheres to surfaces and forms protective biofilms. A kinetic study (0-72 h) demonstrated that the growth of extract-treated bacteria was inhibited up to 9 h after incubation, suggesting bacteriostatic activity. Transmission electron microscopy and fluorescence microscopy showed both viable and nonviable cells, indicating bacterial membrane damage; crystal violet assay and scanning electron microscopy demonstrated that treatment strongly inhibited biofilm formation during 6 and 24 h and that matrix production remained impaired even after growth was restored, at 24 and 48 h of incubation. Herein, we propose that the identified (condensed and hydrolyzable) tannins are able to inhibit biofilm formation via bacteriostatic properties, damaging the bacterial membrane and hindering matrix production. Our findings demonstrate the importance of this abundant class of compounds in higher plants against one of the most challenging issues in the hospital setting: biofilm resilience.

Keywords: *Pseudomonas aeruginosa*, antibiofilm, antibacterial, Caatinga, MALDI-MS, tannin, *Anadenanthera colubrina*, *Commiphora leptophloeos*, *Myracrodruon urundeuva*.

1. Introduction

Adhesion and colonization are prerequisites for the establishment of bacterial infection and pathogenesis. Once adhesion has taken place, on implanted medical devices or damaged tissue, microorganisms may undergo specific molecular changes to become pathogenic and to establish biofilms [1]. It is well known that biofilm formation involves the attachment and accumulation of microbial cells, within a self-produced extracellular matrix, on a solid surface [2]. The inherently defensive character of the biofilm is demonstrated by enhanced persistence of bacteria grown in the sessile mode model *versus* bacteria grown planktonically, which makes most biofilm-associated infections difficult to eradicate, thus contributing to disease chronicity [3,4]. *Pseudomonas aeruginosa*, a ubiquitous bacterium in the nature, is an opportunistic pathogen that adheres to surfaces and forms protective biofilms [3]. In addition, multidrug-resistant *P. aeruginosa* is a leading cause of nosocomial infection worldwide, ranking first among all nosocomial pathogens related to pneumonia in intensive care units in Brazil [5] and in the United States [6].

The challenge and difficulty in finding novel antibacterial agents with innovative mechanisms of action, including anti-adherent compounds, drive the search for antimicrobials toward vegetable sources. This is an appropriate choice because plants play an important role in the biosynthesis of natural products, providing chemical defense against environmental microbes through secondary metabolism, and because they can be considered as a therapeutic alternative in primary health care (ethnopharmacological knowledge). The Caatinga, a xeric shrub-dominated biome of northeastern Brazil, supports a high diversity of plant resources used as folk medicine. This region is known as an area of low economic development, which reflects poor access of the population to pharmaceutical drugs and, consequently, determines the treatment of illnesses based on the use of medicinal plants. The limited scientific basis for the biological properties of these plants prompted an interest in investigating species widespread in the Caatinga in more detail. Our ongoing efforts to evaluate their biological potential have revealed antibacterial and antibiofilm activities against

the Gram-positive bacterium *Staphylococcus epidermidis* for several plant species [7] and against *P. aeruginosa* for a reduced number of plants (see caption 2).

This study aimed to investigate the activity of stem-bark extracts of *Anadenanthera colubrina*, *Commiphora leptophloeos* and *Myracrodruon urundeuva* and their ability to control the growth and prevent biofilm formation of *P. aeruginosa* using bioguided fractionation. The bioactive compounds were purified and further analyzed by MALDI-TOF/TOF in order to identify structures.

2. Materials and methods

2.1. Plant material

Stem barks were collected at a national park, Parque Nacional do Catimbau (PARNA do Catimbau), located in the state of Pernambuco, northeastern Brazil, between July and August 2009. The taxonomic identification was confirmed at the herbarium of Instituto Agronômico de Pernambuco (IPA), where the vouchers were deposited (Table 1). The extracts were prepared as previously described [7].

2.2. Bacterial strain and culture conditions

Pseudomonas aeruginosa ATCC 27853 was grown overnight on Mueller-Hinton (MH) agar (Oxoid Ltd., England, UK) at 37°C. A bacterial suspension of 3 x 10^8 colony-forming units (CFU)/mL in 0.9% NaCl was used in the assays.

2.3. Minimum inhibitory concentration (MIC) and bacterial viability

Bacterial growth was assessed as the difference between optical density at 600 nm (OD_{600}) at the end (6 h) and at the beginning (0 h) of incubation time, in 96-well microtiter plates (Costar 3599, Corning, Inc., USA). In each well, 80 µL of the bacterial suspension, 80 µL of the aqueous extract (concentration ranging from 0.5 to 4.0 mg/mL in the wells) and 40 µL of tryptone soya broth (TSB) (Oxoid Ltd., England, UK) were added. MIC was defined as the lowest concentration of samples able to restrict bacterial growth to a level lower than 0.04 at OD_{600}. Serial dilutions of

the MIC wells were performed and spread on MH agar plates. After incubation (37°C, overnight), the CFU/mL was obtained, in order to determine the viability of bacterial cells. As references for bacterial growth and viability, the extracts were replaced with water (negative control) or with gentamicin sulfate (Sigma-Aldrich Co., USA) (positive control).

2.4. Antibacterial activity kinetics

A kinetic study was performed to assess the effect of extracts (at concentrations of 1/4xMIC, 1/2xMIC, MIC, and 2xMIC) upon *P. aeruginosa* according to the incubation time, as previously described in Section 2.3. OD_{600} was measured at 0, 3, 6, 9, 24, 30, 48, 52, and 72 h after incubation (37°C). Samples were replaced with sterile water as a control for bacterial growth. To avoid the interference of sample color in all results obtained by OD evaluations, the samples were incubated in TSB and sterile saline (without inoculum) and the arithmetic means of OD readings were corrected for each extract (by subtracting OD without inoculum from OD with inoculum for each incubation time). Erythromycin (Sigma-Aldrich Co., USA) was used as a control. The results are expressed as mean ± standard deviation (SD) of 4 wells for each extract concentration and for each incubation time.

2.5. Biofilm formation assay

Biofilm formation was evaluated using the crystal violet assay in 96-well microtiter plates [7]. The incubation period at 37°C was 6, 24, and 48 h. To represent 100% of biofilm formation (untreated sample), the extracts were replaced with sterile water. Values higher than 100% represented stimulation of biofilm formation in comparison with the untreated sample. Since does not exist a commercially available non-biocidal compound possessing antibiofilm activity, we can not apply a positive control to antibiofilm activity.

Table 1 Stem-bark plant species from the Brazilian Caatinga: MIC, CFU/mL and biofilm formation assessed by crystal violet assay (mean ± S.D) by *P. aeruginosa.*

Family	Scientific name	Popular name	Voucher	MIC (mg/mL)	CFU/mL (log)	Biofilm formation at 6 h (%)	Biofilm formation at 24 h (%)	Biofilm formation at 48 h (%)
Fabaceae - Mimosoideae	*Anadenanthera colubrina* (Vell.) Brenan *var. colubrina*	Angico	IPA 84039	2.5	8.7 ± 0.06	13.7 ± 4.4*	35.7 ± 3.2*	108.4 ± 19.1
Burseraceae	*Commiphora leptophloeos* (Mart.) J.B.Gillett	Imburana, amburana, imburana de cambão	IPA 84037	1.0	8.5 ± 0.02	47.4 ± 5.1*	70.6 ± 1.6*	147.0 ± 14.5*
Anacardiaceae	*Myracrodruon urundeuva* Allemão	Aroeira, aroeira-do-sertão	IPA 84059	4.0	8.8 ± 0.06	20.7 ± 2.0*	40.2 ± 7.9*	176.7 ± 15.5*

Experiments were carried out in triplicate. Untreated samples were considered as 100% of biofilm formation and presented log CFU/mL of 8.7 ± 0.2. *represents statistical difference in comparison to the untreated sample

2.6. Microscopic analysis

2.6.1. Scanning electron microscopy (SEM)

Pseudomonas aeruginosa biofilms were grown in 96-well microtiter plates (37°C during 6, 24, and 48 h) with a piece of Permanox™ slide (Nalge Nunc International, USA), as described in Section 2.4. The samples were prepared and examined according to Trentin et al [7].

2.6.2. Transmission electron microscopy (TEM)

Tubes containing 800 µL of the bacterial suspension, 800 µL of the aqueous extract (MIC concentration) and 400 µL of TSB were incubated (37°C, 6 h). In untreated samples, sterile water was added instead of samples. The bacteria were harvested by centrifugation, fixed in glutaraldehyde and paraformaldehyde solution, and, subsequently, in 2% osmium tetroxide. The pellet was dehydrated in an ascending series of acetone concentrations and cells were embedded in acetone:EmBed™ resin, homogenized by rotation and polymerized. Ultrathin sections were contrasted with uranyl acetate and lead citrate and imaged with a JEOL JEM-1200 EX II electron microscope (JEOL Ltd, Tokyo, Japan).

2.6.3. Fluorescence microscopy

Samples were prepared as described in Section 2.6.2. After incubation, they were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, USA). In this assay, the SYTO-9 and propidium iodide (PI) stains compete for binding to the bacterial nucleic acid. SYTO-9 labels cells with both damaged and intact membranes (green cells), whereas PI penetrates only cells with damaged membranes (red cells), reducing the fluorescence of SYTO-9. The samples were observed in the AxioVert 200 fluorescence microscope using the AxioVision AC software (Carl Zeiss MicroImaging Inc, Germany), and image overlays were obtained using ImageJ software.

2.7. Hemolytic assay

This assay was performed as previously described [8], using venous blood of healthy volunteers (ethical approved by UFRGS Ethical Committee, number 19346). The extracts were tested at MIC and, as reference samples, we used water (for baseline values) and Quillaja saponaria saponins (Sigma-Aldrich Co., USA) at 0.25 mg/mL (for 100% hemolysis). To avoid the interference of sample color, a blank sample of extracts and phosphate-buffered saline (PBS) (without erythrocytes) was developed. The assay was calculated as follows: (Abs extracts – Abs blank/ Abs saponin – Abs water) x 100.

2.8. Purification of proanthocyanidins

The crude aqueous extracts (100 mg) were dissolved in water (500 µL) and subjected to column chromatography (10 x 150 mm) packed with Sephadex™ LH-20 (Sigma-Aldrich Co., USA) successively until to obtain the appropriate amount of fractionated sample. Water was used as the first eluent, followed by 30% methanol, 50% methanol, 100% methanol, 10% acetone, 30% acetone, 50% acetone, 70% acetone, and 100% acetone, resulting in fractions coded as F1-F9. Additional data are available as supporting information (Table S1).

2.9. MALDI-MS and MALDI-MS/MS analyses

High-resolution mass spectrometry (MS) analyses were performed using an UltrafleXtreme MALDI-TOF/TOF equipment (Bruker Daltonics, Bremen, Germany). A mixture of peptides was used for external and internal calibration (peptide calibration standard II [Bruker Daltonics]: bradykinin 1-7, angiotensin II and I, substance P, bombesin, renin substrate, ACTH clip 1-17, ACTH clip 18-39, and somatostatin 28). The ions were generated by irradiation with a nitrogen laser (337 nm) and accelerated at 20 kV. For MS analyses, the experimental conditions were: pulsed ion extraction of 100 ns, laser frequency of 1000 Hz, reflectron mode, positive ion mode, and 600 laser shots were averaged to record a mass spectrum. In addition,

the selected ions were accelerated to 19 kV in the LIFT cell for MS/MS analyses. The matrix of choice was DHB (2,5-dihydroxybenzoic acid) at 20 mg/mL (in 30% acetonitrile [ACN] and 70% H_2O with 0.1% trifluoroacetic acid). All samples were suspended in ACN:H_2O (3:7) and mixed with DHB containing 0.1 M solution of NaCl. These mixtures (1 µL) were spotted onto a ground stainless steel MALDI target. The compounds were identified by MS data, fragmentation pathway and accurate mass measurements using the internal calibrant (standard peptide mixture).

2.10 Statistical analysis

Biological assays were carried out in triplicate. Data differences in relation to the untreated samples were analyzed by the Student t test, and $p \leq 0.05$ was considered to be significant.

3. Results

3.1. MIC and viability determinations for stem-bark extracts

The MIC of all three stem-bark aqueous extracts against *P. aeruginosa* was determined (Table 1). CFU counting was used to determine the viability of 6 h-treated cells at MIC. Statistical analysis indicated that previously treated and untreated bacterial suspensions were equivalent regarding CFU/mL, suggesting bacteriostatic activity, i.e., when bacterial growth is inhibited (at MIC), the cells are viable (Table 1).

3.2. Kinetic analysis of antibacterial activity

To observe the effect of extracts upon *P. aeruginosa* growth according to incubation time, a kinetic study was performed (Fig. 1). In this set of experiments, there was a significant decrease in bacterial growth within a short period of exposure to all extract concentrations (except for 1/4xMIC of *A. colubrina* and *C. leptophloeos* – Fig. 1A-B). Bacterial growth remained inhibited or low up to 9 h after incubation. After 24 h, extract-treated bacteria started to grow, achieving values similar to those

obtained with untreated cells. Reinforcing our former result, a similar dynamic profile was observed with erythromycin, a bacteriostatic agent against *P. aeruginosa*.

3.3. Activity upon biofilm formation

The effect of extracts at MIC upon *P. aeruginosa* biofilm formation on the polystyrene surface, using crystal violet assay, is shown in Table 1. Biofilm formation was strongly prevented in 6 h-treated bacteria in relation to untreated cells. After 24 h of treatment, the extracts remained able to inhibit biofilm formation, although with a loss of about 20% in their antibiofilm activity when compared to inhibition at an earlier stage (6 h). According to this assay, no antibiofilm effect was observed at 48 h of incubation. Unlikely, *C. leptophloeos* and *M. urundeuva* extracts stimulated biofilm formation (Table 1). These results were corroborated by morphological features observed using different microscopic techniques. SEM images showed rod-shaped cells of untreated *P. aeruginosa* grown on the Permanox surface, forming a dense and uniform biofilm (untreated biofilms) (Fig. 1A-C), covered with an extracellular matrix (Fig. 1B-C). In contrast, 6 h-treated biofilms displayed lesser adherent bacteria and only slight aggregation, reducing bacterial agglomerates to small clusters (Fig. 2 images 2A, 3A, and 4A). At 24 h of incubation, we could still observe a low number of bacterial clusters deficient in matrix production compared to the untreated sample (Fig. 2 images 2B and 4B), while for *C. leptophloeos*-treated cells a larger amount of aggregated cells was observed, but without the presence of a matrix (Fig. 2 image 3B). After 48 h of treatment, matrix production was extremely poor; the bacterial cluster architecture varied among samples and was different from the typical biofilm shape of the untreated *P. aeruginosa*. However, it was possible to observe that cell agglomerates had completely covered the surface (Fig. 2 images 1C, 2C, 3C, and 4C).

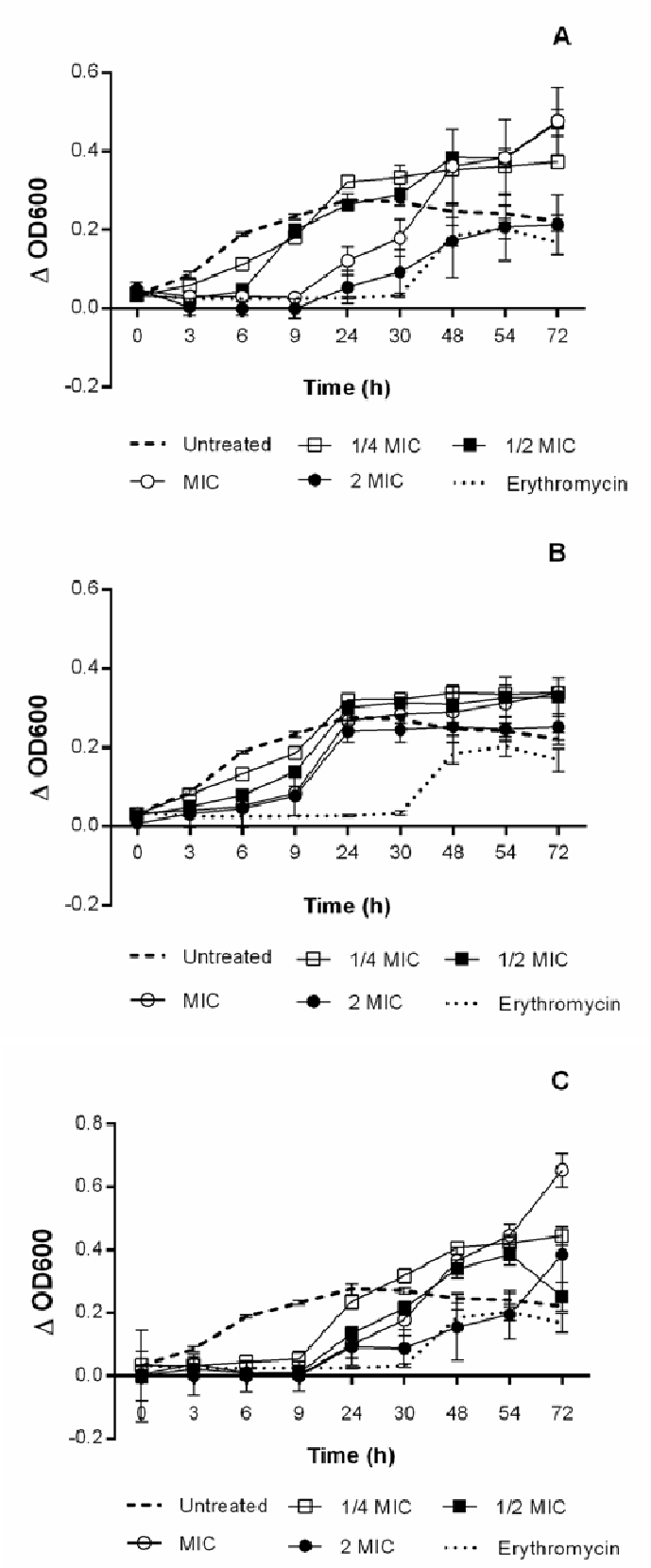


Figure 1 Kinetics of antibacterial activity obtained from *P. aeruginosa* exposed to stem-bark extracts, at four concentrations. (A) *A. colubrina*, (B) *C. leptophloeos*, and (C) *M. urundeuva*.

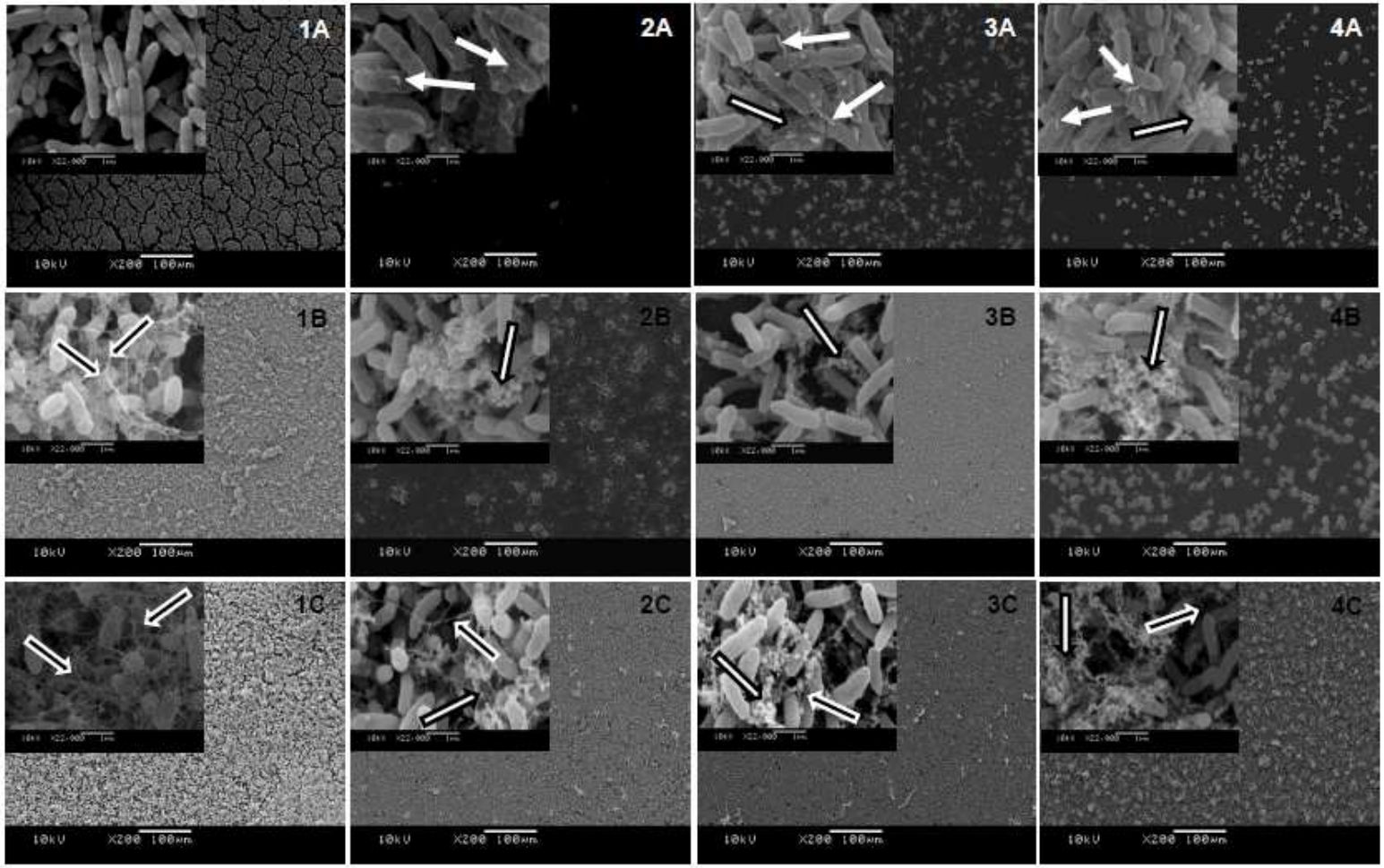


Figure 2 Scanning electron microscopy (SEM) images of biofilms upon Permanox™. Untreated *P. aeruginosa* biofilms (1). Extract-treated biofilms of *A. colubrina* – 2.5 mg/mL (2), *C. leptophloeos* – 1.0 mg/mL (3), and *M. urundeuva* – 4.0 mg/mL (4). Incubation time of 6 h (A), 24 h (B), and 48 h (C). Scale bars: 200X magnification in the images (insert of 22000X magnification). White arrows: morphological changes as stalked nubs; black arrows with white outline: matrix production; white arrows with black outline: extract material.

3.4. Transmission electron microscopy (TEM) and fluorescence microscopy (FM)

On TEM micrographs, untreated cells exhibited an undisturbed cytosol and intact cell envelope (cytoplasmic membrane and cell wall) (Fig. 3A). In contrast, extracts at MIC induced ultrastructural modifications in *P. aeruginosa* cells (Fig. 3B-D). At 15000x magnification, images revealed that all three extracts were able to promote intense vacuolization in several cells (as signaled by black arrows with white outline), although some cells with normal morphology remained present. Regarding *A. colubrina* and *M. urundeuva*, cell deformation and disrupted cell wall could also be observed (Fig. 3B and D, in the inserts). In *C. leptophloeos*-treated cells, in addition to an injured cell wall, we could observe vacuoles dispersed throughout the cytoplasm (black arrows with white outline) and within the periplasm of cells (Fig. 3C, in the inserts). FM images reinforced these results: at MIC, both viable (green) and nonviable (red) cells could be observed, indicating a damaged cytoplasmic membrane in several treated cells (Fig. 4B-D).

3.5. Hemolytic activity

We carried out a simple model to assess injury in human cells and preliminary toxicity of extracts. At MIC, *A. colubrina* and *M. urundeuva* caused 4.3±1.9% and 13.1±0.5% of hemolysis, respectively while *C. leptophloeos* was not hemolytic (0±1.2%). The microscopic analysis showed erythrocyte integrity and absence of erythrocyte aggregation (data not shown).

3.6. Bioguided fractionation

The aqueous extracts were fractionated on Sephadex LH-20. The eluted fractions (F1-F9) were tested for *P. aeruginosa* antibiofilm and antibacterial activities at three concentrations: MIC, 1/2xMIC and 1/4xMIC of the crude extracts, at 6 h of incubation. For all plants, the F7 fraction provided the same activity observed for the crude extracts (Fig. 5). The fraction obtained from *A. colubrina* allowed a biofilm formation of 14, 20, and 32% at 2.5, 1.25, and 0.625 mg/mL, respectively, with growth inhibition in all tested concentrations (Fig. 5A). The same profile was observed for F7 obtained from *M. urundeuva* at all concentrations tested, in which biofilm formation

was limited to 23%, accompanied by absence of growth (Fig. 5C). Regarding F7 from *C. leptophloeos*, at 1.0 mg/mL, bacterial growth was suppressed, with biofilm formation of about 35%. In *C. leptophloeos*, when fraction concentration decreased to 0.25 mg/mL, *P. aeruginosa* began to grow and biofilm inhibition decreased (54% of biofilm formation) (Fig. 5B). The number of CFU/mL after F7 treatments was determined and bacteriostatic effects were observed in all concentrations tested for the three plants evaluated (Fig. 5A-C).

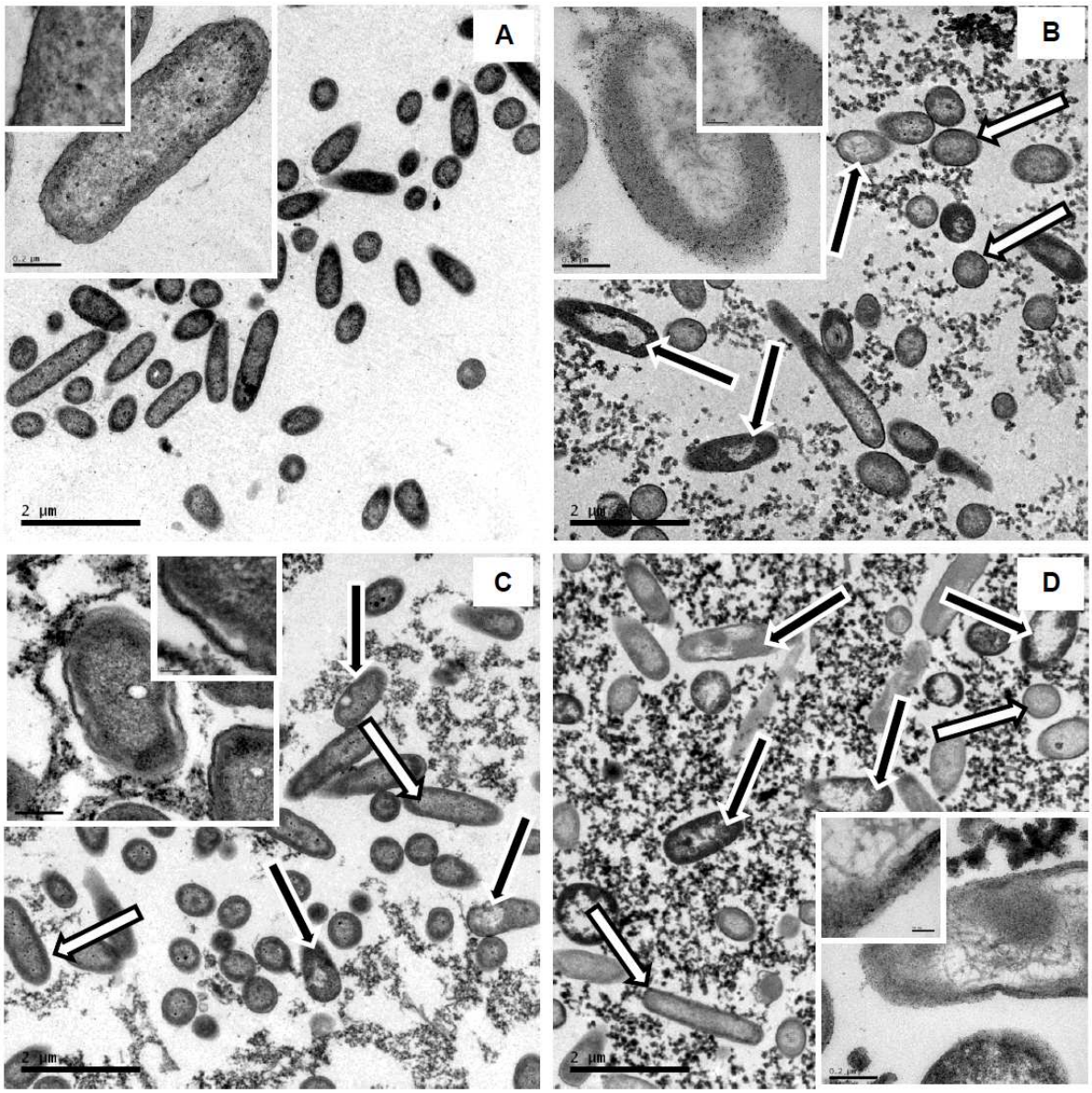


Figure 3 Transmission electron microscopy (TEM). (A) untreated and extract-treated *P. aeruginosa* cells, at minimum inhibitory concentration (MIC), of (B) *A. colubrina*, (C) *C. leptophloeos*, and (D) *M. urundeuva*. Scale bars: 15000X magnification in the images (inserts: 120000X magnification and 500000X magnification). Black arrows with white outline: vacuoles in the cells; white arrows with black outline: normal morphology.

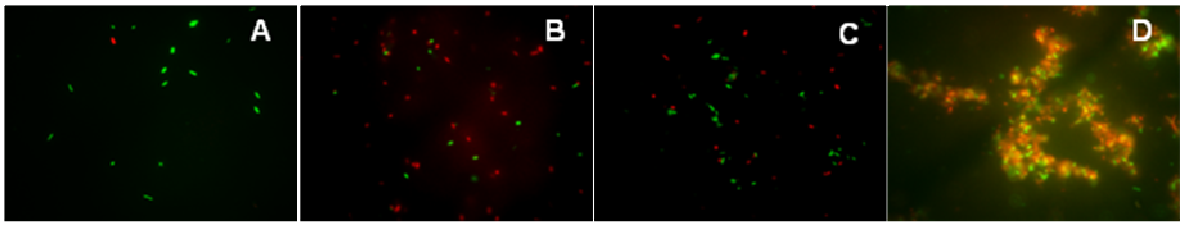


Figure 4 Fluorescence microscopy (FM) images. (A) untreated and extract-treated *P. aeruginosa* cells, at minimum inhibitory concentration (MIC), of (B) *A. colubrina*, (C) *C. leptophloeos*, and (D) *M. urundeuva* (1000X magnification).

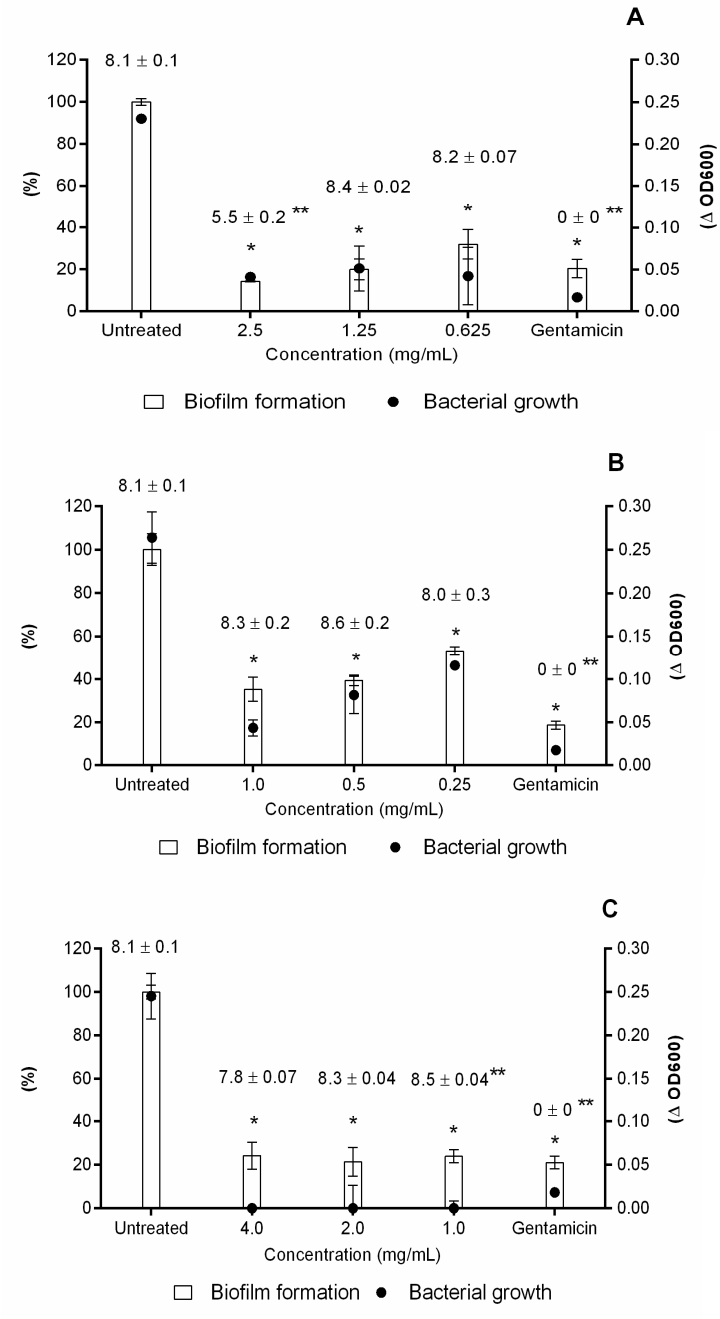


Figure 5 Effect of tannins (F7) on *P. aeruginosa* biofilm formation, bacterial growth, and viability. (A) *A. colubrina*, (B) *C. leptophloeos*, and (C) *M. urundeuva*. Gentamicin was used as a positive control. Numbers on top of bars are the mean values ± standard deviation (SD) of log CFU/mL. *Represents statistical difference in growth and biofilm formation compared to the untreated sample and **represents statistical difference in CFU/mL compared to the untreated sample.

Figure 6 shows the MALDI-TOF spectra of the polymeric tannin mixture of the F7 fractions from *A. colubrina*, *C. leptophloeos*, and *M. urundeuva*. The molecular formulae were obtained from the mass data, and the MS/MS data were important to establish the units of flavan-3-ol or galloyl bound in oligomers. All ions and fragment ions were cationized by sodium. Proanthocyanidins (or condensed tannins) were identified from *A. colubrina* and *C. leptophloeos* fractions, yielding units mainly of profisetinidin and prorobinetinidin types, up to 10 and 13 units, respectively (Table 2, Fig. 7). In addition, *M. urundeuva* fraction was composed of hydrolyzable tannins that were gallic acid derivatives, being esterified to glucose carbohydrate.

The mass spectra of *A. colubrina* fraction showed a series mostly composed of units of profisetinidin (272 *u*) (Table 2), such as the majoritarian polymeric series (857, 1129, 1401, 1673, 1945, 2217, 2489, 2761 *u*) (Fig. 6A and Table 2 – series B). The main differences between the series occurred in lowest-mass oligomers. The MS/MS of *m/z* 857 yielded the fragment ions *m/z* 585 and 295, the latter confirmed profisetinidin as the starter unit and the former was related to procyanidin addition (see supporting information). The fragment ions *m/z* 705 and 433 represented the loss of 152 *u* and confirmed the B-ring substituents of the proposed units. Therefore, the MS/MS data confirmed profisetinidin units through the C-ring fragmentation pathway, such as the fragments yielding the loss of 152 *u* and other units. Other important polymers of this fraction were bound to one or two procyanidins (288 *u*) and/or to one prorobinetinidin (288 *u*) with repeat units of profisetinidin (272 *u*) (Table 2- series C and D, respectively). The polymeric series of *A. colubrina* showed differences in 16 *u*, which represents oxygen (hydroxyl); additionally, all compounds were linked by B type (Fig. 7A).

In the fraction from *C. leptophloeos*, the main polymeric series was yielded with repeat prorobinetinidin units (288 *u*) (Fig. 6B and Table 2 – series A). All these compounds were determined through MS/MS data (see supporting information), and this series of compounds exhibited a B-type linkage. In series B, the peak *m/z* 1159 yielded the fragments *m/z* 1007, 989, 871, 719, 581, 429, and 311 in the MS/MS spectrum, similarly to what was observed for the fragmentation pathway of ion *m/z* 871. The compound of *m/z* 871 was produced by coupling one profisetinidin and two

prorobinetidins, the second unit with an A-type linkage (Fig. 7B). Thus, the compounds of this polymeric series have a difference of 2 *u*. Series C (Table 2) was formed by repeat units of prorobinetinidin and only one prodelphinidin, which was confirmed by MS/MS data, with the loss of 168 *u* confirming this unit.

The fraction from *M. urundeuva* was also analyzed by MALDI-TOF, and hydrolyzable tannins were observed in its composition. The MS spectrum showed peaks with 152 *u* increment (Fig. 6C) and MS/MS spectra showed consecutive losses of gallic acid units attached to glucose, as observed in a recent study [9], confirming the presence of gallotannins in this fraction. However, the structural elucidation of gallotannin series was not possible, since the MS/MS data were inconclusive.

4. Discussion

The ability to impair bacterial adhesion represents an ideal strategy to combat bacterial pathogenesis, given its importance in the early stages of the infectious process. In addition, bacterial adhesion blockade is suitable as a prophylactic intervention to prevent infection [10]. Therefore, the use of natural agents that can successfully inhibit cell attachment is a promising tool for reduction of bacterial colonization on several surfaces [11]. In recent years, studies have reported anti-adhesive or antibiofilm activities of compounds, which are related to their antimicrobial properties [12-16].

Plants produce many compounds that are biologically active, either as part of their normal program of growth and development or in response to pathogen attack or stress. Traditionally, *A. colubrina*, *C. leptophloeos* and *M. urundeuva* have been used by communities in the Caatinga to treat infectious diseases, such as cough, bronchitis, influenza, urinary/liver diseases, ulcerative external lesions, and ovarian inflammation [17,18].

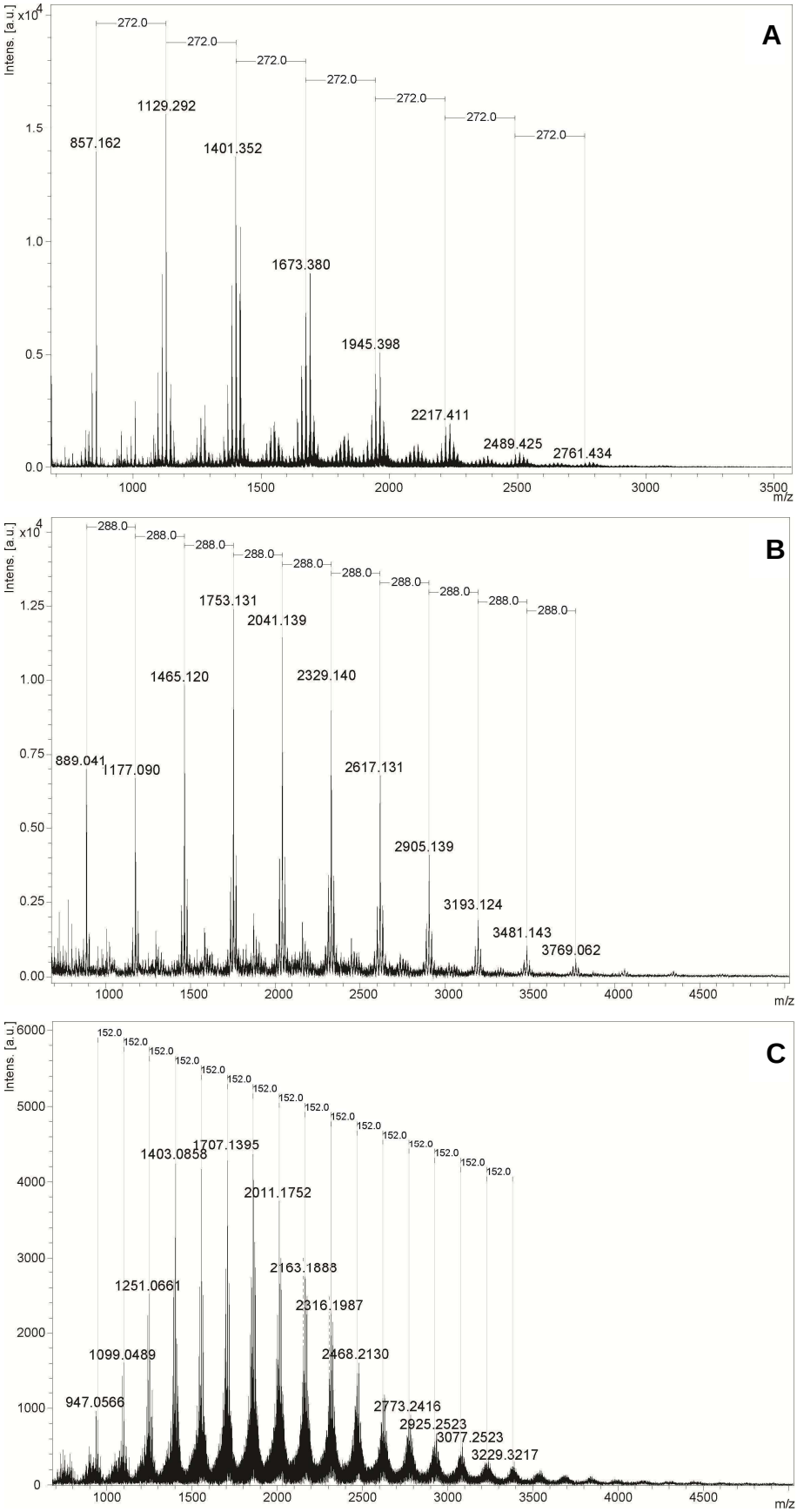
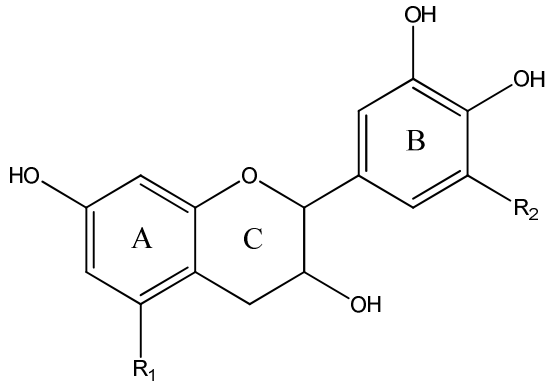


Figure 6 Mass spectra (positive ion mode) of F7 fractions. (A) *A. colubrina*, (B) *C. leptophloeos*, and (C) *M. urundeuva*. In the images, the majoritarian series of each fraction is highlighted.

Table 2. Distribution of polyflavanoid oligomers by MALDI-TOF for tannin F7 fractions of *A. colubrina* and *C. leptophloeos*.

Fraction F7		$[M+Na]^+$ **(error)**	**MF**	**Compound**
A. colubrina	Series A	841.2139 (4.3 ppm)	$C_{45}H_{38}O_{15}$	3 PFI
		1113.2831 (3.9 ppm)	$C_{60}H_{50}O_{20}$	4 PFI
		1385.3545 (5.2 ppm)	$C_{75}H_{62}O_{25}$	5 PFI
		1657.4131 (1.6 ppm)	$C_{90}H_{74}O_{30}$	6 PFI
		1929.4889 (2.4 ppm)	$C_{105}H_{86}O_{35}$	7 PFI
		2201.5526 (6.2 ppm)	$C_{120}H_{98}O_{40}$	8 PFI
		2473.6316 (4.2 ppm)	$C_{135}H_{110}O_{45}$	9 PFI
	Series B	857.2089 (4.3 ppm)	$C_{45}H_{38}O_{16}$	PFI – PCY - PFI
		1129.2660 (6.0 ppm)	$C_{60}H_{50}O_{21}$	PFI - PCY - 2 PFI
		1401.3444 (1.6 ppm)	$C_{75}H_{62}O_{26}$	PFI - PCY - 3 PFI
		1673.4018 (5.3 ppm)	$C_{90}H_{74}O_{31}$	PFI - PCY - 4 PFI
		1945.4764 (1.4 ppm)	$C_{105}H_{86}O_{36}$	PFI - PCY - 5 PFI
		2217.5378 (4.4 ppm)	$C_{120}H_{98}O_{41}$	PFI - PCY - 6 PFI
		2489.6035 (5.0 ppm)	$C_{135}H_{110}O_{46}$	PFI - PCY - 7 PFI
		2761.6966 (4.4 ppm)	$C_{150}H_{122}O_{51}$	PFI - PCY - 8 PFI
	Series C	1417.3433 (4.4 ppm)	$C_{75}H_{62}O_{27}$	PFI - PCY - 2 PFI - PCY
		1689.3964 (5.4 ppm)	$C_{90}H_{74}O_{32}$	PFI - PCY - 2 PFI - PCY - PFI
		1961.4630 (5.6 ppm)	$C_{105}H_{86}O_{37}$	PFI - PCY - 2 PFI - PCY - 2 PFI
		2233.5266 (7.1 ppm)	$C_{120}H_{98}O_{42}$	PFI - PCY - 2 PFI - PCY - 3 PFI
		2505.6230 (4.8 ppm)	$C_{135}H_{110}O_{47}$	PFI - PCY - 2 PFI - PCY - 4 PFI
	Series D	873.2014 (1.5 ppm)	$C_{45}H_{38}O_{17}$	PFI - PCY - PRO
		1145.2718 (2.8 ppm)	$C_{60}H_{50}O_{22}$	PFI - PCY - PRO - PFI
C. leptophloeos	Series A	889.1903 (5.3 ppm)	$C_{45}H_{38}O_{18}$	3 PRO
		1177.2547 (3.2 ppm)	$C_{60}H_{50}O_{24}$	4 PRO
		1465.3119 (6.8 ppm)	$C_{75}H_{62}O_{30}$	5 PRO
		1753.3925 (4.2 ppm)	$C_{90}H_{74}O_{36}$	6 PRO
		2041.4558 (3.5 ppm)	$C_{105}H_{86}O_{42}$	7 PRO
		2329.5124 (0.2 ppm)	$C_{120}H_{98}O_{48}$	8 PRO
		2617.5827 (2.8 ppm)	$C_{135}H_{110}O_{54}$	9 PRO
		2905.6428 (1.4 ppm)	$C_{150}H_{122}O_{60}$	10 PRO
		3193.7053 (0.1 ppm)	$C_{165}H_{134}O_{66}$	11 PRO
		3481.7596 (1.7 ppm)	$C_{180}H_{146}O_{72}$	12 PRO
		3769.8211 (2.1 ppm)	$C_{195}H_{158}O_{78}$	13 PRO
	Series B	871.1876 (3.6 ppm)	$C_{45}H_{36}O_{17}$	PFI - PRO - PRO*
		1159.2381 (8.4 ppm)	$C_{60}H_{48}O_{23}$	PFI - PRO - 2 PRO*
		1447.3182 (4.8 ppm)	$C_{75}H_{60}O_{29}$	PFI - PRO - 3 PRO*
		1735.3695 (3.0 ppm)	$C_{90}H_{72}O_{35}$	PFI - PRO - 4 PRO*
		2023.4327 (2.6 ppm)	$C_{105}H_{84}O_{41}$	PFI - PRO - 5 PRO*
		2311.5065 (2.2 ppm)	$C_{120}H_{96}O_{47}$	PFI - PRO - 6 PRO*
		2599.5567 (3.1 ppm)	$C_{135}H_{108}O_{53}$	PFI - PRO - 7 PRO*
		2887.6290 (0.3 ppm)	$C_{150}H_{120}O_{59}$	PFI - PRO - 8 PRO*
		3175.7055 (4.4 ppm)	$C_{165}H_{132}O_{65}$	PFI - PRO - 9 PRO*
		3463.7^{NO}	$C_{180}H_{144}O_{71}$	PFI - PRO - 10 PRO*
		3751.8^{NO}	$C_{195}H_{156}O_{77}$	PFI - PRO - 11 PRO*
	Series C	889.1903 (5.3 ppm)	$C_{45}H_{38}O_{18}$	3 PRO
		1193.2623 (7.5 ppm)	$C_{60}H_{50}O_{25}$	3 PRO - PDE
		1481.3236 (4.6 ppm)	$C_{75}H_{62}O_{31}$	3 PRO - PDE - PRO
		1769.3789 (0.7 ppm)	$C_{90}H_{74}O_{37}$	3 PRO - PDE - 2 PRO
		2057.4309 (6.1 ppm)	$C_{105}H_{86}O_{43}$	3 PRO - PDE - 3 PRO
		2345.4921 (6.3 ppm)	$C_{120}H_{98}O_{49}$	3 PRO - PDE - 4 PRO
		2633.5869 (6.3 ppm)	$C_{135}H_{110}O_{55}$	3 PRO - PDE - 5 PRO
		2921. 6171(5.3 ppm)	$C_{150}H_{122}O_{61}$	3 PRO - PDE - 6 PRO
		3209.6808 (5.1 ppm)	$C_{165}H_{134}O_{67}$	3 PRO - PDE - 7 PRO
		3497.8^{NO}	$C_{180}H_{146}O_{73}$	3 PRO - PDE - 8 PRO
		3785.8^{NO}	$C_{195}H_{158}O_{79}$	3 PRO - PDE - 9 PRO

MF: molecular formula, PFI: Profisetinidin, PCY: Procyanidin, PRO: Prorobinetinidin, PDE: Prodelphinidin. * A-type, NO: not observed with internal calibrant (low intensity).



R_1	R_2	
H	H	Profisetinidin (PFI) – 274 *u*
OH	H	Procyanidin (PCY) – 290 *u*
H	OH	Prorobinetinidin (PRO) – 290 *u*
OH	OH	Prodelphinidin (PDE) – 306 *u*

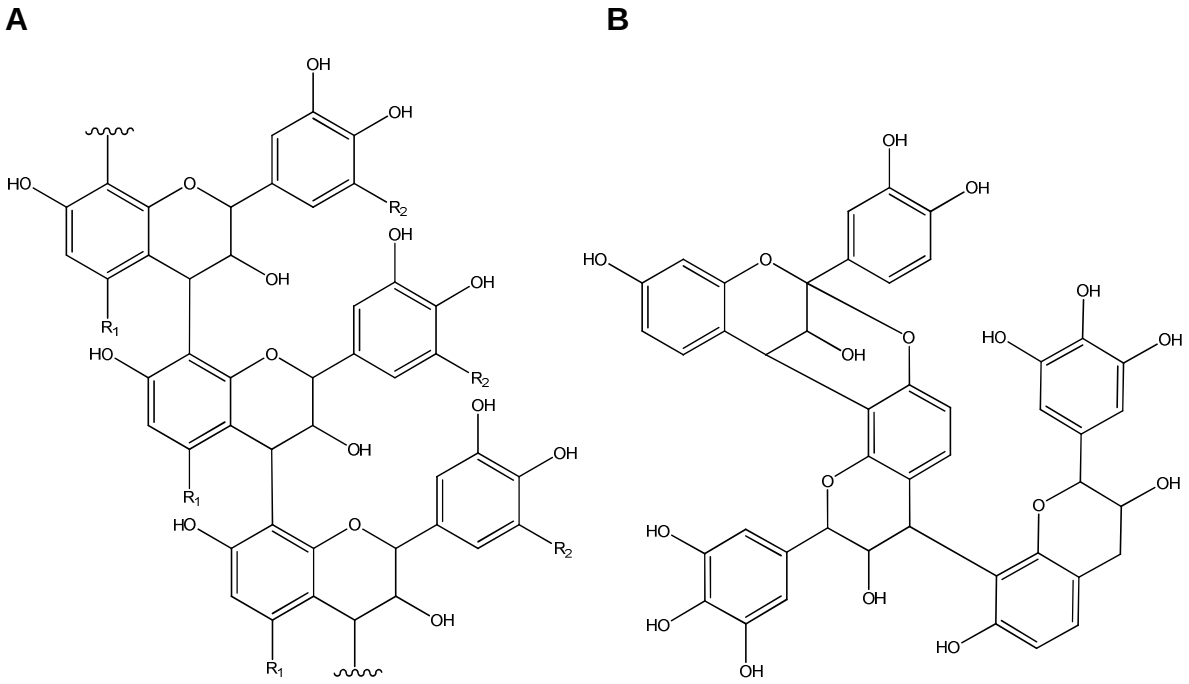


Figure 7 Polymeric structure of the condensed tannin purified from the plants studied. Typical linear condensed tannin with a B-type linkage (A) and an A-type linkage (*m/z* 871 $[M+Na]^+$) identified from *C. leptophloeos* (B). Profisetinidin (PFI), procyanidin (PCY), prorobinetinidin (PRO), and prodelphinidin (PDE).

In this study, we demonstrated that highly complex structures (Fig. 6 and 7 and Table 2) of proanthocyanidins (mostly composed of profisetinidin in *A. colubrina* and prorobinetinidin in *C. leptophloeos*) and hydrolyzable tannins (consisting of gallic acid units in *M. urundeuva*) induced *P. aeruginosa* damage, providing bacteriostatic and anti-adhesive effects. Bacterial growth kinetic experiments revealed that inhibition of bacterial growth persisted up to 9 h post-incubation (Fig. 1). The bacteriostatic property was confirmed by cell counting (CFU/mL) after F7 tannin and extract treatments (Fig. 5 and Table 1) and by recovering bacterial growth of extract- and erythromycin-treated cells, as indicated by OD_{600} measurements in the kinetic experiment (Fig. 1). At MIC, the extracts were also able to inhibit bacterial adhesion and prevent biofilm formation on the polystyrene surface for 6 and 24 h after incubation. However, this activity was lost after 48 h of incubation (Table 1). SEM was employed to improve understanding of the qualitative impact of the extracts upon the behavior of bacteria. We could observe that 6 h-treated cells presented morphological changes, such as stalked nubs (white arrows with black outline in Fig. 2), a phenotype that is also induced by amikacin and oxytetracycline [19]. This finding supports the hypothesis that morphological changes were due to the mechanisms of bacteria involved in protection against aggression from extracts. At 24 and 48 h of incubation (the latter characterizing the mature stage of biofilm development), untreated *P. aeruginosa* was enclosed by extracellular matrix, while just after 48 h of treatment cells were surrounded by a very discrete matrix (Fig. 2). Flemming and Wingender [20] demonstrated that matrix is essential for biofilm formation, which allows a lifestyle that is entirely different from the planktonic state, and concluded that there is no biofilm without a matrix. Based on these observations, we may suggest that *P. aeruginosa* agglomerates as visualized by SEM at 24 and 48 h of treatment could not be considered biofilm structures.

The level of bacterial membrane dysfunction could potentially result in cell death and may explain the rapid loss of viability observed in the kinetic experiments. Although TEM and FM showed no viable cells, these analyses also indicated the presence of cells without morphological alterations and with an intact membrane. These findings are consistent with Pankey and Sabath report [21], who highlighted that

most agents characterized as bacteriostatic agents are able to kill some bacteria – often more than 90-99% of the inoculum, but this is not sufficient (>99.9%) to characterize them as bactericidal agents. Although *in vitro* bacteriostatic/bactericidal data may provide information on the potential action of antimicrobial agents, this is only one of many factors required to predict a favorable clinical outcome. Bacteriostatic agents have been effectively used in the treatment of endocarditis, meningitis, and osteomyelitis – indications that are often considered to require bactericidal activity [21]. Additionally, the bacterial membrane may be compromised during antimicrobial treatments, such as due to exposure to a bacteriostatic agent. The ultrastructural analysis of *P. aeruginosa* (Fig. 3) showed that extract-exposed cells presented excess vacuoles and a disrupted cell wall, when compared to untreated samples. FM results (Fig. 4) suggest that the extracts have anti-membrane activity, resulting in the disturbance of membrane structure in a large amount of cells, while displaying absence or very low toxicity against human erythrocytes, which corroborates the idea of a selective action of these tannins upon *P. aeruginosa* membrane.

The programmed death of some damaged cells may be beneficial to a multicellular bacterial community [22]. Thus, the occurrence of both viable and dying cells after extracts treatment could be understood as a suicide mission that would contribute to the maintenance of a population. There is strong evidence that genomic DNA released during *P. aeruginosa* lysis is a structural component of the biofilm matrix, supporting the idea that cell lysis contributes to the stability of the biofilm structure [23]. These findings are in agreement with our data, which show the development of *P. aeruginosa* clusters at 48 h of incubation, at the same time point when the potential of extracts to impair bacterial adhesion is decreased. The immune system is capable of eliminating pathogens that would otherwise persist in the presence of bacteriostatic agents, although the elimination of persister cells from biofilms by the immune system has not been specifically studied yet [22].

As a Gram-negative bacterium, *P. aeruginosa* has a cell wall consisting of a peptidoglycan layer and an additional outer membrane [24]. It should be noted that, in order to reach the cell membrane, tannins must cross the bacterial cell wall. Scalbert [25] has suggested that the cell wall probably fixes part of tannins, contributing to

increase their MIC values. This observation is in agreement with our results, since MIC values for the extracts ranged from 1.0 to 4.0 mg/mL (Table 1) and for purified tannins (F7) the value was 1.0 mg/mL for *C. leptophloeos* and at least 0.625 mg/mL and 1.0 mg/mL for *A. colubrina* and *M. urundeuva*, respectively (Fig. 5).

This study represents an unprecedented report on phytochemical analysis, identifying tannins from three important plants used as folk medicine in Brazil: *A. colubrina*, *C. leptophloeos*, and *M. urundeuva*. It is also the first work to elucidate the tannin structure of plants from the genera *Anadenanthera* and *Commiphora*. Considering the origin of the plant material (stem barks), the extraction method (aqueous maceration) and the data about the tannin content of these species [26], the achievement of tannins by bioguided fractionation was as expected. It is worth mentioning that, as previously reported by Almeida et al [27], Caatinga plants are exposed to high solar radiation in a semiarid environment, which favors the synthesis of phenolic compounds, reinforcing the medicinal potential of plants from this biome.

In summary, we propose that tannins are able to inhibit biofilm formation by damaging the bacterial membrane and hindering matrix production, displaying bacteriostatic properties. Therefore, biofilm formation is prevented during minimal bacterial growth; when cells start to grow, they are able to attach to the surface and develop matrix-deficient cell clusters. The bacteriostatic activity against *P. aeruginosa* observed in this study provides a scientific basis which may justify some uses of these plants in traditional medicine. To the best of our knowledge, this is the first study to show the modulation of *P. aeruginosa* biofilm formation by the herein described bacteriostatic tannins and the first to report the identification of tannins from *A. colubrina* and *C. leptophloeos*. This fact highlights the importance of this widespread and abundant class of secondary metabolites in higher plants against one of the most challenging issues in the hospital setting: biofilm resilience.

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Authors' contributions

Conceived and designed the experiments: DST DBS KRZ MWA RBG NPL AJM. Performed the experiments: DST DBS KRZ MWA RBG. Analyzed the data: DST DBS KRZ MWA RBG. Contributed with reagents/materials/analysis tools: MVS NPL AJM. Drafted the manuscript: DST DBS RBG AJM. Revised the paper critically for important intellectual content: KRZ NPL AJM.

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Supporting Information

Table S1 Yield (mg/% w/w) of each fraction obtained from Sephadex column in relation to the powdered plant material.

	A. colubrina	*C. leptophloeos*	*M. urundeuva*
F1 (water)	3.60	5.96	8.17
F2 (30% methanol)	0.33	0.14	0.32
F3 (50% methanol)	1.57	0.44	0.52
F4 (methanol)	5.26	1.33	2.61
F5 (10% acetone)	0.3	0.09	0.1
F6 (30% acetone)	0.07	0.01	0.03
F7 (50% acetone)	0.22	0.29	0.37
F8 (70% acetone)	0.04	0.10	0.07
F9 (acetone)	0.33	0.24	0.01

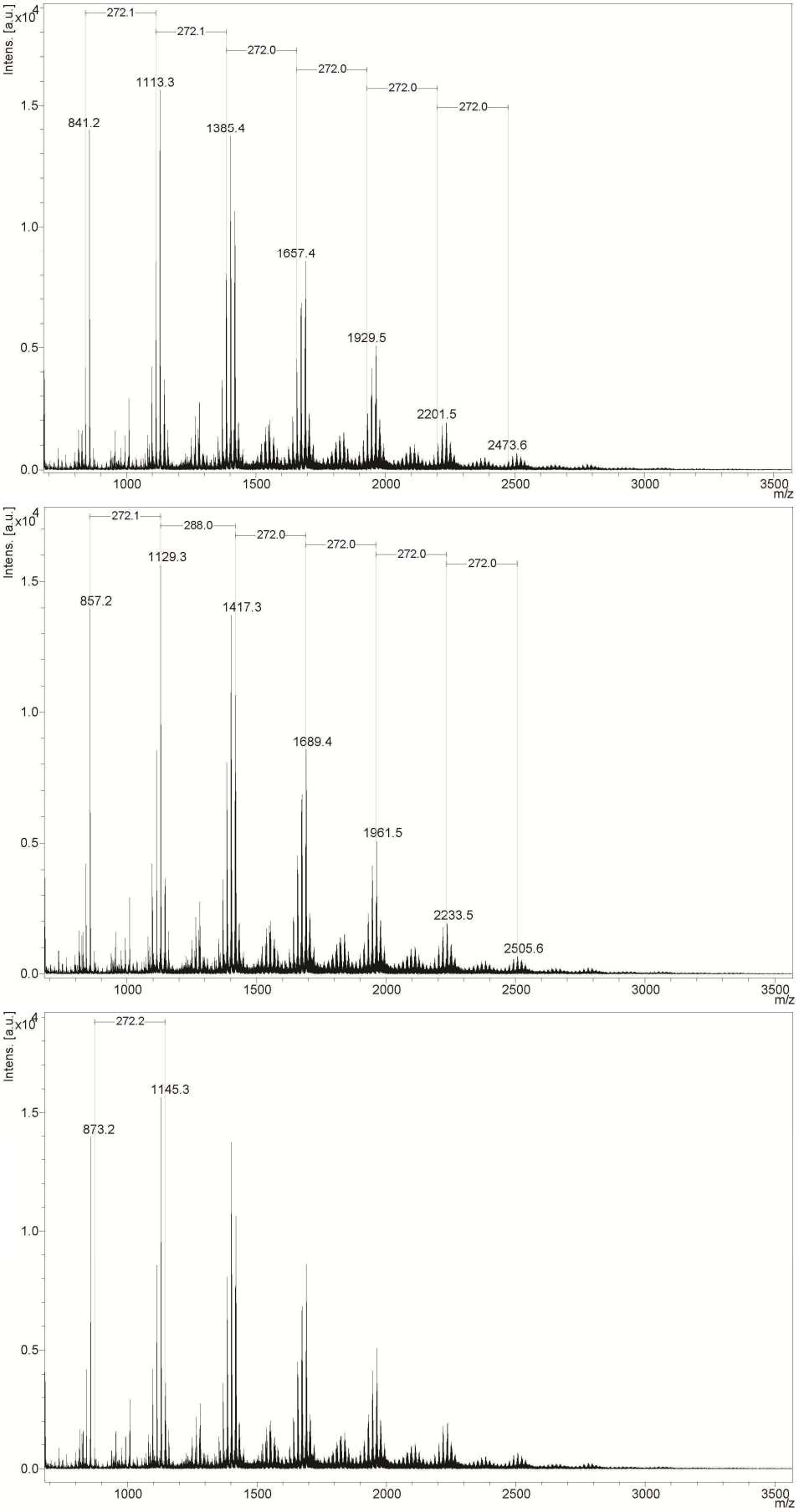

Figure S1 Mass spectra (positive ionization mode) of the fraction obtained from *A. colubrina*.

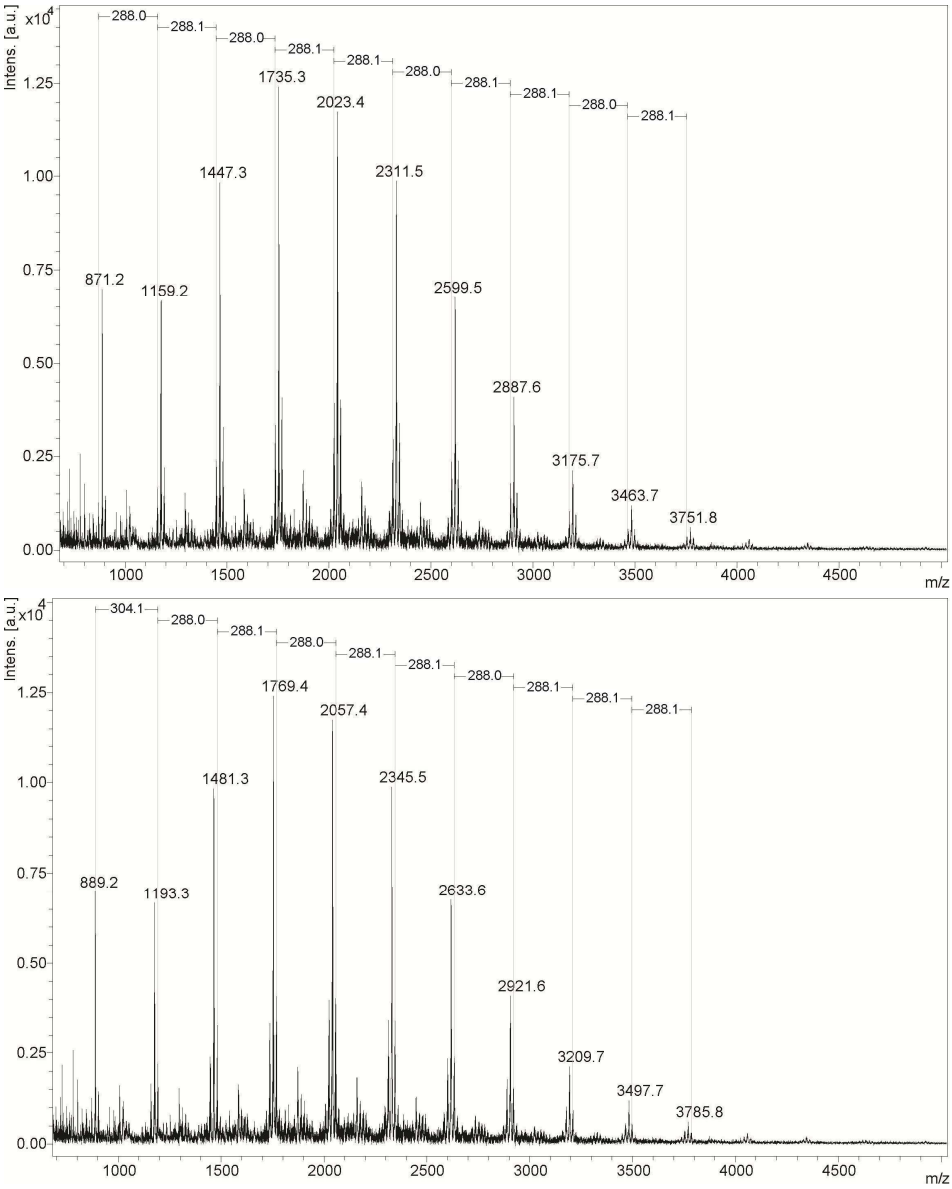

Figure S2 Mass spectra (positive ionization mode) of the fraction obtained from *C. leptophloeos*.

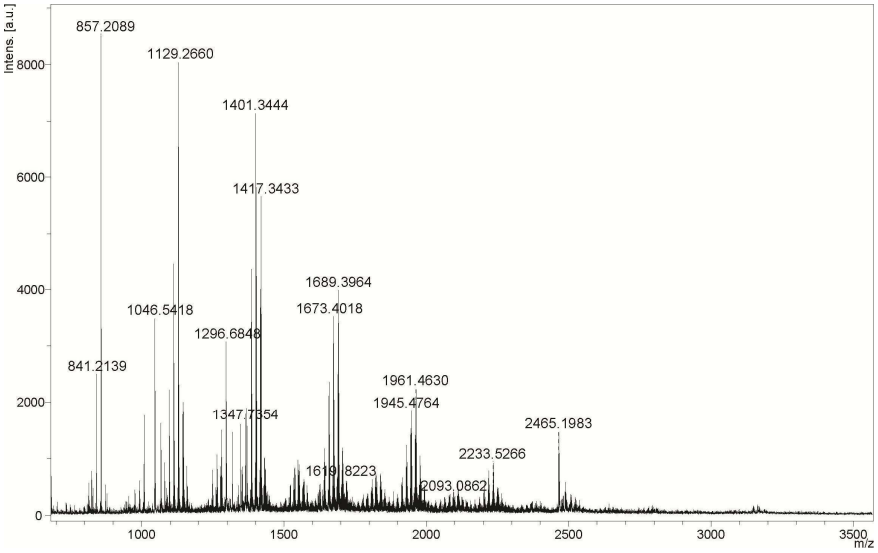

Figure S3 Mass spectra (positive ionization mode) of the fraction obtained from *A. colubrina* with internal calibrant (peptide calibration standard II).

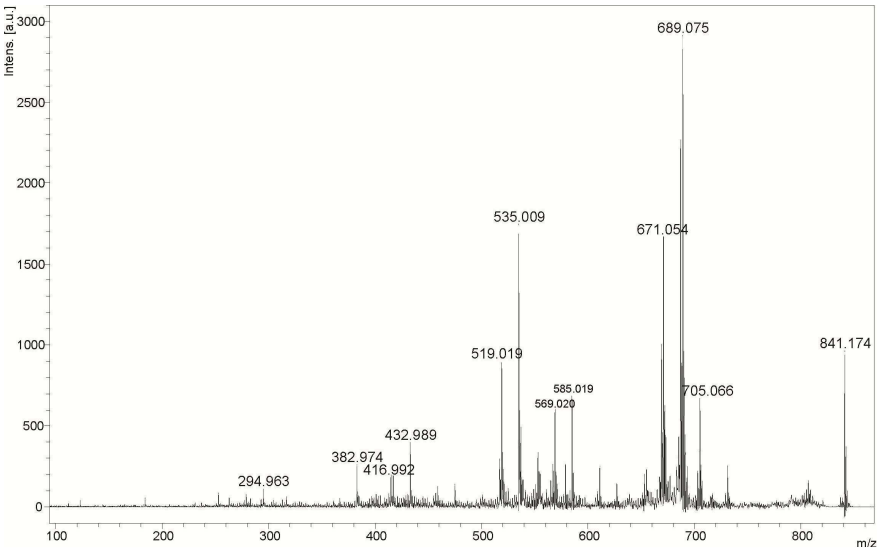

Figure S4 MS/MS spectrum of ion *m/z* 841 from *A. colubrina*.

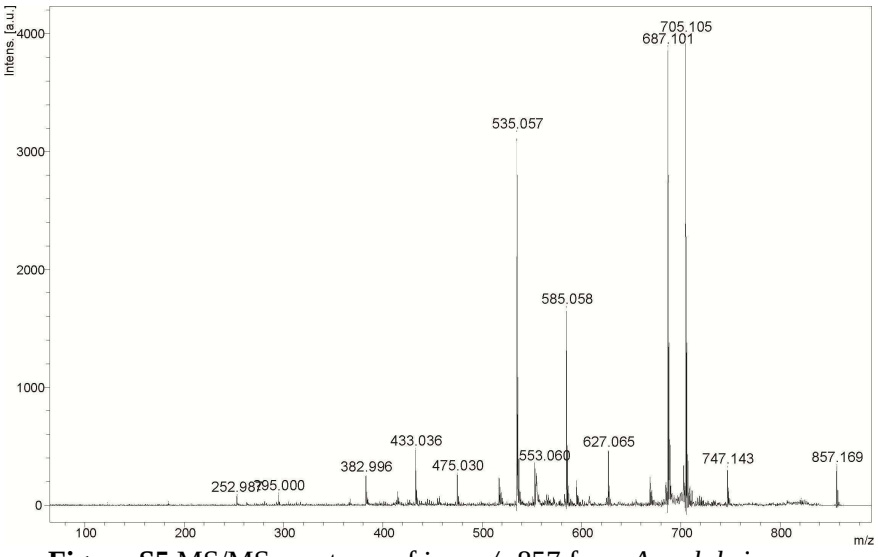

Figure S5 MS/MS spectrum of ion *m/z* 857 from *A. colubrina*.

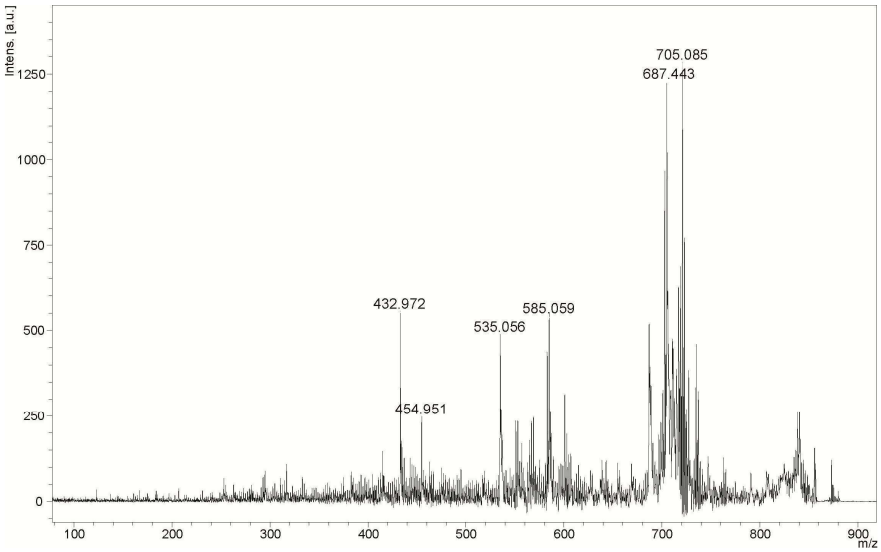

Figure S6 MS/MS spectrum of ion *m/z* 873 from *A. colubrina*.

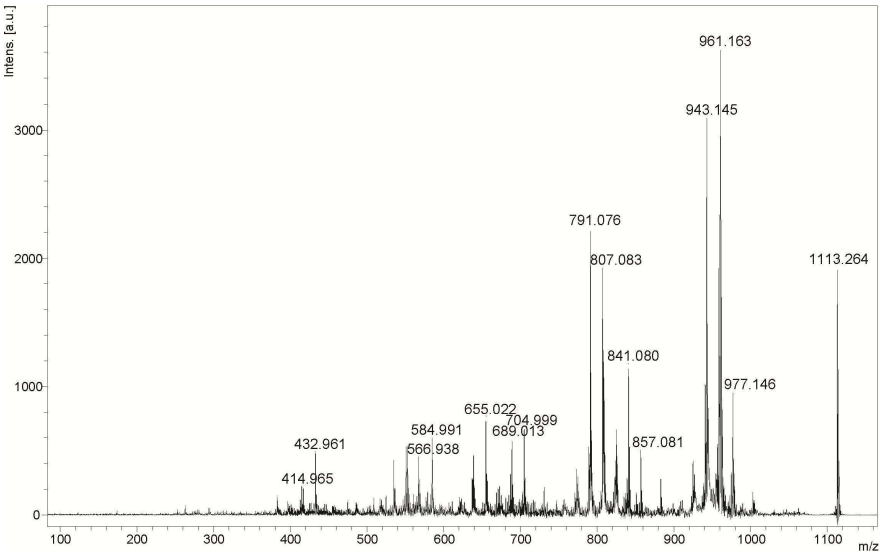

Figure S7 MS/MS spectrum of ion *m/z* 1113 from *A. colubrina*.

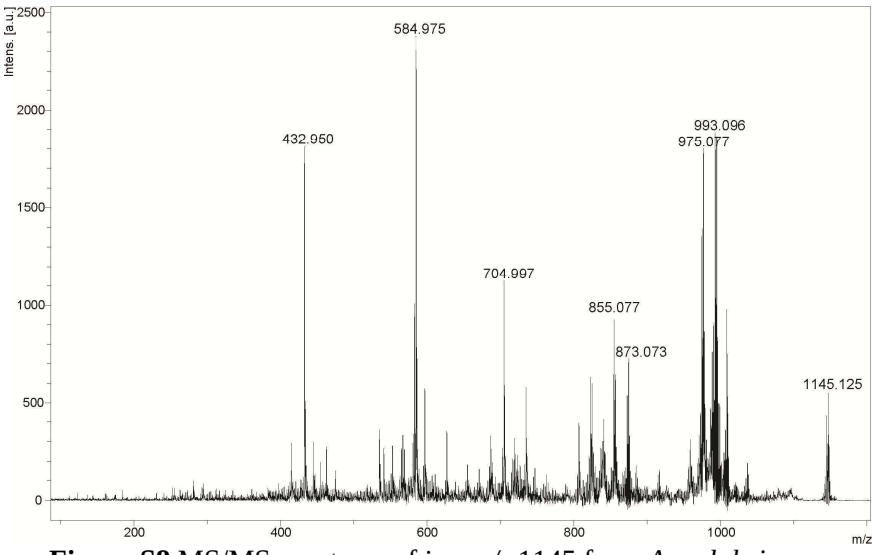

Figure S8 MS/MS spectrum of ion *m/z* 1145 from *A. colubrina*.

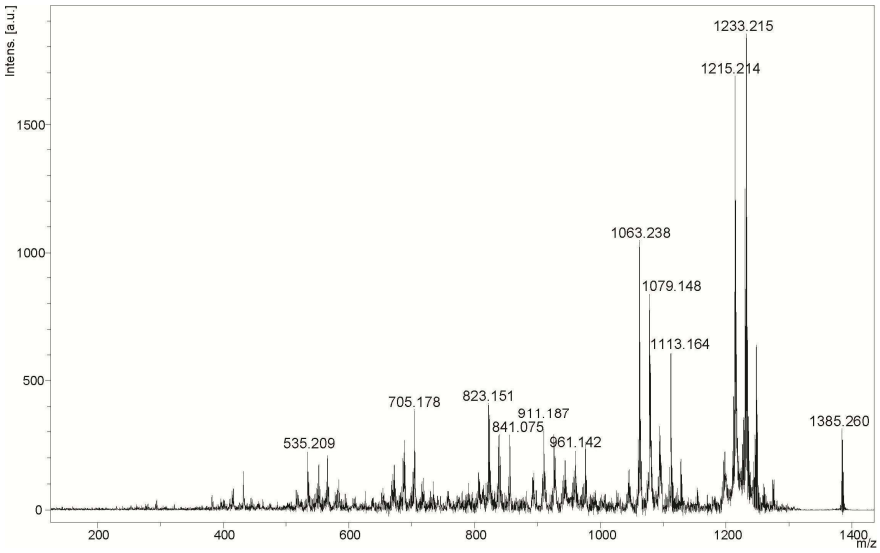

Figure S9 MS/MS spectrum of ion *m/z* 1385 from *A.colubrina*.

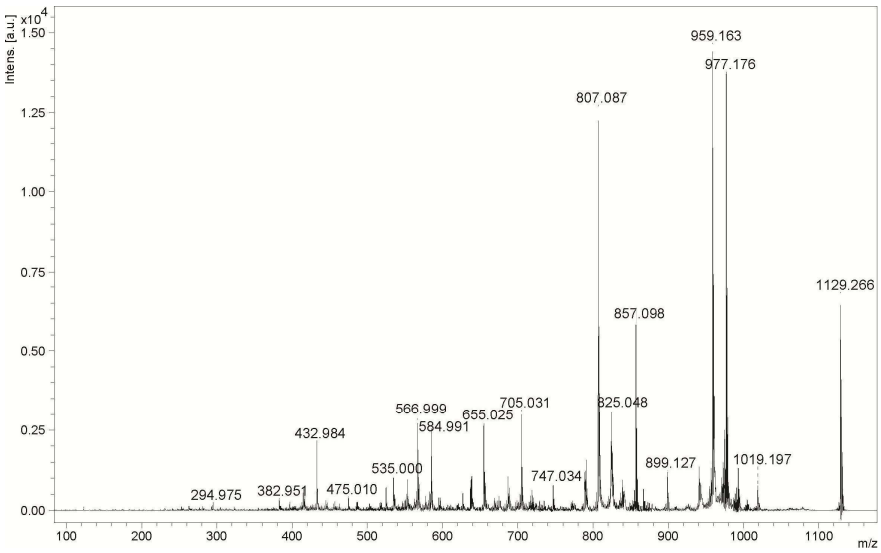

Figure S10 MS/MS spectrum of ion *m/z* 1129 from *A. colubrina*.

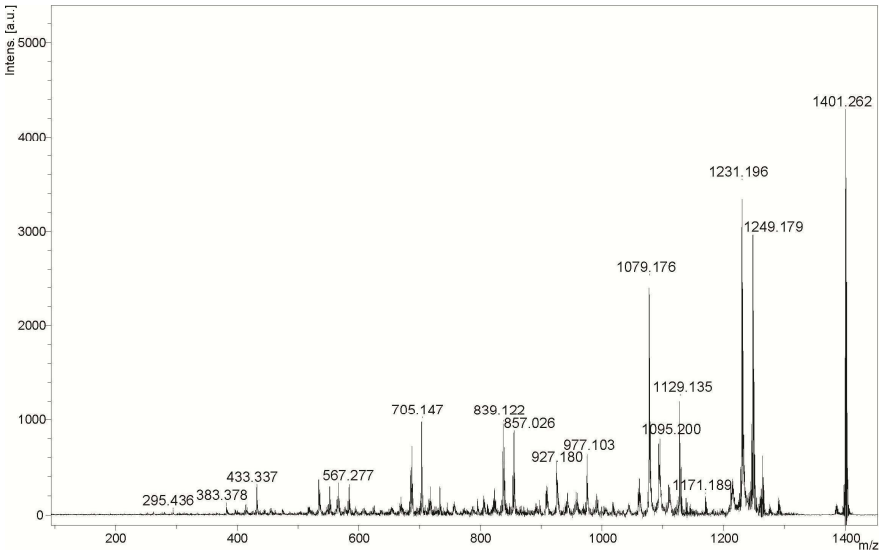

Figure S11 MS/MS spectrum of ion *m/z* 1401 from *A. colubrina*.

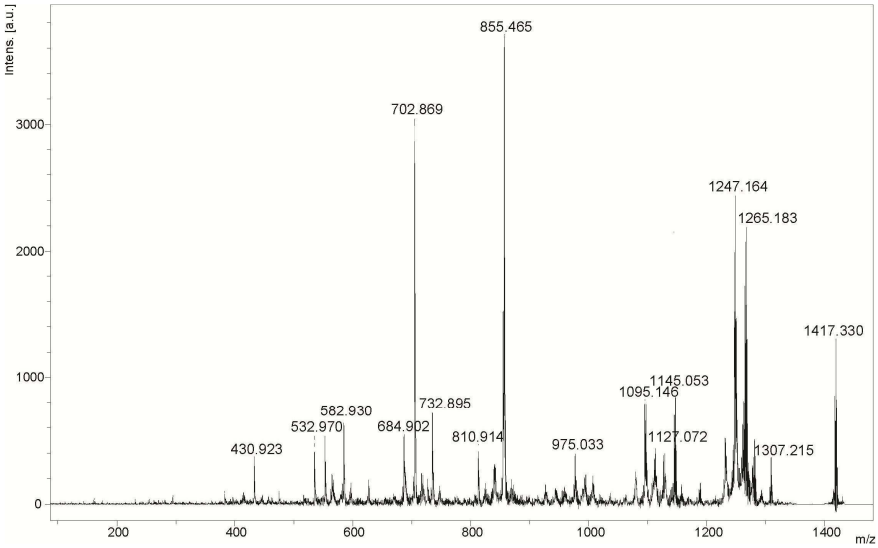

Figure S12 MS/MS spectrum of ion *m/z* 1417 from *A. colubrina*.

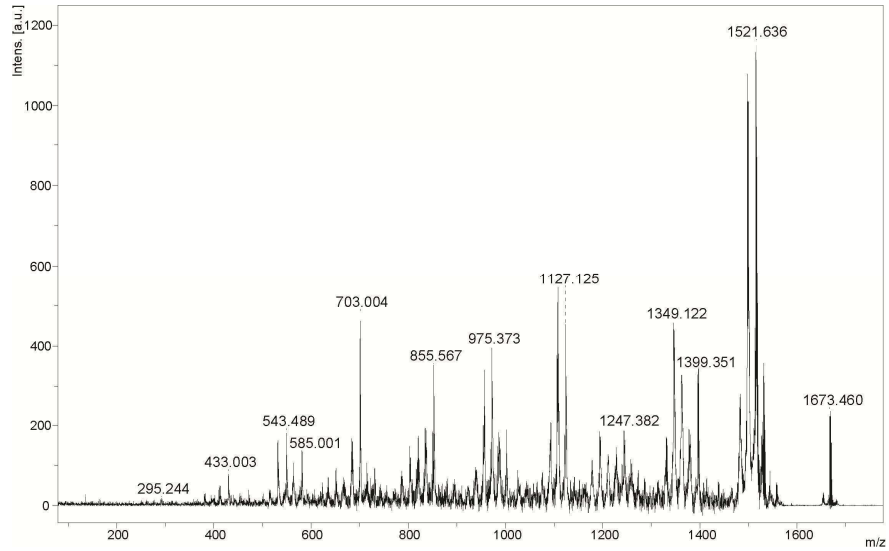

Figure S13 MS/MS spectrum of ion *m/z* 1673 from *A. colubrina*.

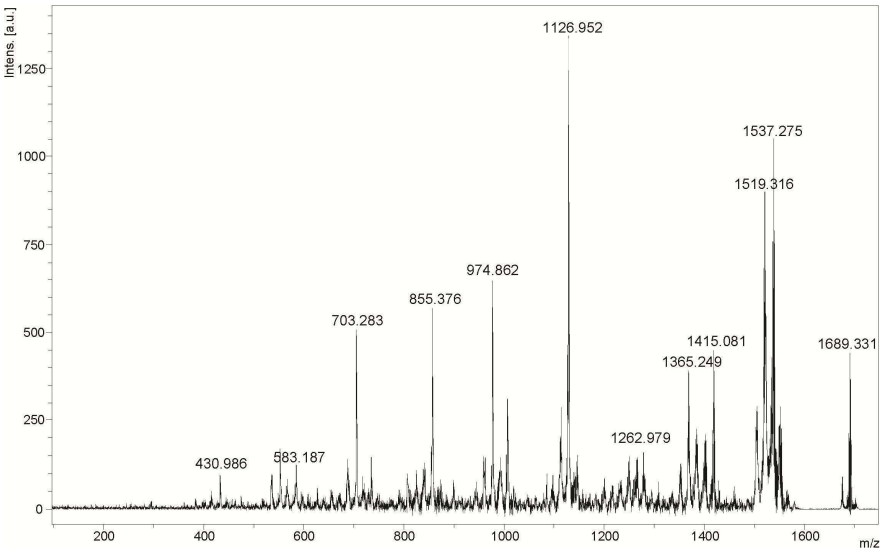

Figure S14 MS/MS spectrum of ion *m/z* 1689 from *A. colubrina*.

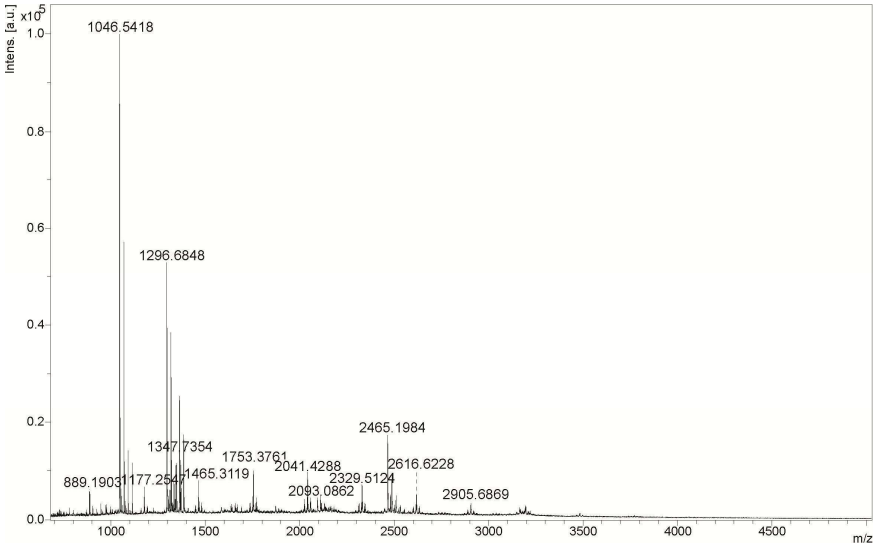

Figure S15 Mass spectra (positive ionization mode) of the fraction obtained from *C. leptophloeos* with internal calibrant (peptide calibration standard II).

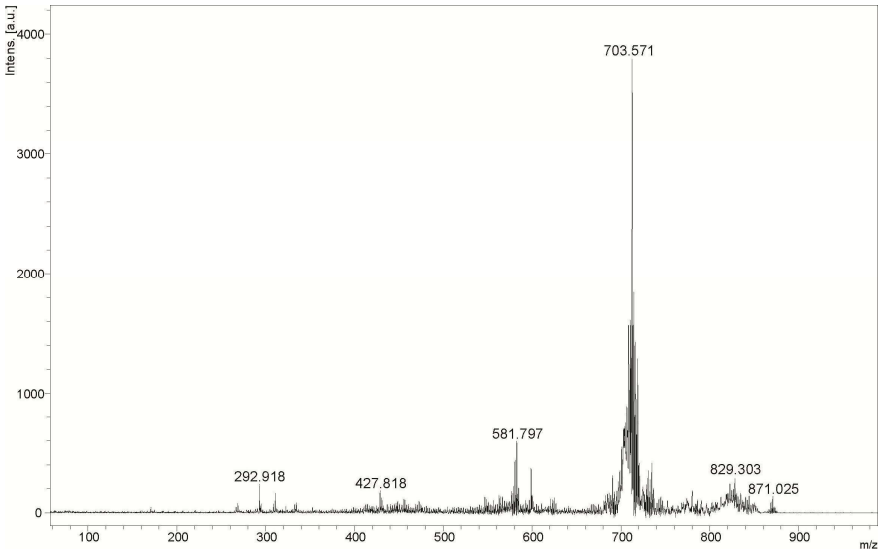

Figure S16 MS/MS spectrum of ion *m/z* 871 from *C. leptophloeos*.

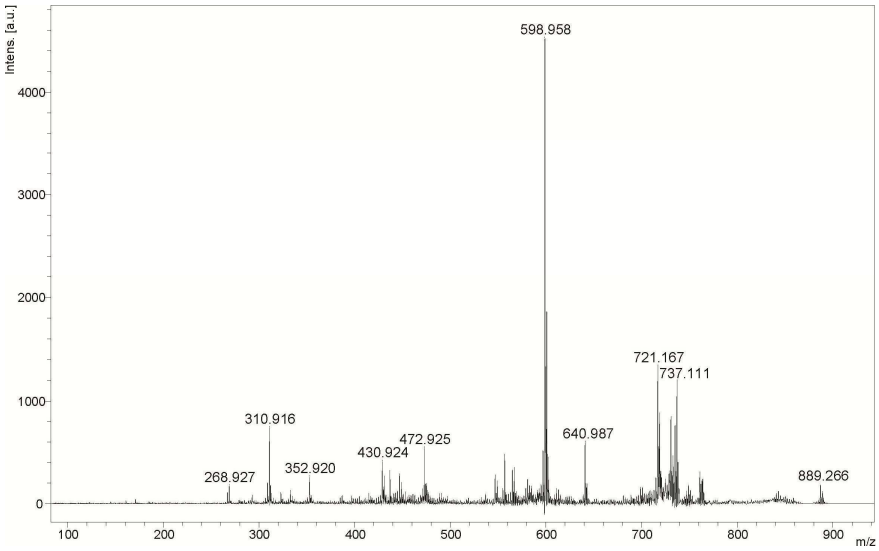

Figure S17 MS/MS spectrum of ion *m/z* 889 from *C. leptophloeos*.

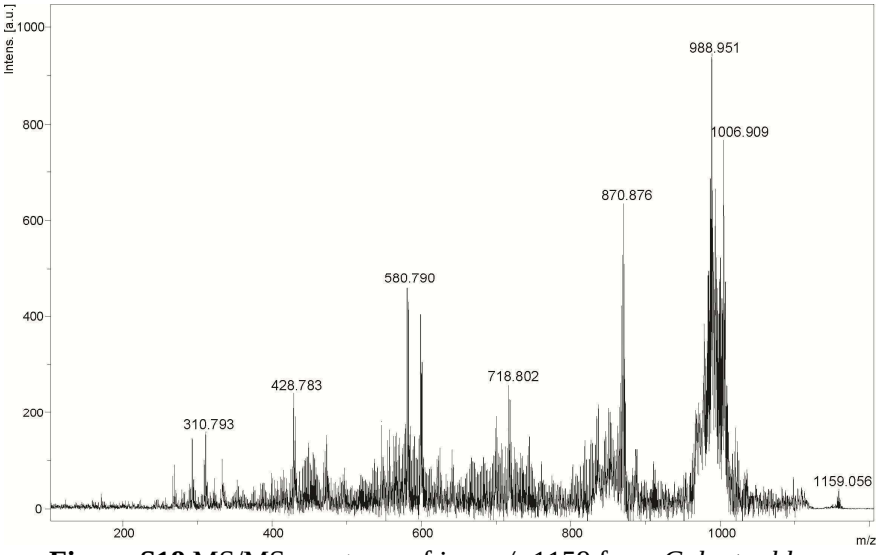

Figure S18 MS/MS spectrum of ion *m/z* 1159 from *C. leptophloeos*.

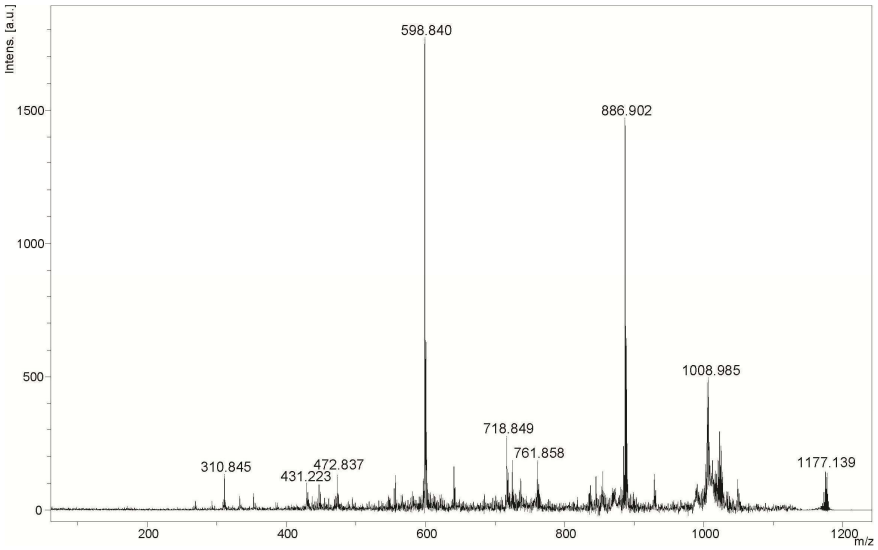

Figure S19 MS/MS spectrum of ion *m/z* 1177 from *C. leptophloeos*.

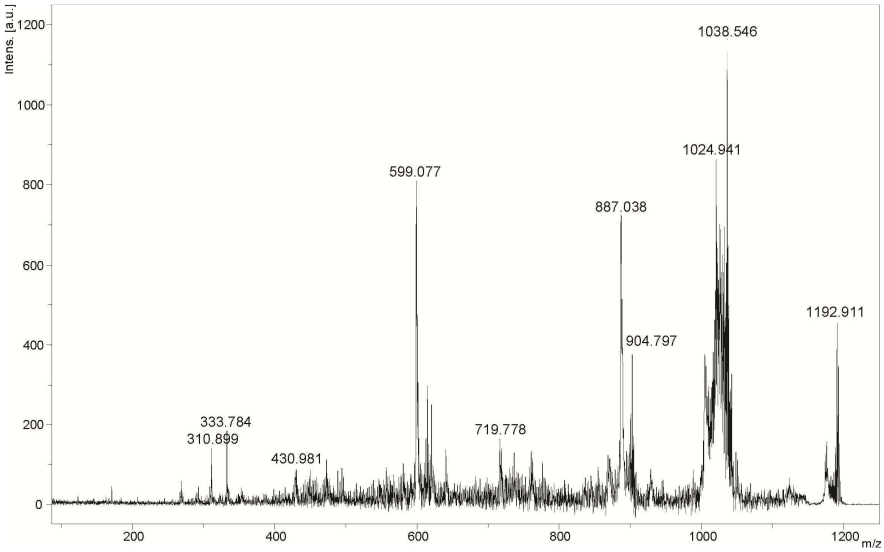

Figure S20 MS/MS spectrum of ion *m/z* 1193 from *C. leptophloeos*.

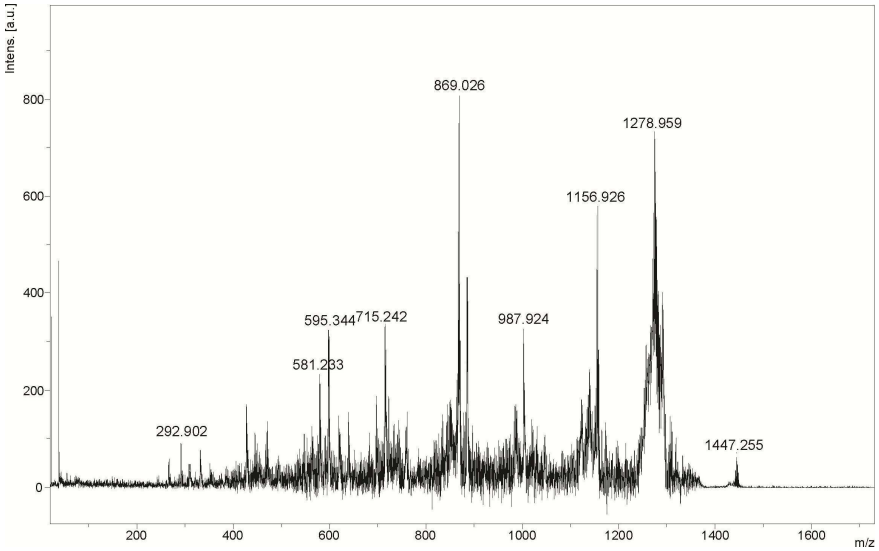

Figure S21 MS/MS spectrumof ion *m/z* 1447 from *C. leptophloeos*.

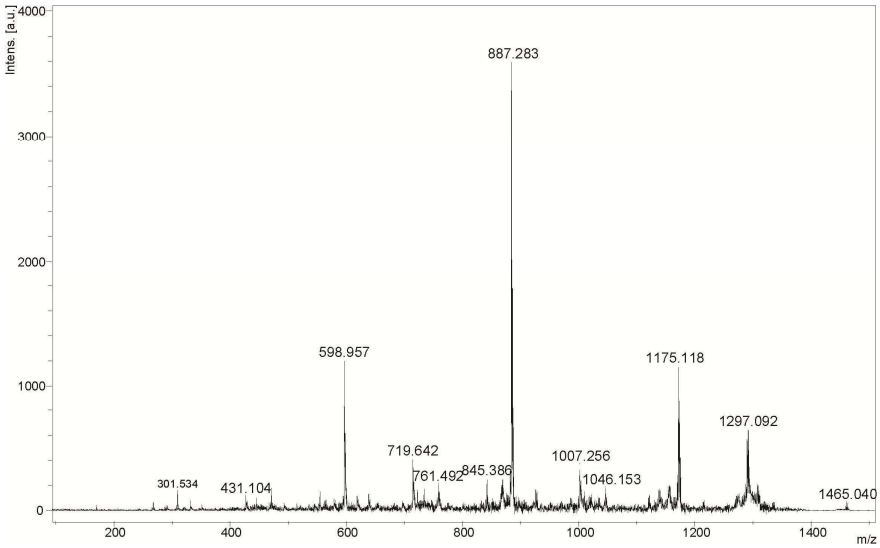

Figure S22 MS/MS spectrum of ion *m/z* 1465 from *C. leptophloeos*.

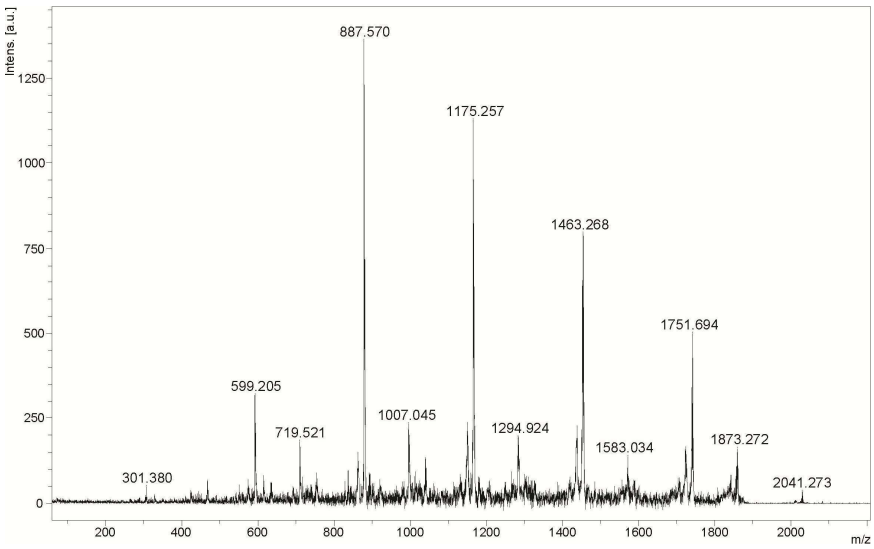

Figure S23 MS/MS spectrum of ion *m/z* 2041 from *C. leptophloeos*.

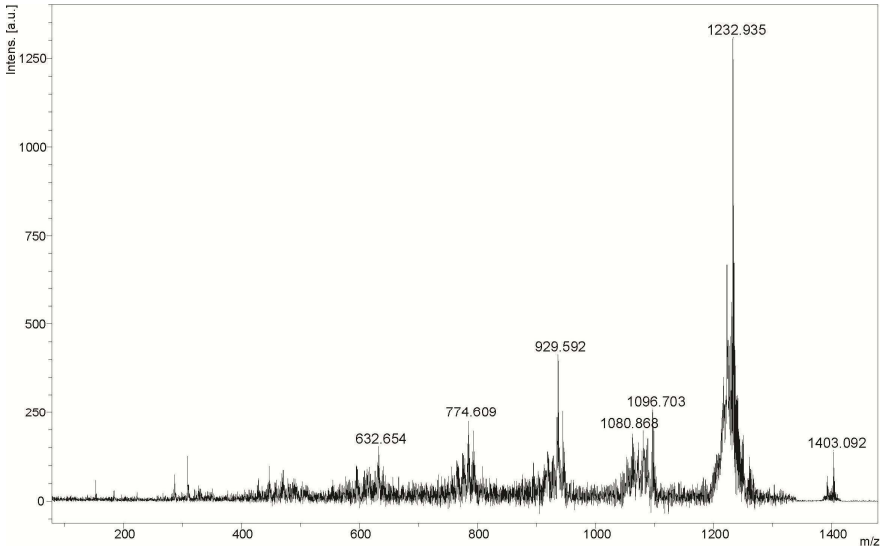

Figure S24 MS/MS spectrum of ion *m/z* 1403 from *M. urundeuva*.

IV - CAPÍTULO 4 - Proanthocyanidins with high prodelphinidin content from *Pityrocarpa moniliformis* prevent *Staphylococcus epidermidis* biofilm formation

Manuscrito a ser submetido para o periódico **Phytochemistry**

Proanthocyanidins with high prodelphinidin content from *Pityrocarpa moniliformis* prevent *Staphylococcus epidermidis* biofilm formation

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Abstract

Investigation of leaves extract of *Pityrocarpa moniliformis* (Benth.) Luckow & R. W. Jobson by bioguided fractionation led to obtaining 9 fractions and the identification of active proanthocyanidins. The structures of these non-biocidal antibiofilm proanthocyanidins against *Staphylococcus epidermidis*, a permanent colonizer of human skin and one of the most frequent cause of indwelling medical devices infections, which characteristically involve biofilms, were elucidated by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). *P. moniliformis* condensed tannins consist predominantly of prodelphinidin units, presenting all B-type linkage and completely linear structures. Proguibourtinidol, procyanidin and galloyl units were the substituents found in the heteropolyflavan-3-ols structures. The spectra illustrated a series of peaks corresponding to the presence of the prodelphinidin monomer (*m/z* 329 $[M+Na]^+$) until oligomers of up to 13 flavan-3-ol units (*m/z* 3929 $[M+Na]^+$). *P. moniliformis* is widespread in dry forests of semi-arid Northeastern Brazil (Caatinga), where it has been used by local community as medicine. Despite the abundance of this species in the Brazilian semi-arid region, phytochemical studies remain scarce and little is known about its biological activities. We combined phytochemistry and microbiological studies to establish, for the first time, that *P. moniliformis* is a source of complex flavan-3-ol oligomers able to prevent *S. epidermidis* biofilm formation without interfere in bacterial growth. Finally, our results highlighted a homogeneity regarding the intermolecular bonds (linear B-type interflavanyl linkages) and the pattern of hydroxylation (riched in prodelphinidin), but a heterogeneity concerning on the degree of oligomers polymerization (DP) of 1 to 13 of the bioactive proanthocyanidins derived from *P. moniliformis*.

Keywords: tannin, MALDI, *Pityrocarpa moniliformis***,** delphinidin, biofilm, *Staphylococcus epidermidis*

1. Introduction

Bacterial adhesion plays a pivotal role in the microbial surface colonization, constituting the first step in the development of structured surface-associated communities of bacteria called biofilms (Costerton, 1999; Dunne, 2002; Otto, 2012). Living as biofilms, bacterial cells are protected by a self produced-matrix, which enhances resistance to antimicrobials agents when compared to the free-floating cells (planktonic lifestyle) (Hoiby et al., 2010; Otto, 2012). The reduced permeability of antimicrobial agents and the stationary phase growth of some bacterial cells, both biofilm resistance traits, have been postulated as reasons for recalcitrance of biofilms to treatments (Pozo and Patel, 2007; Hoiby et al., 2010).

Therefore, adherent communities are very problematic in clinical settings. They have been shown to be associated with several human diseases, such as native valve endocarditis and cystic fibrosis, and to colonize a wide variety of medical devices (Donlan and Costerton, 2002; Burmolle et al., 2012), accounting for over 80% of all human bacterial infections according to US National Institutes of Health (Davies, 2003). The genus *Staphylococcus* ranks first among the causative agents of nosocomial infections. In particular, *S. epidermidis* represents the most frequent cause of indwelling medical devices infections, which characteristically involve biofilms (Otto, 2009). Hence, the identification of compounds that target bacterial virulence (such as biofilm formation) rather than cell growth represents an alternative approach to control pathogens. The greatest advantage to targeting the virulence factors of pathogens is that there is milder evolutionary pressure for the development of resistance, differently of antimicrobials agents (Cegelski et al., 2008; Rasko and Sperandio, 2010). In this sense, the utility of natural products as sources of novel structures, but not necessarily the final drug entity, is still alive and it may serve as the leads and scaffolds for elaboration of needed efficacious drugs (Newmann and Cragg, 2012).

Pityrocarpa moniliformis (Benth.) Luckow & R. W. Jobson is a mimosoid legume widespread in dry forests of semi-arid Northeastern Brazil (Caatinga) and in Venezuela. It presents a rapidly-growing, possess small to medium sized (Azeredo et al., 2010) and is popularly known in Caatinga as canzenzo, angico de bezerro or quipembe. *P. moniliformis* is used by local community as source of wood, as forage

for cattle and goat (Azeredo et al., 2010) and as medicine due its healing properties (Trentin et al., 2011). Despite the abundance of this species in the Brazilian semi-arid region, phytochemical studies remain scarce and little is known about its biological activities. The insecticidal effect of seeds ethanolic extract (Souza et al., 2011), the antioxidant effect potential, DNA protection abilities and anti- *Staphylococcus aureus* action of hydroalcoholic extract from fruits (Silva et al., 2011, 2012), as well as our previous work demonstrating the antibiofilm activity of leaves aqueous extract against *S. epidermidis* (Trentin et al., 2011) are examples of biological potential of *P. moniliformis*.

In order to improve the knowledgement on the phytochemistry of Brazilian semi-arid species, herein we describe the bioguided phytochemical investigation regarding antibiofilm properties of the aqueous extracts from *P. moniliformis* leaves and the identification of proanthocyanidins, by MALDI-MS/MS analysis, for the first time. Our results demonstrated that the high-prodelphinidin condensed tannins presenting all B-type linkage and heterogeneous degree of polymerization (DP) are responsible by the non-biocidal antibiofilm activity against *S. epidermidis*.

2. Results and Discussion

The leaves aqueous extract resembles the popular use of *P. moniliformis* in Brazilian semi-arid, thus it was the extraction procedure chosen in this work. Polyphenol derivatives, such as flavonoids, are examples of natural products easily extracted by water, however in light of our results the biological activity was showed only by an insoluble methanol fraction which was, subsequently, extracted with 70% acetone (AE). Sephadex LH20 was used to fractionate the AE, where the bioactive fractions were eluted with methanol or gradient of aqueous acetone (F4-F9). Here we demonstrated that the biofilm formation by *S. epidermidis* on a hydrophobic model surface (polystyrene) was strongly prevented when purified fractions of proanthocyanidins (F4-F9) were used (Fig. 1a). In addition, they were able to avoid biofilm formation independently of action on bacterial growth, in concentrations of 4.0, 2.0 and 1.0 mg/mL (Fig. 1b).

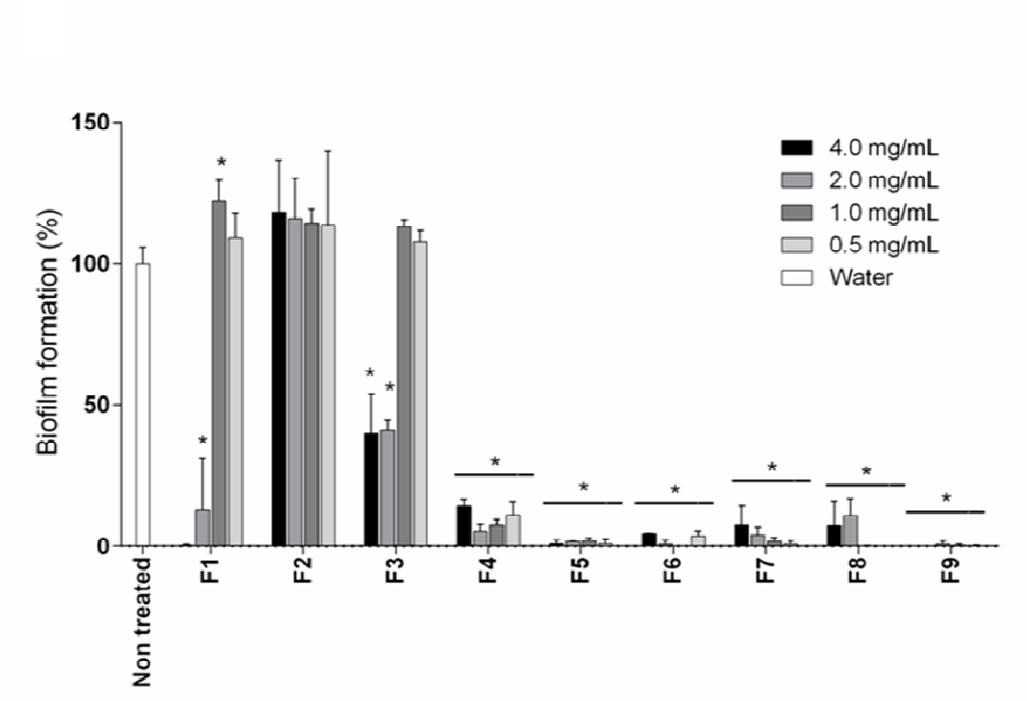


Fig. 1a. Effect of *P. moniliformis* fractions on *S. epidermidis* biofilm formation. * represents statistical difference in relation to the untreated samples.

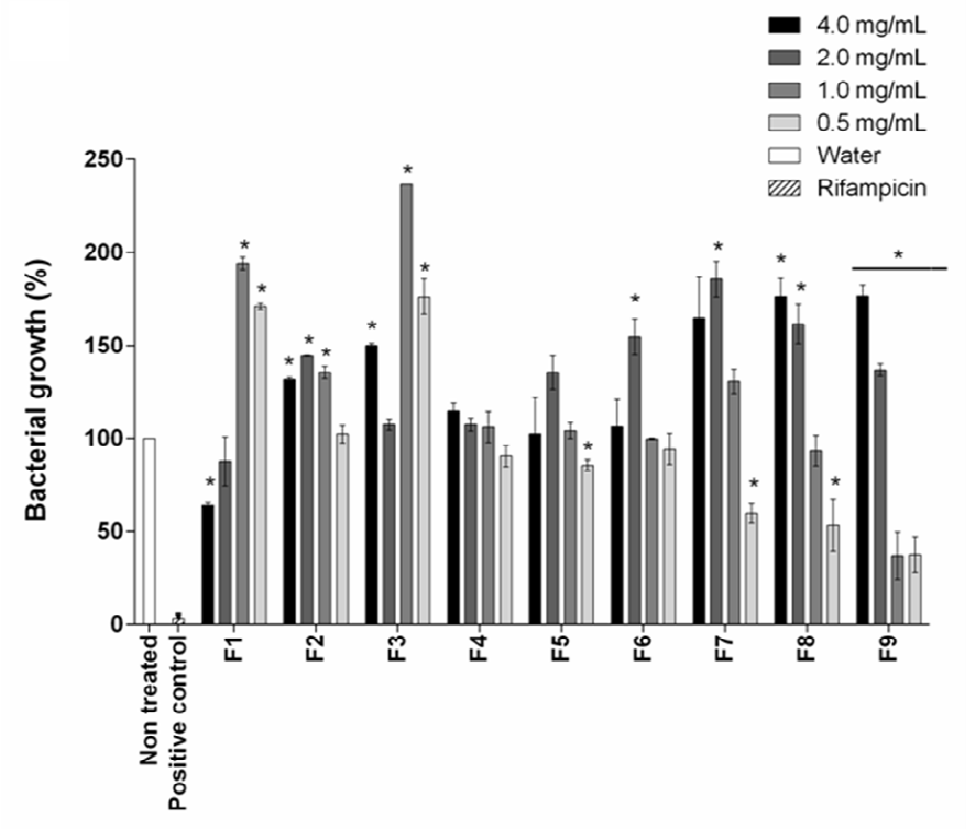


Fig. 1b. Effect of *P. moniliformis* fractions on *S. epidermidis* growth. * represents statistical difference in relation to the untreated samples.

The chromatographic and chemical properties observed for the bioactive fractions (data not shown) indicated that large polyphenolic molecules, like tannins could be the responsible ones by the activity. To characterize and to determine the chemical composition of the active samples against *S. epidermidis* biofilm formation, we employed MALDI-MS/MS, a very suited tool for the analysis of highly polydisperse and heterogeneous proanthocyanidins (Monagas et al., 2010). This technique is able to distribute a complex mixture of compounds into portions of different *m/z* values and tend to produce single charged species, which is of great importance for identify the molecular ion of high molecular weight compounds (El-Aneed et al., 2009). Although MALDI-TOF spectra of polydispersive oligomers present a characteristic pattern of decreasing signal intensity with increasing degree of polymerization (DP), due to the detector saturation with lower masses that reach the detector first (Reed et al., 2005), the intensity of each peak could be related to the amount of the corresponding oligomer presents in the sample by a semi-quantitative way, enable us to deduce the majority series of each fraction.

The bioactive fractions were characterized as series of polyflavan-3-ol oligomers based on a mostly prodelphinidin unit repeating structure (losses of 304 Da) having B-type interflavanyl linkages, in which monomers are linked through the C-4 position of the top unit to the C-8 position of the terminal unit, leading to linear structures (Table 1, Fig. 2 and Scheme 1). The retro Diels Alder fragmentation of the C-ring flavanol confing the loss of *m/z* 168 or 152 indicate the presence of a three or di-hidroxilated B-ring, respectively; confiming the presence of prodelphinidin or procyanidin structures. MALDI-MS/MS analyses of *P. moniliformis* proanthocyanidins allowed the determination of the starter and the extender units of each selected oligomer series and the detection of oligomers with a variable DP in all fractions (Table 1). With exception of F9, in all fractions we detected one series formed by prodelphinidin (*m/z* 304) homopolymer and other series constituted by heteropolyflavan-3-ols, presenting prodelphinidin units bonded to one proguibourtinidin, procyanidin or galloyl residues (Table 1 and Scheme 1 and 2). In the oligomeric series that present proguibourtinidin, the starter unit was a proguibourtinidin residue with interflavanyl bonds via the C-4 to C-8 of the

prodelphinidin (the extender unit). However, when the procyanidin or the galloyl residues are present, the precursor unit was the prodelphinidin, following by the C4-C8 linkages of the procyanidin or by the linkage of the galloyl residue on the hydroxyl of C3 of the second prodelphinidin unit (Scheme 2). Since no configurational information can be gleaned from MS, we can not unequivocally assign the specific stereochemistry of interflavanyl bonds.

Table 1 Distribution of polyflavan-3-ol oligomers by MALDI-TOF for tannins from *P. moniliformis*

Fraction		**$[M+Na]^+$**	**MF**	**Compound**
F4		937	$C_{45}H_{38}O_{21}$	3 PDE
F5	Series 1	889	$C_{45}H_{38}O_{18}$	1 PGU – 2 PDE
		1193	$C_{60}H_{50}O_{25}$	1 PGU – 3 PDE
		1497	$C_{75}H_{62}O_{32}$	1 PGU – 4 PDE
		1801	$C_{90}H_{74}O_{39}$	1 PGU – 5 PDE
		2105	$C_{105}H_{86}O_{46}$	1 PGU – 6 PDE
		2409	$C_{120}H_{98}O_{53}$	1 PGU – 7 PDE
		2713	$C_{135}H_{110}O_{60}$	1 PGU – 8 PDE
		3017	$C_{150}H_{122}O_{67}$	1 PGU – 9 PDE
		3321	$C_{165}H_{134}O_{74}$	1 PGU – 10 PDE
	Series 2	329	$C_{15}H_{14}O_7$	PDE
		633	$C_{30}H_{26}O_{14}$	2 PDE
		937	$C_{45}H_{38}O_{21}$	3 PDE
		1241	$C_{60}H_{50}O_{28}$	4 PDE
		1545	$C_{75}H_{62}O_{35}$	5 PDE
		1849	$C_{90}H_{74}O_{42}$	6 PDE
		2153	$C_{105}H_{86}O_{49}$	7 PDE
		2457	$C_{120}H_{98}O_{56}$	8 PDE
F6	Series 1	329	$C_{15}H_{14}O_7$	PDE
		633	$C_{30}H_{26}O_{14}$	2 PDE
		937	$C_{45}H_{38}O_{21}$	3 PDE
		1241	$C_{60}H_{50}O_{28}$	4 PDE
		1545	$C_{75}H_{62}O_{35}$	5 PDE
		1849	$C_{90}H_{74}O_{42}$	6 PDE
		2153	$C_{105}H_{86}O_{49}$	7 PDE
		2457	$C_{120}H_{98}O_{56}$	8 PDE
	Series 2	329	$C_{15}H_{14}O_7$	PDE
		617	$C_{30}H_{26}O_{13}$	PDE - PCY
		921	$C_{45}H_{38}O_{20}$	PDE - PCY - PDE
		1225	$C_{60}H_{50}O_{27}$	PDE - PCY – 2 PDE
		1529	$C_{75}H_{62}O_{34}$	PDE - PCY – 3 PDE
		1833	$C_{90}H_{74}O_{41}$	PDE - PCY – 4 PDE
		2137	$C_{105}H_{86}O_{48}$	PDE - PCY – 5 PDE
		2441	$C_{120}H_{98}O_{55}$	PDE - PCY – 6 PDE
		2745	$C_{135}H_{110}O_{62}$	PDE - PCY – 7 PDE
	Series 3	329	$C_{15}H_{14}O_7$	PDE
		633	$C_{30}H_{26}O_{14}$	2PDE
		785	$C_{37}H_{30}O_{18}$	2 PDE - GAL
		1089	$C_{52}H_{42}O_{25}$	2 PDE – GAL - PDE
		1393	$C_{67}H_{54}O_{32}$	2 PDE – GAL – 2 PDE
		1697	$C_{82}H_{66}O_{39}$	2 PDE – GAL – 3 PDE
		2001	$C_{97}H_{78}O_{46}$	2 PDE – GAL – 4 PDE
		2305	$C_{112}H_{90}O_{53}$	2 PDE – GAL – 5 PDE

F7	Series 1	329	$C_{15}H_{14}O_7$	PDE
		633	$C_{30}H_{26}O_{14}$	2 PDE
		937	$C_{45}H_{38}O_{21}$	3 PDE
		1241	$C_{60}H_{50}O_{28}$	4 PDE
		1545	$C_{75}H_{62}O_{35}$	5 PDE
		1849	$C_{90}H_{74}O_{42}$	6 PDE
		2153	$C_{105}H_{86}O_{49}$	7 PDE
		2457	$C_{120}H_{98}O_{56}$	8 PDE
		2761	$C_{135}H_{110}O_{63}$	9 PDE
		3065	$C_{150}H_{122}O_{70}$	10 PDE
	Series 2	617	$C_{30}H_{26}O_{13}$	PDE - PCY
		921	$C_{45}H_{38}O_{20}$	PDE - PCY - PDE
		1225	$C_{60}H_{50}O_{27}$	PDE - PCY – 2 PDE
		1529	$C_{75}H_{62}O_{34}$	PDE - PCY – 3 PDE
		1833	$C_{90}H_{74}O_{41}$	PDE - PCY – 4 PDE
		2137	$C_{105}H_{86}O_{48}$	PDE - PCY – 5 PDE
		2441	$C_{120}H_{98}O_{55}$	PDE - PCY – 6 PDE
		2745	$C_{135}H_{110}O_{62}$	PDE - PCY – 7 PDE
	Series 3	329	$C_{15}H_{14}O_7$	PDE
		633	$C_{30}H_{26}O_{14}$	2PDE
		785	$C_{37}H_{30}O_{18}$	2 PDE – GAL
		1089	$C_{52}H_{42}O_{25}$	2 PDE – GAL - PDE
		1393	$C_{67}H_{54}O_{32}$	2 PDE – GAL – 2 PDE
		1697	$C_{82}H_{66}O_{39}$	2 PDE – GAL – 3 PDE
		2001	$C_{97}H_{78}O_{46}$	2 PDE – GAL – 4 PDE
		2305	$C_{112}H_{90}O_{53}$	2 PDE – GAL – 5 PDE
		2609	$C_{127}H_{102}O_{60}$	2 PDE – GAL – 6 PDE
		2913	$C_{142}H_{114}O_{67}$	2 PDE – GAL – 7 PDE
F8	Series 1	889	$C_{45}H_{38}O_{18}$	1 PGU – 2 PDE
		1193	$C_{60}H_{50}O_{25}$	1 PGU – 3 PDE
		1497	$C_{75}H_{62}O_{32}$	1 PGU – 4 PDE
		1801	$C_{90}H_{74}O_{39}$	1 PGU – 5 PDE
		2105	$C_{105}H_{86}O_{46}$	1 PGU – 6 PDE
		2409	$C_{120}H_{98}O_{53}$	1 PGU – 7 PDE
		2713	$C_{135}H_{1104}O_{60}$	1 PGU – 8 PDE
		3017	$C_{150}H_{122}O_{67}$	1 PGU – 9 PDE
		3321	$C_{165}H_{134}O_{74}$	1 PGU – 10 PDE
		3625	$C_{180}H_{146}O_{81}$	1 PGU – 11 PDE
		3929	$C_{195}H_{158}O_{88}$	1 PGU – 12 PDE
	Series 2	329	$C_{15}H_{14}O_7$	PDE
		633	$C_{30}H_{26}O_{14}$	2 PDE
		937	$C_{45}H_{38}O_{21}$	3 PDE
		1241	$C_{60}H_{50}O_{28}$	4 PDE
		1545	$C_{75}H_{62}O_{35}$	5 PDE
		1849	$C_{90}H_{74}O_{42}$	6 PDE
		2153	$C_{105}H_{86}O_{49}$	7 PDE
		2457	$C_{120}H_{98}O_{56}$	8 PDE
F9	Series 1	889	$C_{45}H_{38}O_{18}$	1 PGU – 2 PDE
		1193	$C_{60}H_{50}O_{25}$	1 PGU – 3 PDE
		1497	$C_{75}H_{62}O_{32}$	1 PGU – 4 PDE
		1801	$C_{90}H_{74}O_{39}$	1 PGU – 5 PDE
		2105	$C_{105}H_{86}O_{46}$	1 PGU – 6 PDE
		2409	$C_{120}H_{98}O_{53}$	1 PGU – 7 PDE
		2713	$C_{135}H_{110}O_{60}$	1 PGU – 8 PDE
		3017	$C_{150}H_{122}O_{67}$	1 PGU – 9 PDE
		3321	$C_{165}H_{134}O_{74}$	1 PGU – 10 PDE
		3625	$C_{180}H_{146}O_{81}$	1 PGU – 11 PDE
		3929	$C_{195}H_{158}O_{88}$	1 PGU – 12 PDE

MF: molecular formula, PGU: Proguibourtinidin, PDE: Prodelphinidin; PCY: Procyanidin, GAL: Galloyl

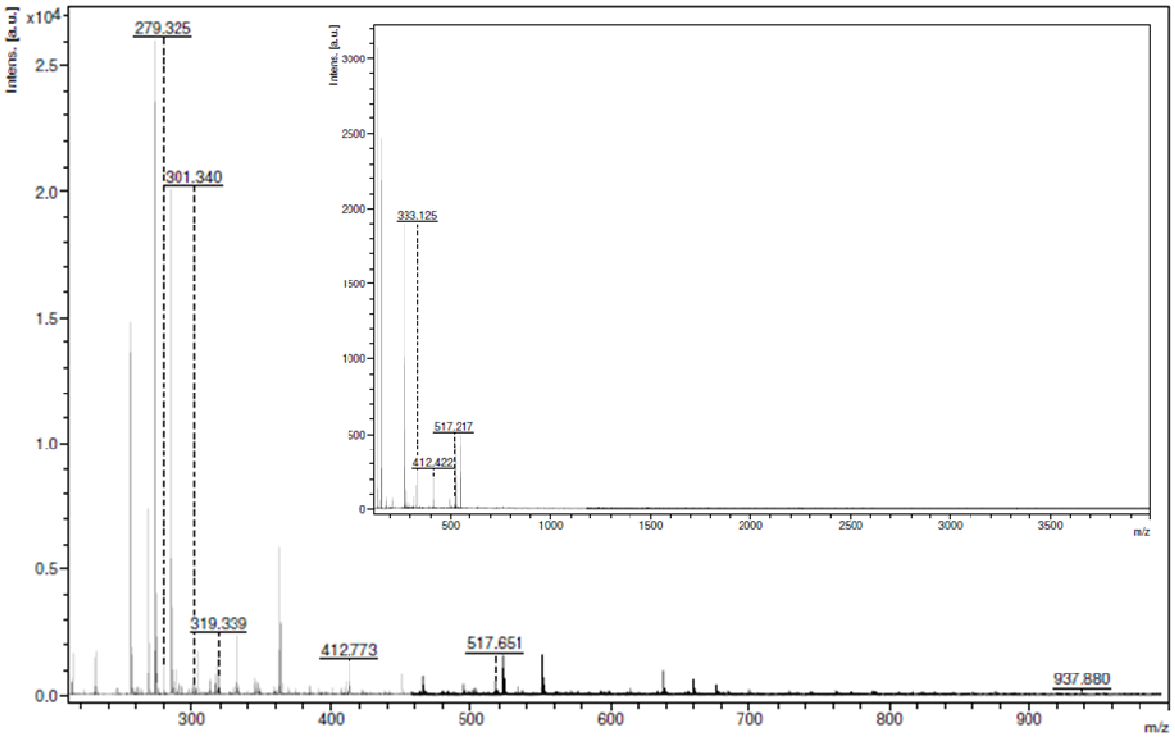


Fig. 2a. Mass spectra (positive ionization mode) of F4 fraction obtained from *P. moniliformis* leaves: presence of a trimer with 3 prodelphinidin units (*m/z* 937). The insert shows the absence of high molecular weight peaks in the spectra up to *m/z* 4000.

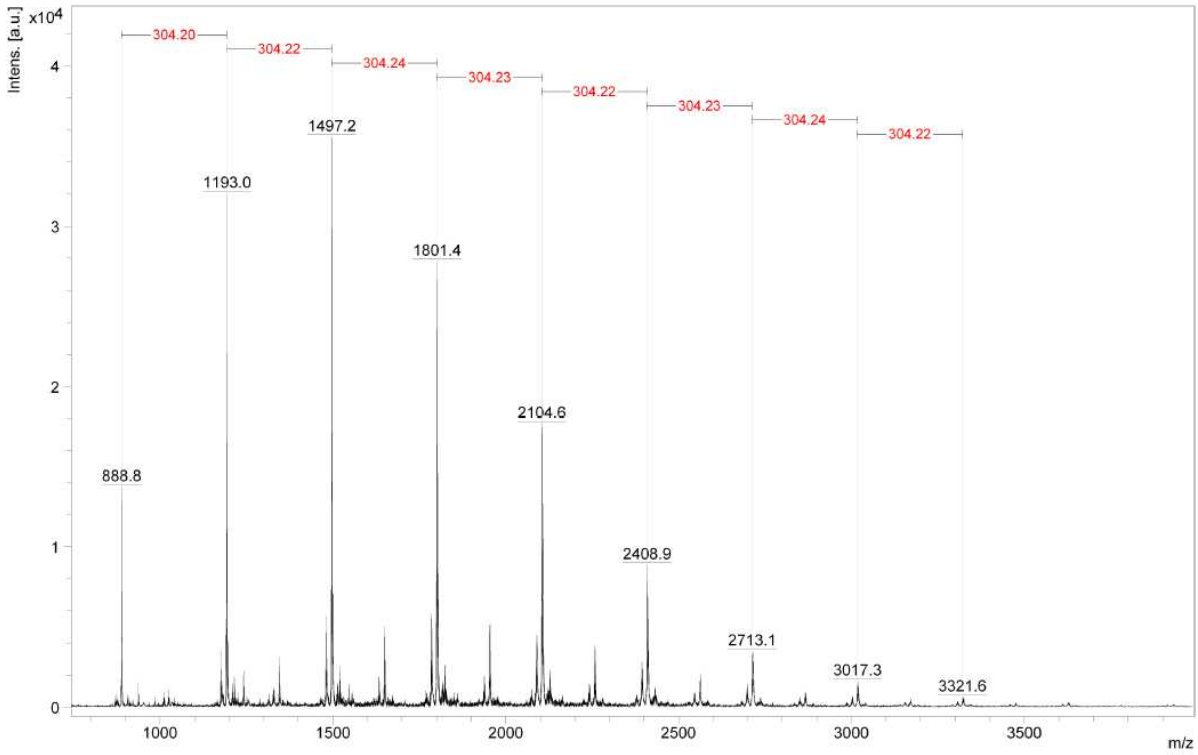


Fig. 2b. Mass spectra (positive ionization mode) of F5 fraction from *P. moniliformis* leaves: the majority series (series 1) is highlighted, showing the presence of oligomers riched in prodelphinidin including one proguibourtinidin unit, with DP up to 8.

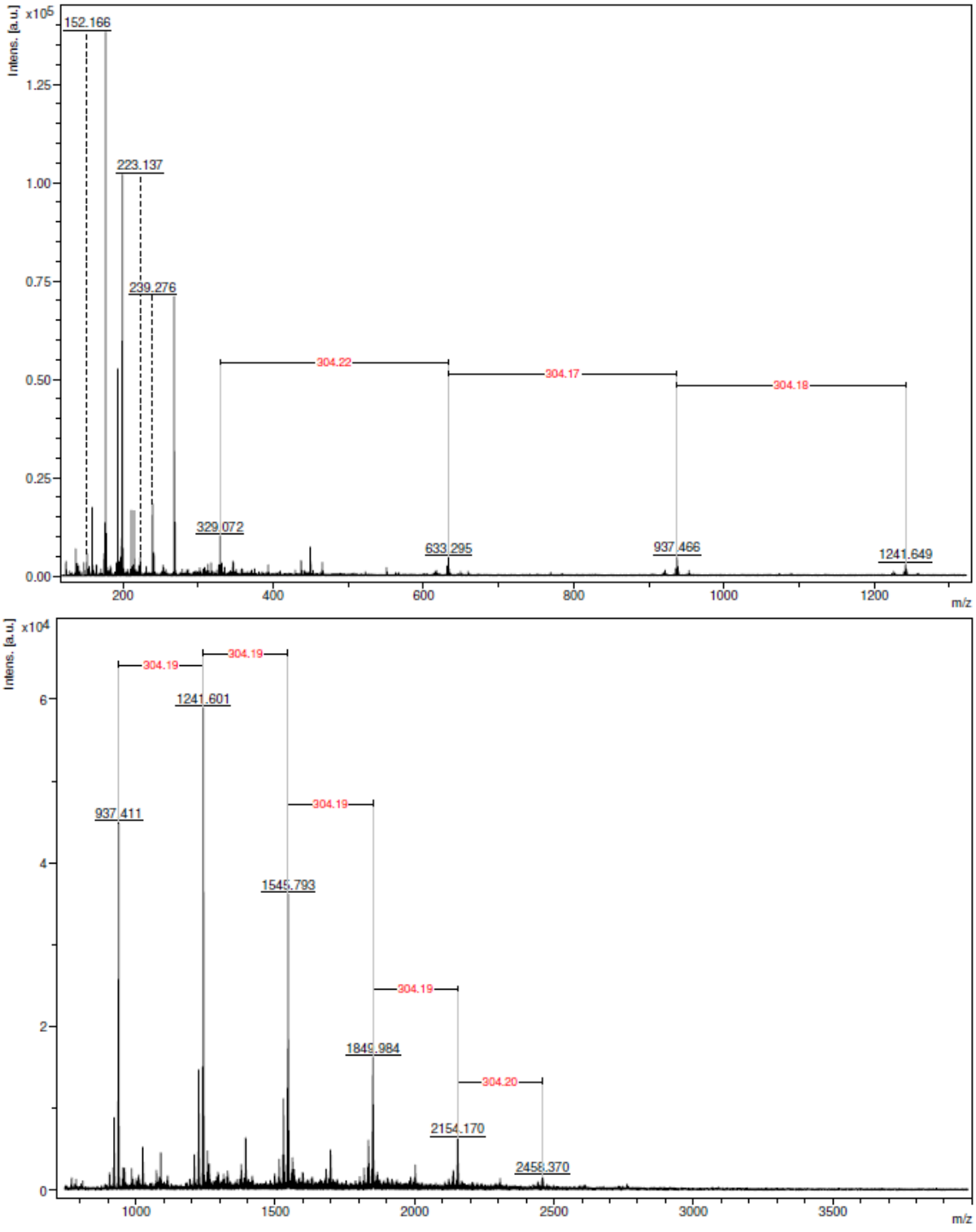


Fig. 2c. Mass spectra (positive ionization mode) of F6 fraction from *P. moniliformis* leaves: the majority series (series 1) is highlighted. The top (up to *m/z* 1300) and the bottom (up to *m/z* 4000) figures show the oligomers with DP of 1-8 prodelphinidin units.

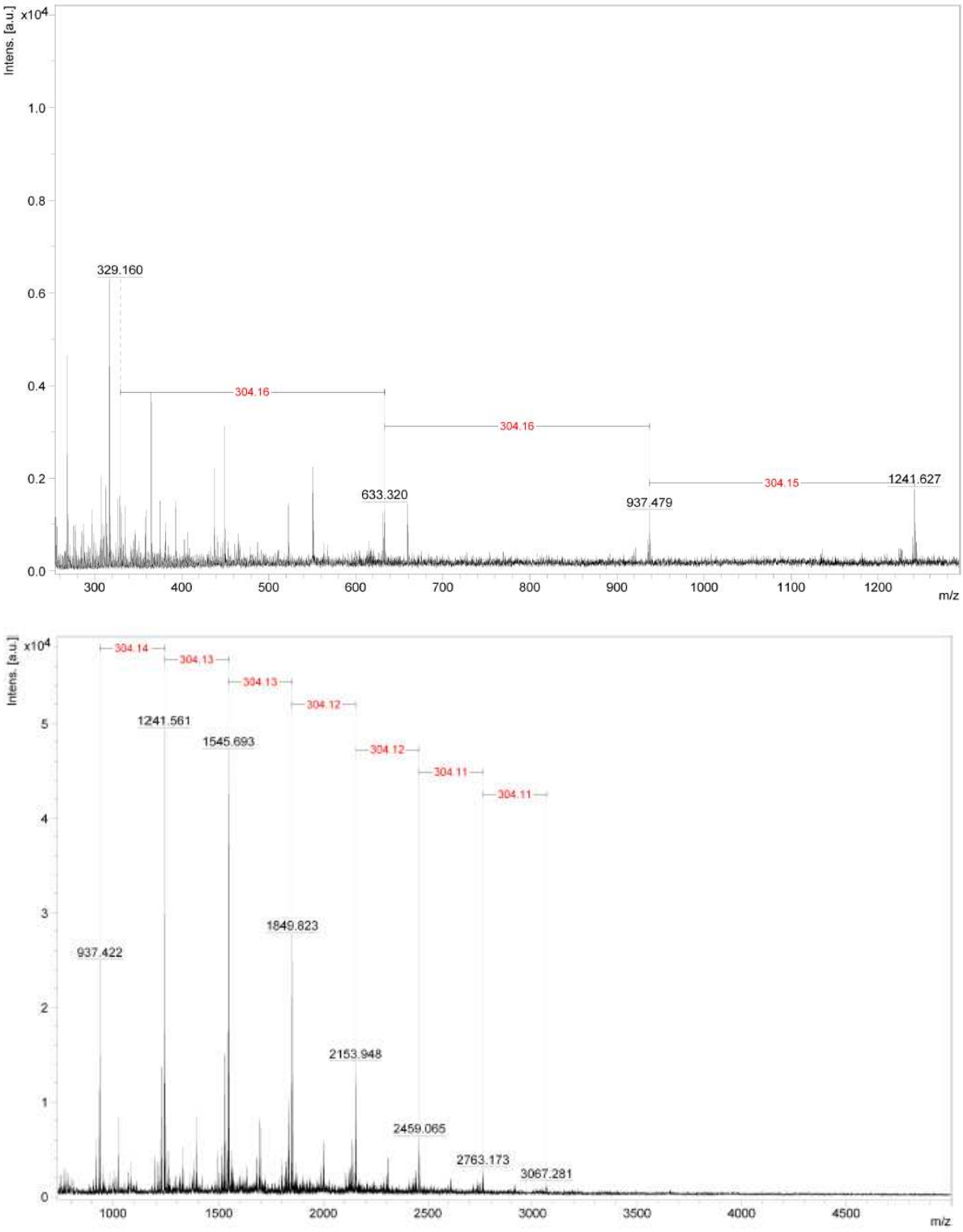


Fig. 2d. Mass spectra (positive ionization mode) of F7 fraction from *P. moniliformis* leaves: majority series (series 1) is highlighted. The top (up to *m/z* 1300) and the bottom (up to *m/z* 4000) figures show the oligomers with DP of 1-10 prodelphinidin units.

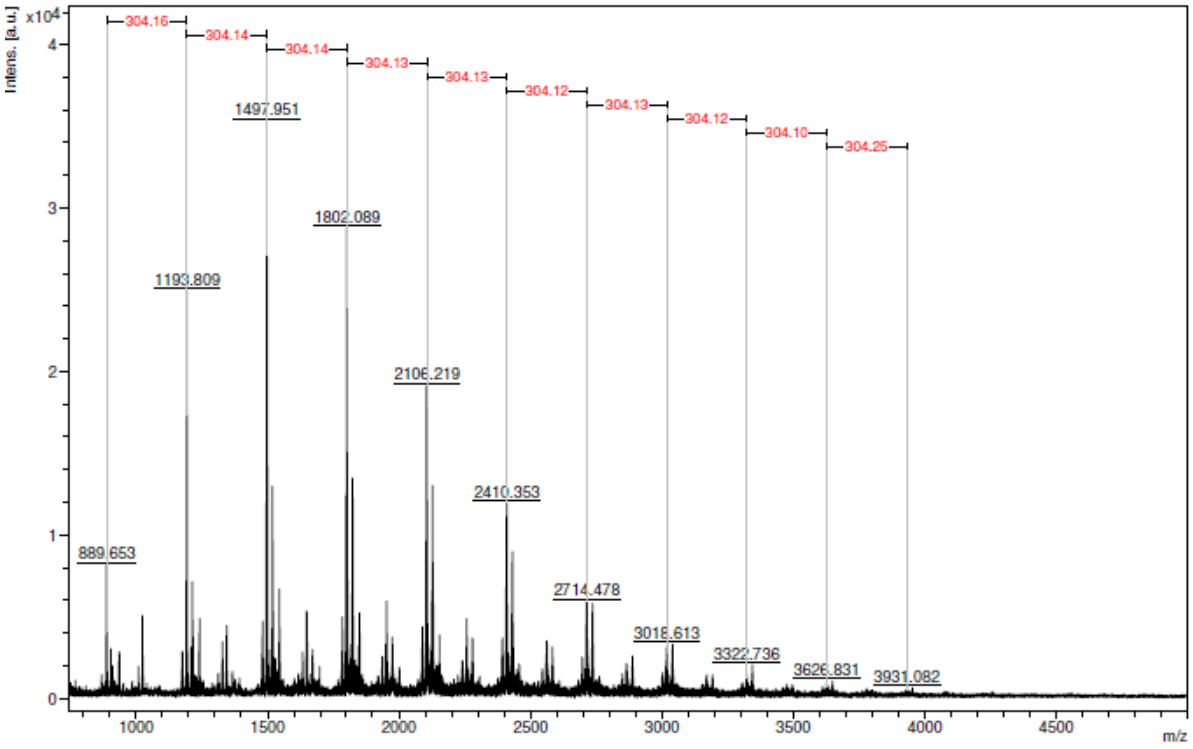


Fig. 2e. Mass spectra (positive ionization mode) of F8 fraction from *P. moniliformis* leaves: its majority series (series 1) is highlighted, showing the presence of oligomers riched in prodelphinidin including one proguibourtinidin unit, with DP up to 13.

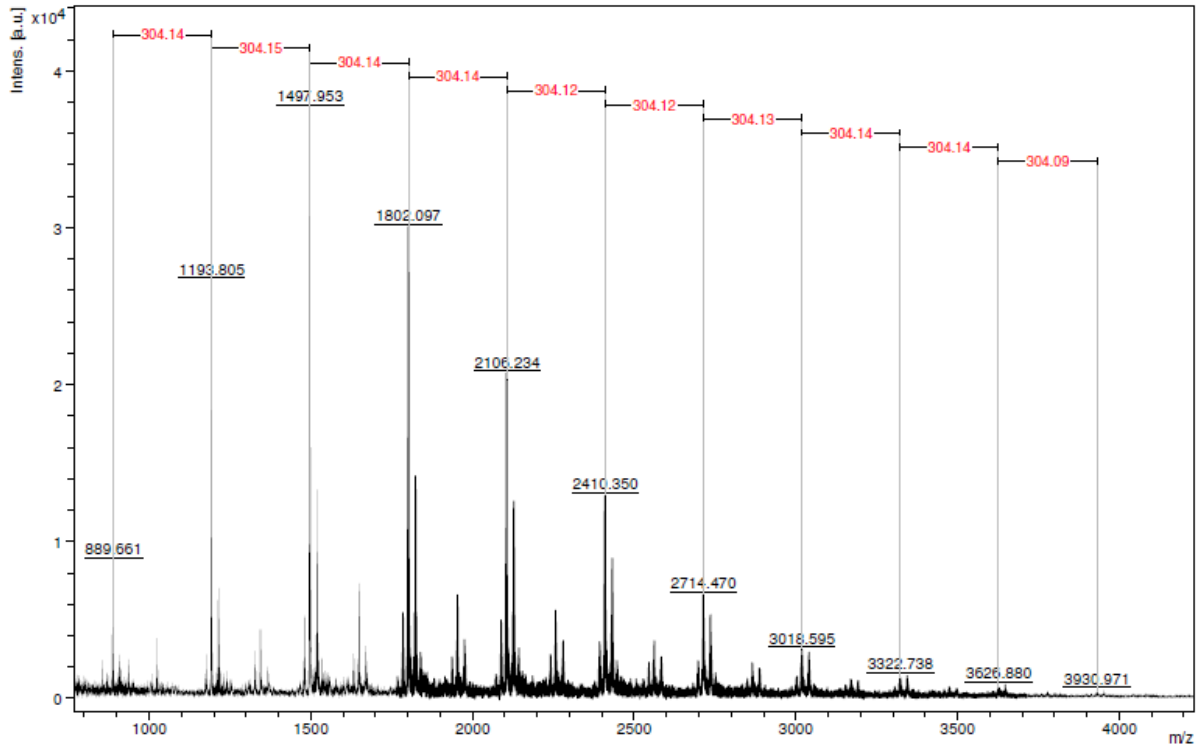
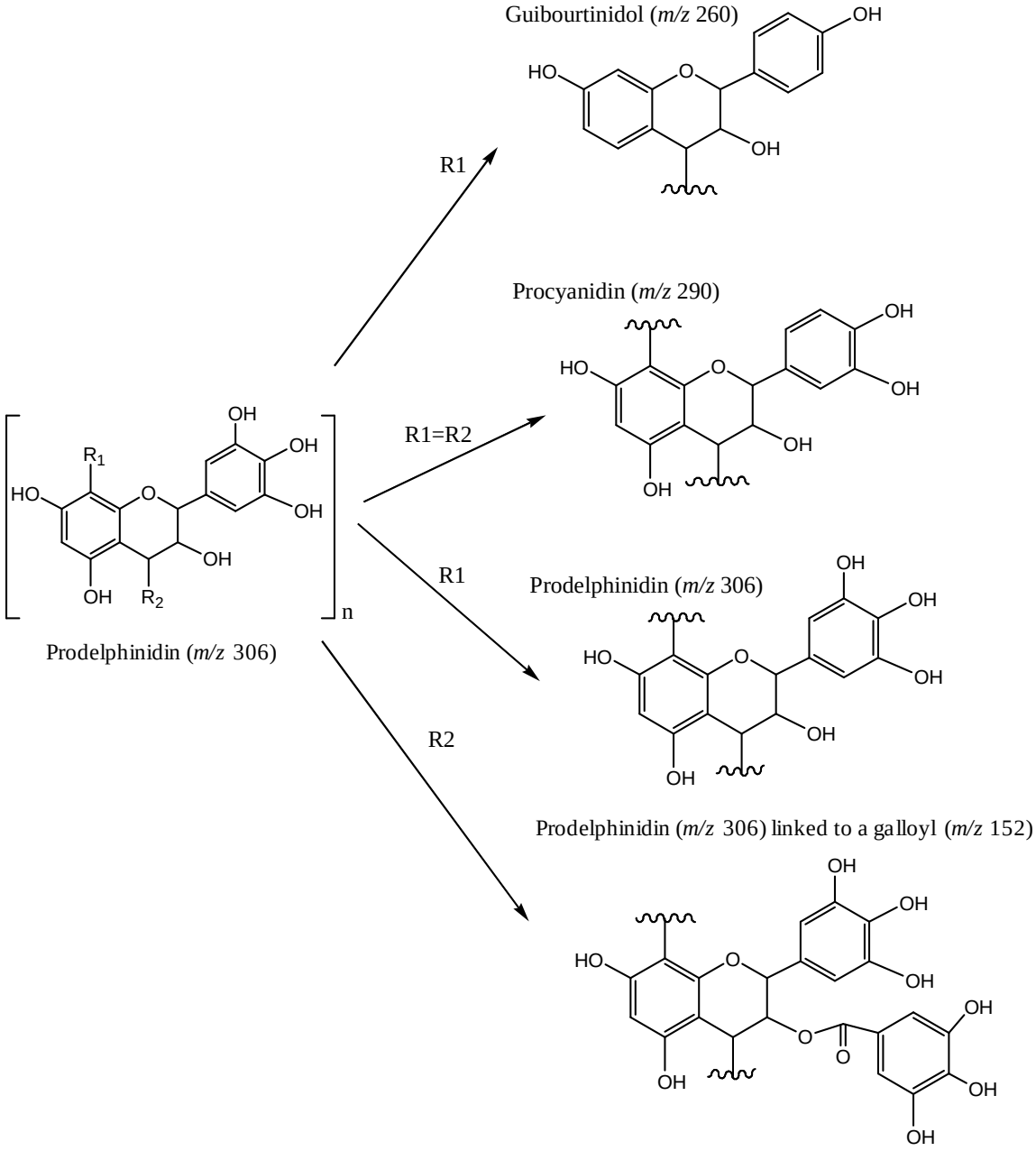
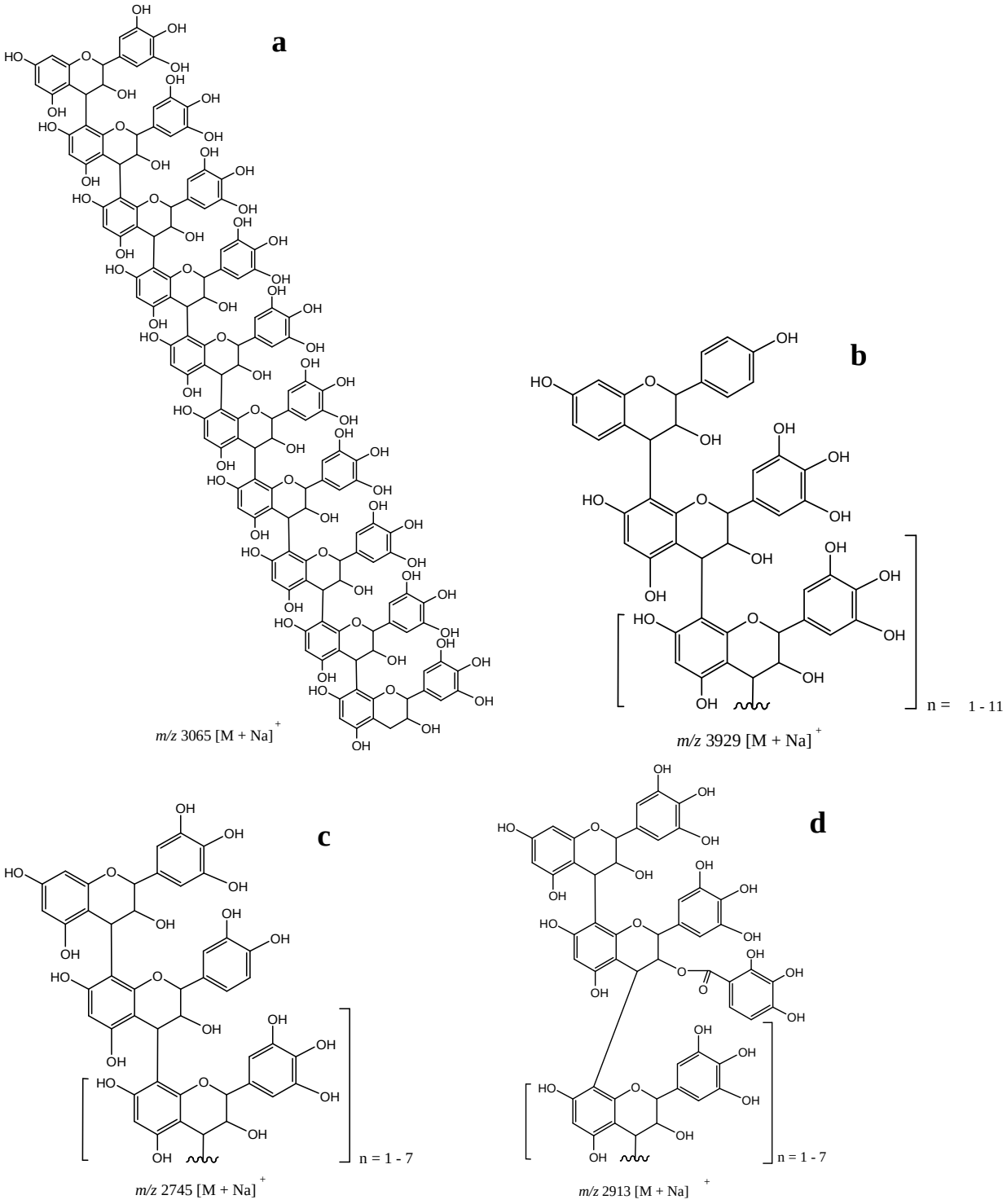


Fig. 2f. Mass spectra (positive ionization mode) of F9 fraction from *P. moniliformis* leaves: its majority series (series 1) is highlighted, showing the presence of oligomers riched in prodelphinidin including one proguibourtinidin unit, with DP up to 13.



Scheme 1. Schematic representation of monomeric units that form the proanthocyanidins obtained from *P. moniliformis* leaves. R1 and R2 represent the binding site between the units.



Scheme 2. Schematic representation of proanthocyanidins obtained from *P. moniliformis* leaves. a) Homopolymeric structure constituted by C4-C8 bounded prodelphinidin units. b) Proguibourtinidin C4-C8 bounded up to 12 prodelphinidin extender units. c) Procyanidin residue linked to prodelphinidins though C4 and C8, reaching up to 7 prodelphinidin extender units. The prodelphinidin monomer and the dimer prodelphinidin-procyanidin also were found. d) The galloyl substitution on the hydroxyl of C3 of the second prodelphinidin units, reaching up to 7 prodelphinidin extender units. The prodelphinidin monomer, dimer and the dimer linked to the galloyl also were found.

The occurrence of proguibourtinidin in Fabaceae is described for the first time in light of our results. Otherwise, procyanidin and prodelphinidin residues are widespread in this family. In such way, a study developed with *Parapiptadenia rigida*, taxonomic related to *P. moniliformis* demonstrated dimeric prodelphinidin and procyanidin from bark (Schmidt et al., 2011). The most known anti-adherence and antibiofilm properties of proanthocyanidins are exhibited by condensed tannins derived from the North American cranberry (*Vaccinium macrocarpon*) or cranberry juice, mainly against *Escherichia coli* (Habash et al., 1999; Allison et al., 2000; Eydelnant and Tufenki, 2008). They consist predominantly of procyanidin (epicatechin units) containing at least one A-type of linkage (Foo et al., 2000). In 2007, Delehanty and co-works demonstrated that proanthocyanidins from cranberry are able to bind to the lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, and that this LPS-binding activity was not limited only to *E. coli* LPS, having comparable affinities for LPS preparations from *Salmonella, Shigella* and *Pseudomonas*. The importance of the A-type linkage as a prerequisite for *E. coli* anti-adhesion activity when compared with other food containing all B-linked proanthocyanidins also was demonstrated by Howell et al. (2005). Also, Idowu et al. (2010) isolated a doubly linked A-type proanthocyanidins from leaves of *Ixora coccinea* that present antibacterial properties against *Bacillus subtilis*.

Regarding the biological activity that directed this investigation, we can observe that antibiofilm fractions that eluted from Sephadex LH20 from 10% to 100% acetone (F5 to F9) present a series of closely related compounds reaching complex structures of up to *m/z* 3929 $[M+Na]^+$ (DP of 13) constituted predominantly by B-linked prodelphinidin units. In addition, the fraction eluted with methanol (F4) presented just 15% of biofilm formation and was chemically characterized as possessing lower molecular weight peaks up to *m/z* 937 $[M+Na]^+$ (prodelphinidin trimer). This is an important observation, but the complete inhibition of *S. epidermidis* biofilm formation was obtained with fractions presenting higher DP (F5-F9) (Fig. 1a). One motivation for using *P. moniliformis* derived proanthocyanidins in the prevention of *S. epidermidis* biofilm formation lies in its potential action as antiadhesive compounds

independent of antibacterial mechanism as shown in Fig. 1b, which may hamper the selective development of resistant strains. It is possible to verify that some concentrations of tested *P. moniliformis* fractions led to a significant stimulation of *S. epidermidis* growth when compared to non treated control, which could be understand as a bacterial defensive response against the presence or action of the fractions (Fig. 1b). This similar profile of growth stimuli and biofilm formation inhibition by *S. epidermidis* also was observed to the crude aqueous extract of *P. moniliformis* (Trentin et al., 2011).

So far there is no study in the literature reporting the antiadhesion and antibiofilm formation activity of proanthocyanidins against *S. epidermidis*. This Gram positive bacterium is one of the most frequent causes of biofilm-associated infections on indwelling medical devices, which has been recognized as a microorganism reservoir of genes that, after horizontal transfer, facilitate the pathogenicity of *Staphylococcus aureus* (Otto, 2012, 2013). Thereby, the results presented herein allow us to propose that, in contrast to studies on other bacteria genus, the A-type linkage is not essential for proanthocyanidins antibiofilm activity against *S. epidermidis*. Likewise, Janecki and Kolodziej (2010) provided evidences that support our findings, showing that reduced adherence by the Gram-positive bacterium *Streptococcus* was observed exclusively for prodelphinidins, suggesting that pyrogallol-type elements, *i.e.*, (epi)gallocatechin units are important structural features.

In addition, the traditional use of *P. moniliformis* could be justified by scientific basis, corroborating with the evidences of a correlation between tannin content and the effects popularly attributed to wound healing, anti-inflammatory and antimicrobial of Caatinga medicinal plants (Araújo et al., 2008; Siqueira et al., 2012). In this sense, a better understanding of the constituents of vegetable tannins will not only lead to a better understanding of their biosynthesis and biological function in plants, but will also enhance industrial and biotechnology applications (Venter et al., 2012).

3. Concluding Remarks

As part of our ongoing research on antibiofilms from Caatinga plants, this report concerns the structural elucidation of the bioactive proanthocyanidins from the

aqueous extract of *P. moniliformis* leaves using bioguided phytochemical investigation followed by MALDI-MS MS analysis. In this context, the identification of compounds that interfere in a bacterial virulence target, e.g., limiting biofilm formation (and not impairing on its growth) offers the potential application to control pathogens with a reduced selection pressure for drug-resistant mutations. Importantly, we have shown that tannins with high prodelphinidin content act in such a way that viability of the microorganisms is not negatively affected. The present investigation is a pioneer study using the plant *P. moniliformis* and demonstrates the important action of B-type linkage proanthocyanidins in preventing *S. epidermidis* biofilm formation.

4. Experimental

4.1. General

Plant material was grounded into particles using a blender (Waring Laboratory, Torrington, USA). Sterile 96-well polystyrene flat-bottom microtiter plates (Costar 3599) purchased from Corning Inc. (New York, USA). The absorbances were measured on a Spectramax M2e Multimode Microplate Reader (Molecular Devices, Sunnyvale, USA). Column chromatography was performed over Sephadex™ LH-20 (Sigma-Aldrich Co., St. Loius, USA). The high resolution MS analyses were done at an UltrafleXtreme MALDI-MS/MS equipment (Bruker Daltonics, Bremen, Germany). The internal and external calibration was conducted with a mixture of peptides (peptide calibration standard II of Bruker: bradykinin 1-7, angiotensin II and I, substance P, bombesin, renin substrate, ACTH clip 1-17, ACTH clip 18-39 and somatostatin 28). The ions were yielded by a nitrogen laser (337 nm) and accelerated at 20 kV. 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. Besides, the ions selected were accelerated to 19 kV in the LIFT cell for MS/MS analyses.

4.2. Chemicals

Solvents for extraction and purification procedures were obtained from Merck (Darmstadt, Germany). Mueller Hinton agar and tryptone soya broth (TSB) were

purchased from Oxoid Ltd. (Basingstoke, England). DMSO used in crystal violet assay was from Sigma-Aldrich Co. (St. Loius, USA). Aqueous plant extract, solvents mixtures and culture medium were prepared with a Milli-Q water system (Millipore Bedford, MA, USA).

4.3. Plant material

The leaves of *P. moniliformis* were collected between July and August 2009 in the Parque Nacional do Catimbau (PARNA do Catimbau), Pernambuco, Brazil. A voucher specimen number (IPA 84048) was deposited at the herbarium of the Instituto Agronômico de Pernambuco, Brazil.

4.4. Extraction and purification of proanthocyanidins

The dried leaves of *P. moniliformis* were grounded into particles using a grinder followed by a blender. Aqueous extracts were prepared by maceration, during 24 h, and lyophilized.

The bioguided fractionation was used to investigate the active compounds present in the crude aqueous extracts of *P. moniliformis* leaves. The powder of aqueous extract (15 g) was suspended in methanol (75 mL) providing two samples: a soluble fraction, inactive in the biological assay and a bioactive and insoluble fraction (12.9 g). This methanol insoluble fraction was dried and further extracted with 70% aqueous acetone (3 x 60 mL). The active acetone soluble fraction was concentrated on a rotatory evaporator at about 40 °C and the resulting residue was freeze dried to give a brown powder (8.8 g). The acetone fraction (8.7 g) was subjected to column chromatography through Sephadex™ LH-20 eluted with 50 mL of: H_2O (4360 mg), 70:30 [H_2O:MeOH, v/v] (223 mg), 50:50 [H_2O:MeOH, v/v] (314 mg), 100% MeOH (600 mg), 90:10 [H_2O:$(CH_3)_2CO$, v/v] (167 mg), 70:30 [H_2O:$(CH_3)_2CO$, v/v] (51 mg), 50:50 [H_2O:$(CH_3)_2CO$, v/v] (329 mg), 30:70 [H_2O:$(CH_3)_2CO$, v/v] (159 mg) and 100% $(CH_3)_2CO$ (25 mg). This procedure resulted in nine fractions, which were coded from F1 to F9.

4.5. MS analyses

According to Monagas et al (2010), we selected the 2,5-dihydroxibenzoic acid (DHB) as matrix to analyze the active fractions from *P. moniliformis*. To minimize the background ionization of matrix at *m/z* <1000 *u*, we also carried out analyses using DHB ionic matrix, as proposed by Pavarini et al (2012). The samples (2 mg) were dissolved in 200 uL of ACN:H_2O (30:70, v/v). The DHB matrix (20 mg/mL) and DHB ionic matrix (0.5 µL ionic matrix: 100 µL of solvent) in ACN:H_2O (30:70) with 0.1% trifluoracetic acid were supplemented with 0.1 M of NaCl solution to increase the detection of $[M+Na]^+$ adducts. The sample solution and the matrix were mixed in equal amounts. These mixtures (1 µL) were spotted onto a ground stainless steel MALDI target and dried at room temperature. The ratio of sample:matrix:cationizant agent employed was 1:1:0.1 (v/v/v). The compounds were identified by MS data, fragmentation pathway and accurate mass measures using the internal calibrant (mixture of standard peptides and ions from DHB matrix).

4.6. Bacterial strain and culture conditions

Staphylococcus epidermidis ATCC 35984 was grown in Mueller Hinton agar overnight, at 37°C, and a bacterial suspension in 0.9% NaCl, corresponding to 1 McFarland scale (3×10^8 CFU/mL), was used in the assays.

4.7. In vitro bacterial antibiofilm activity

Fractions obtained from Sephadex LH-20 were dissolved in water (10 mg/mL), and a 4-fold dilution series (final concentration of 4.0, 2.0, 1.0 and 0.5 mg/mL) were evaluated for activity against biofilm formation. The crystal violet assay was used as previously described (Trentin et al., 2011). In this assay the adherent biofilm layer formed after 24 h of incubation is stained with crystal violet and the absorbance is measured. The biofilm formation control represents 100% of biofilm formation, replacing the samples by water. Since there is not a commercially available non-biocidal compound possessing antibiofilm activity, we can not apply a positive control to antibiofilm activity. Values higher than 100% represent a stimulation of biofilm formation in comparison to the untreated control.

4.8. Bacterial growth assay

The bacterial growth was evaluated by the difference between the OD_{600} absorbance measured at the end (24 h) and at the beginning (0 h) of the incubation time in polystyrene 96-well microtiter plates. As a control for bacterial growth the extracts were replaced by water, to represent 100% of planktonic bacterial growth or by rifampicin (8.0 µg/mL) to represent a positive control of antibacterial activity. To avoid the interference of the samples color in the results, they were incubated with TSB and sterile saline (without inoculum) and the arithmetic means of the OD lectures were corrected for each substance (by the subtraction of OD without innoculum from the OD with innoculum). Values higher than 100% represent a stimulation of bacterial growth in comparison to the untreated control.

4.9. Statistical analysis

Biological activity values represent the mean ± standard deviation (SD) of three independent experiments. The data were statistically compared using Student's t-test, and a p-value < 0.05 was considered significant.

Acknowledgments

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Supplementary material

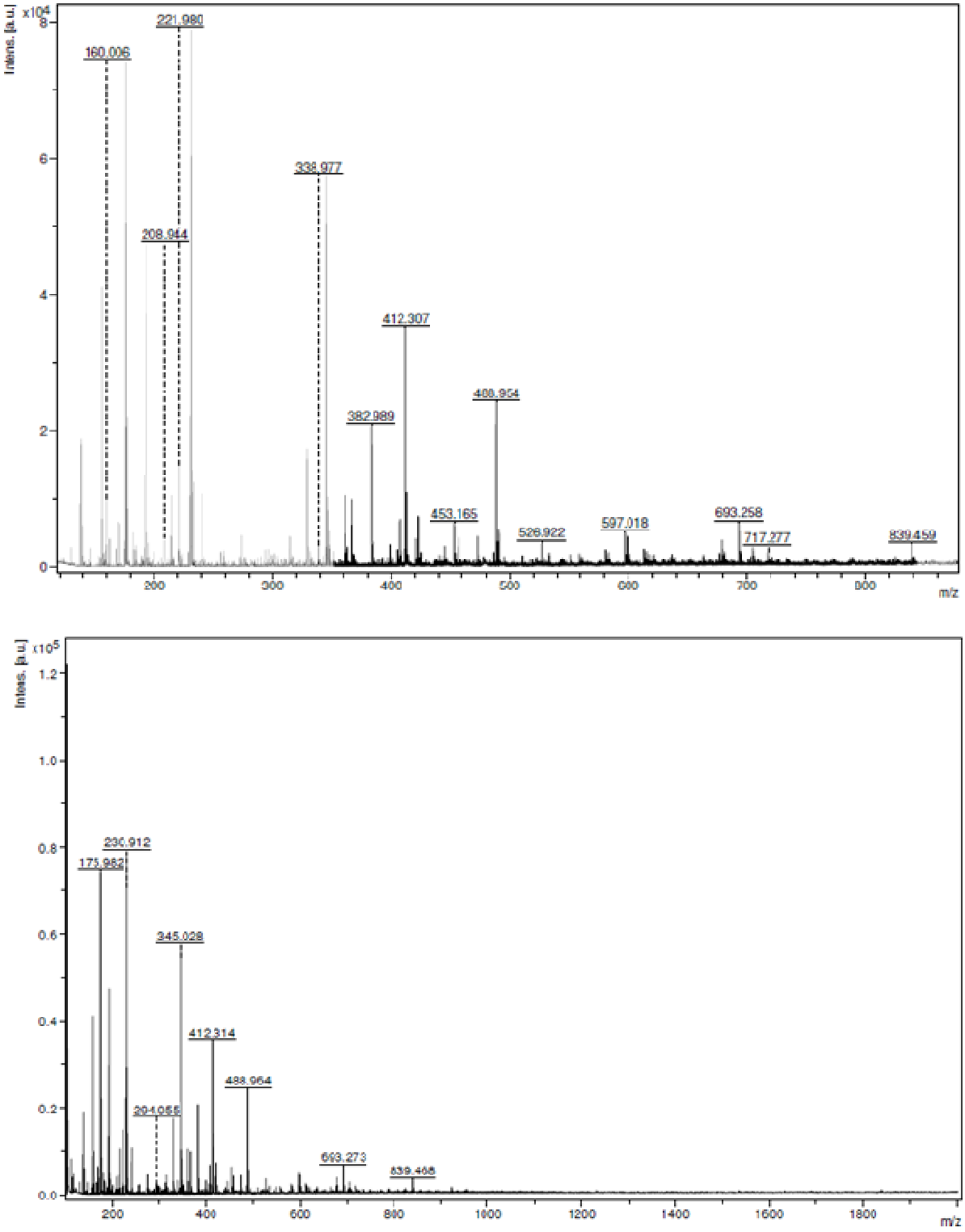

Fig. 1S. Mass spectra (positive ionization mode) of the F1 obtained from *P. moniliformis* leaves. In the top the low molecular weight peaks and in the bottom peaks between *m/z* 200 to 2000. The *m/z* values are shown to the sample peaks, excluding matrix peaks interfering.

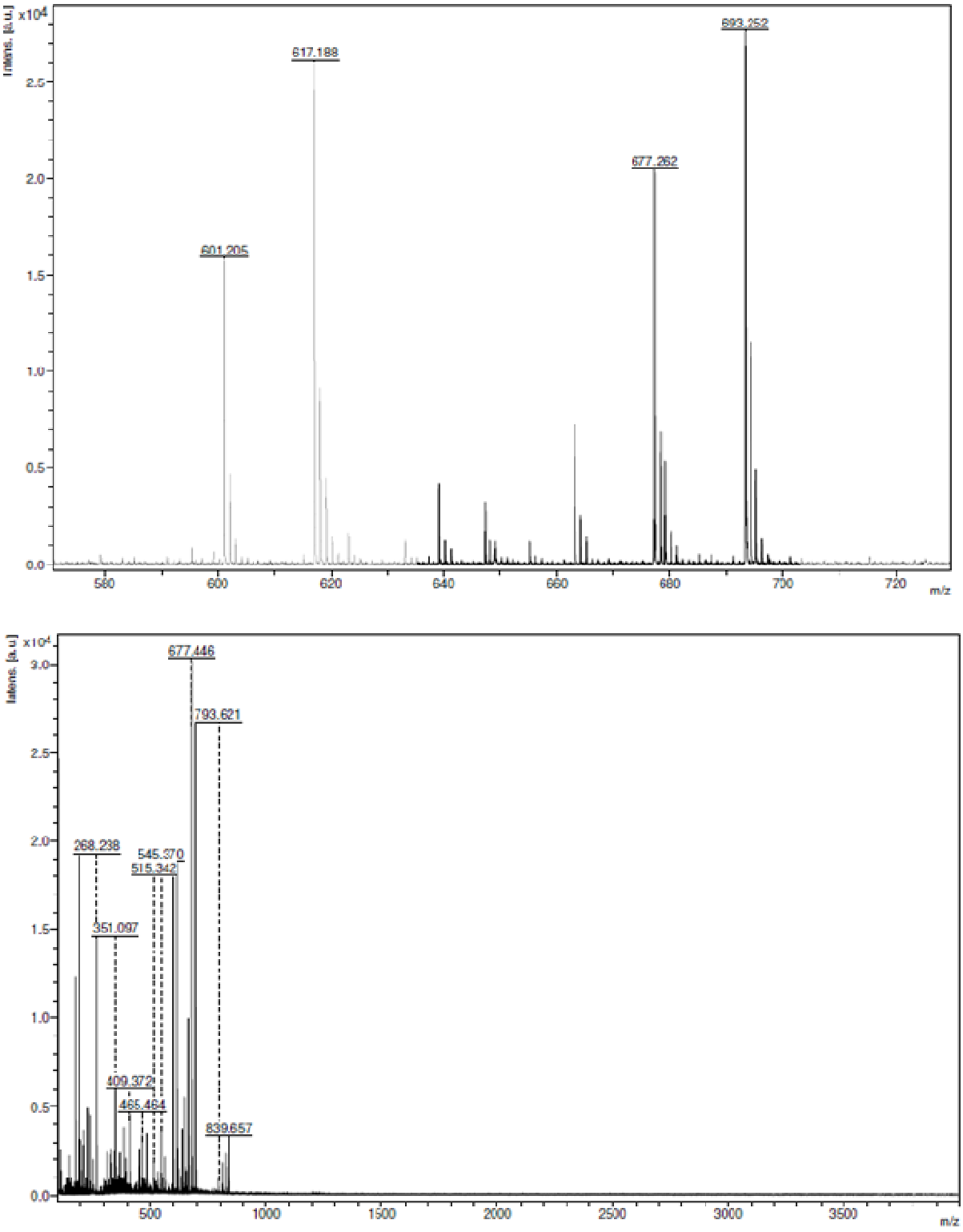

Fig. 2S. Mass spectra (positive ionization mode) of the F2 obtained from *P. moniliformis* leaves. In the top the low molecular weight peaks and in the bottom peaks up to *m/z* 4000. The *m/z* values are shown to the sample peaks, excluding matrix peaks interfering.

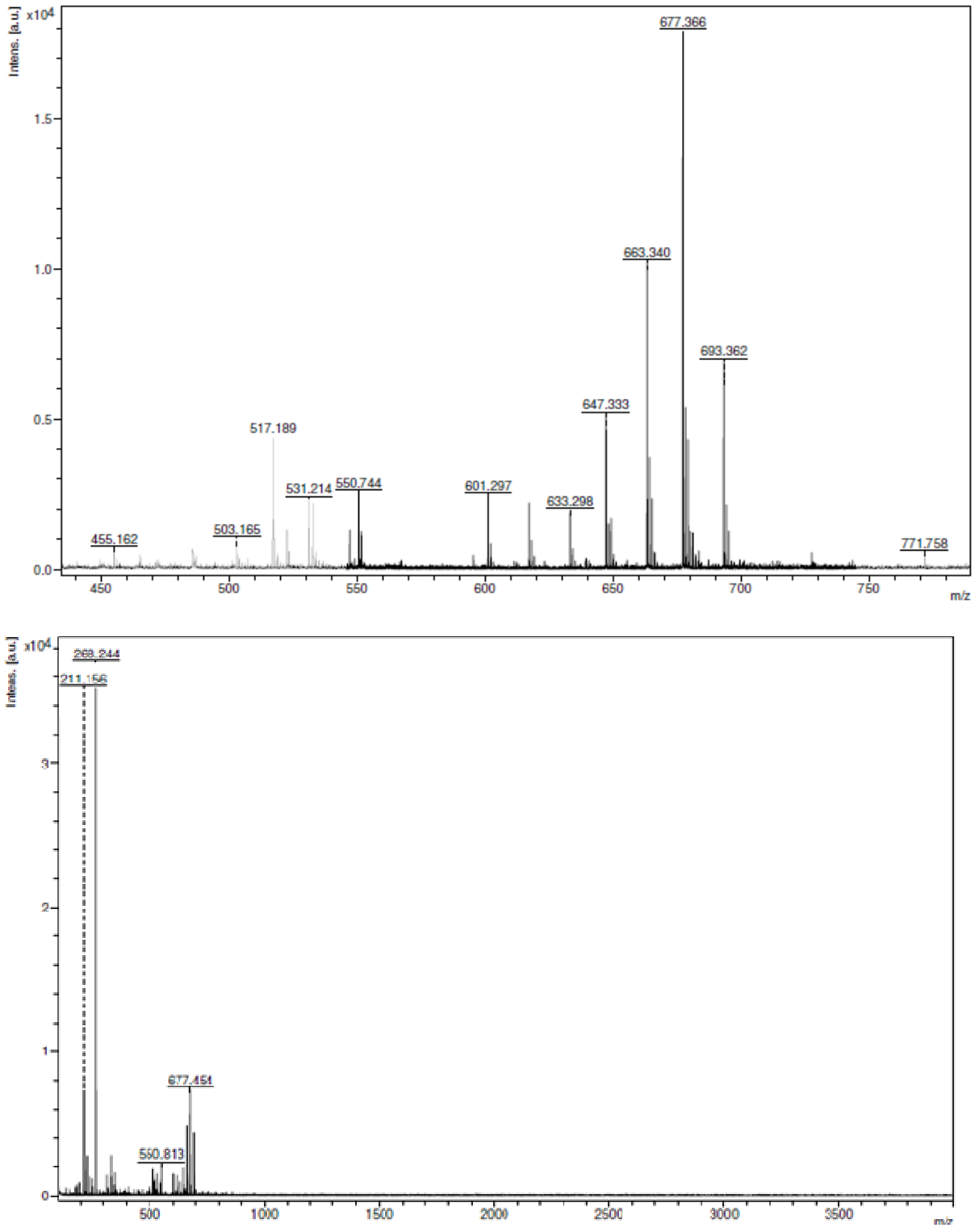

Fig. 3S. Mass spectra (positive ionization mode) of the F3 obtained from *P. moniliformis* leaves. In the top the low molecular weight peaks and in the bottom peaks up to *m/z* 4000. The *m/z* values are shown to the sample peaks, excluding matrix peaks interfering.

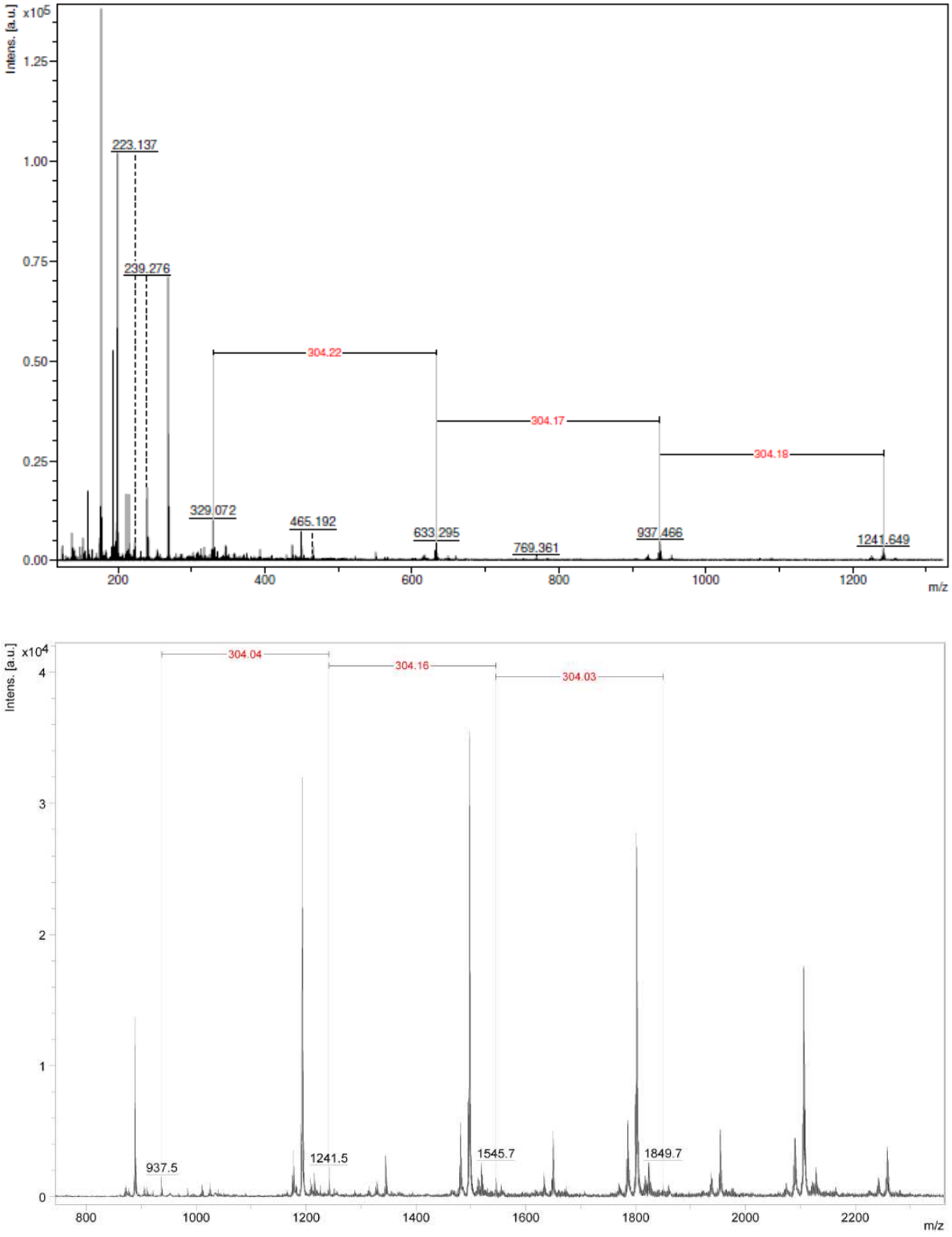

Fig. 4S. Mass spectra (positive ionization mode) of the F5 obtained from *P. moniliformis* leaves, highlighting the series 2. In the top the low molecular weight peaks and in the bottom peaks between *m/z* 800 to 2500.

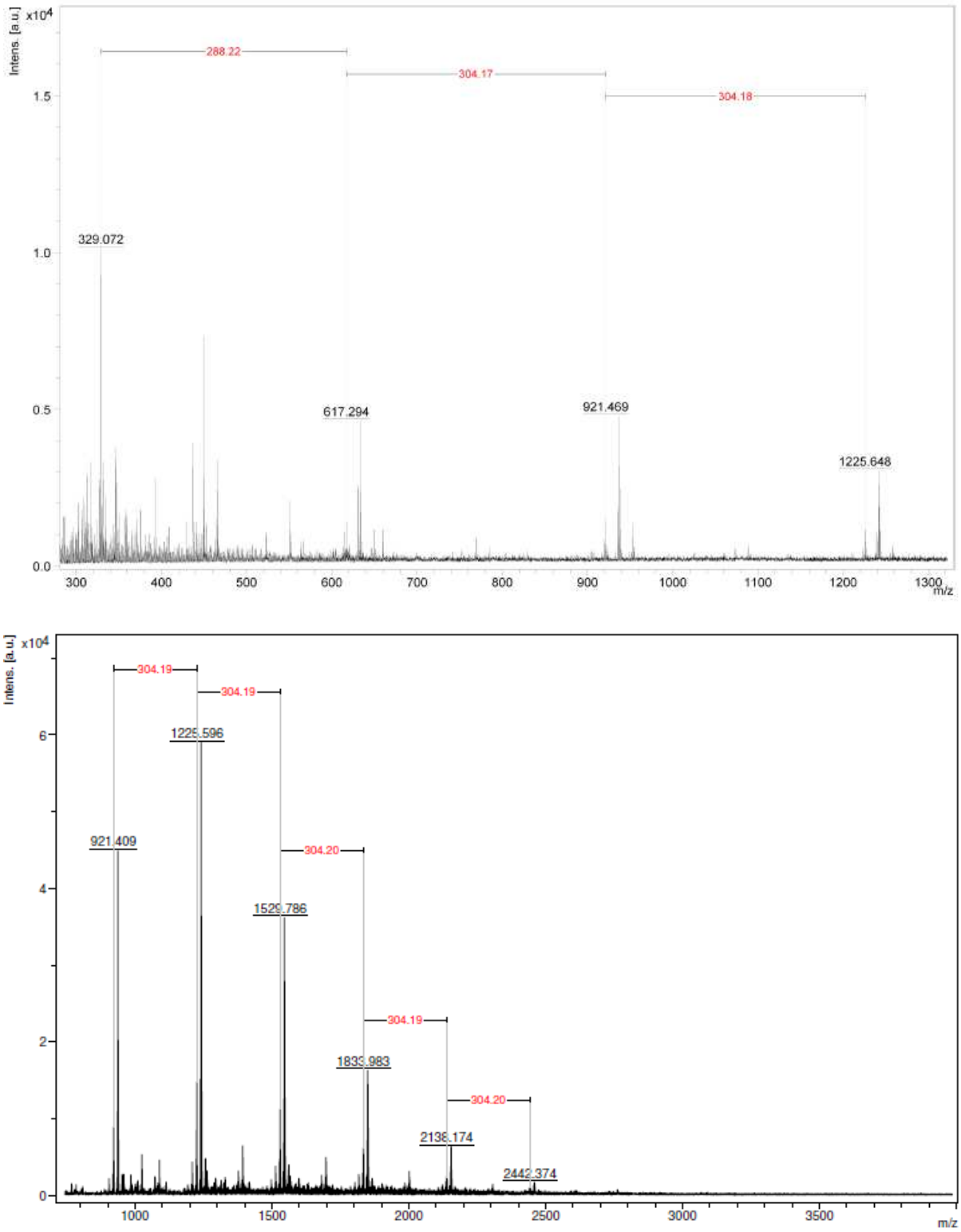

Fig. 5S. Mass spectra (positive ionization mode) of the F6 obtained from *P. moniliformis* leaves, highlighting the series 2. In the top the low molecular weight peaks and in the bottom peaks between *m/z* 800 to 4000.

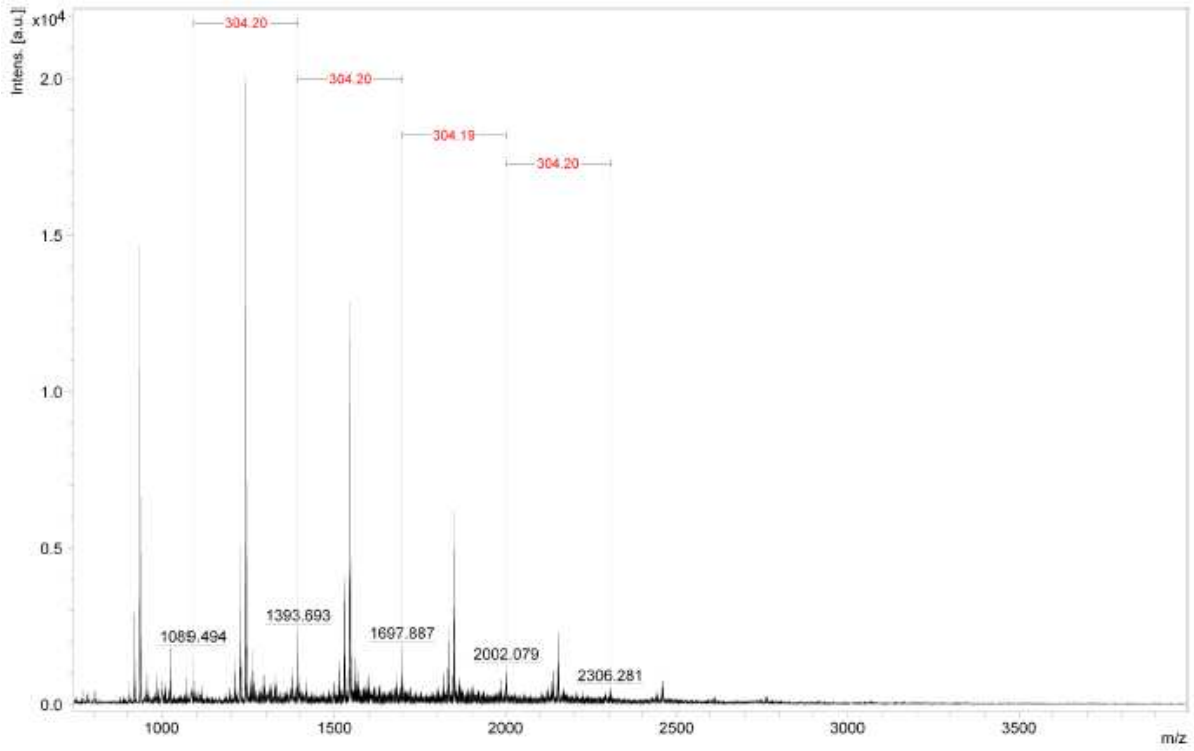

Fig. 6S. Mass spectra (positive ionization mode) of the F6 obtained from *P. moniliformis* leaves, highlighting the series 3.

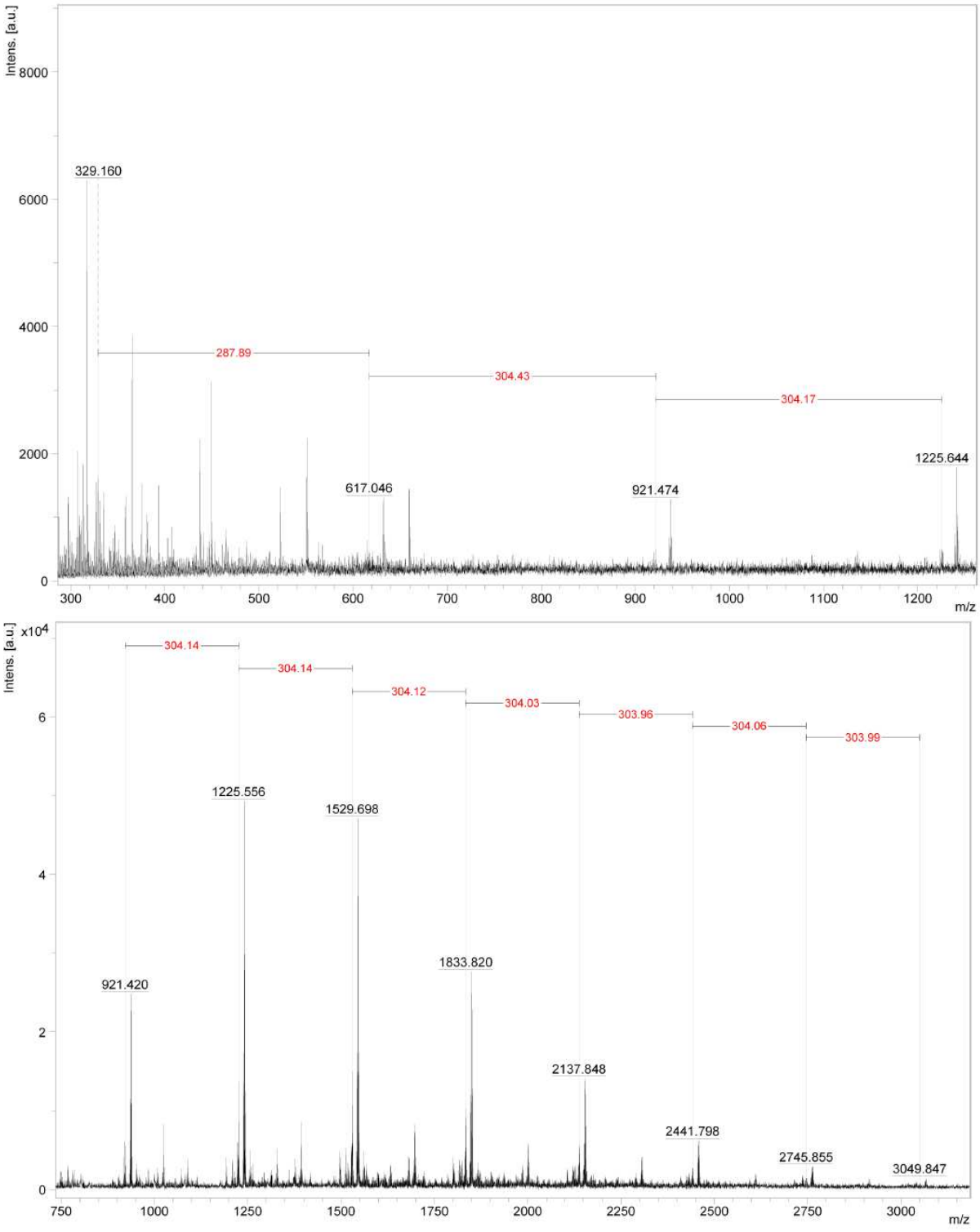

Fig. 7S. Mass spectra (positive ionization mode) of the F7 obtained from *P. moniliformis* leaves, highlighting the series 2. In the top the low molecular weight peaks and in the bottom peaks between *m/z* 750 to 3250.

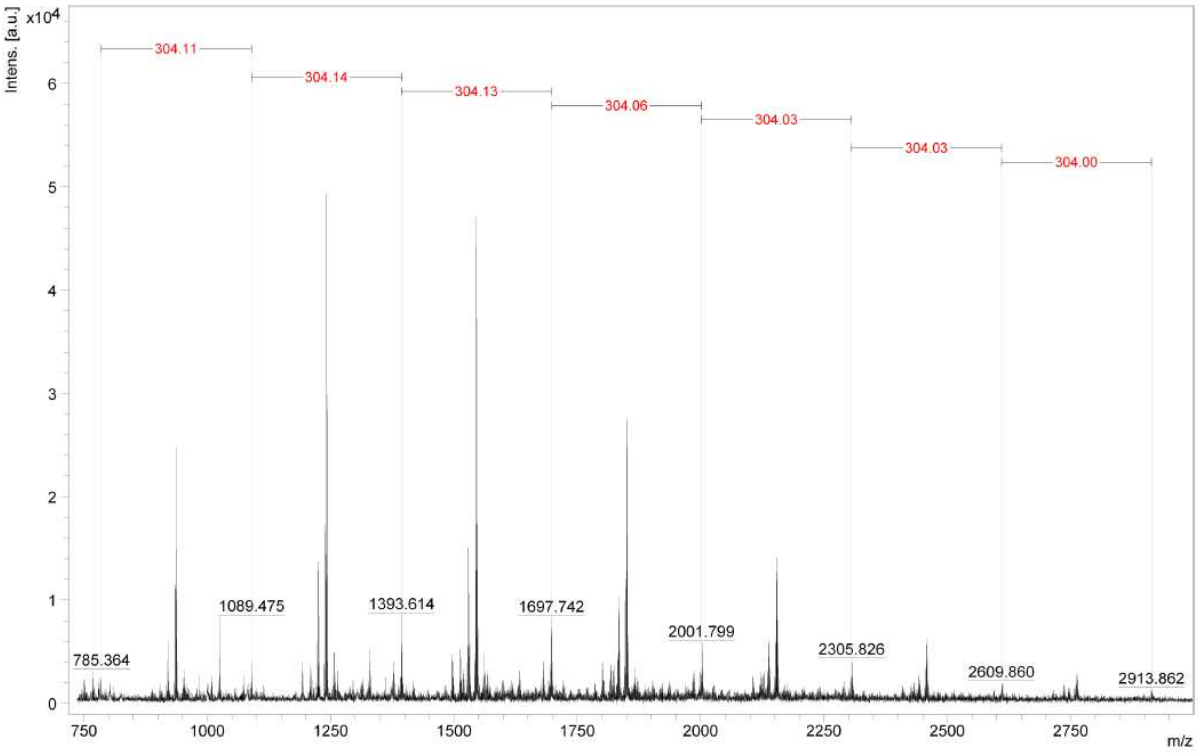

Fig. 8S. Mass spectra (positive ionization mode) of the F7 obtained from *P. moniliformis* leaves, highlighting the series 3.

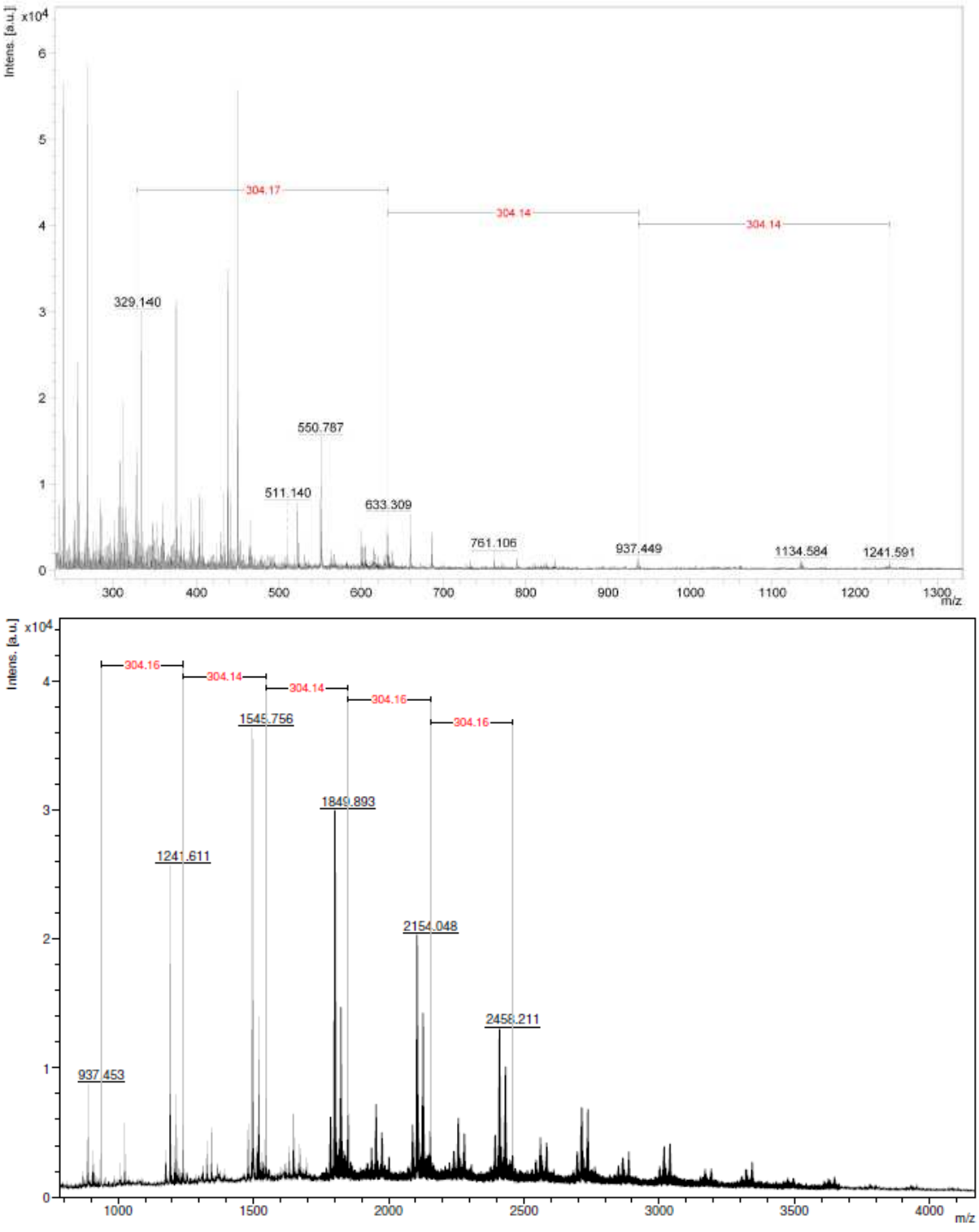

Fig. 9S. Mass spectra (positive ionization mode) of the F8 obtained from *P. moniliformis* leaves, highlighting the series 2. In the top the low molecular weight peaks and in the bottom peaks between *m/z* 800 to 4000.

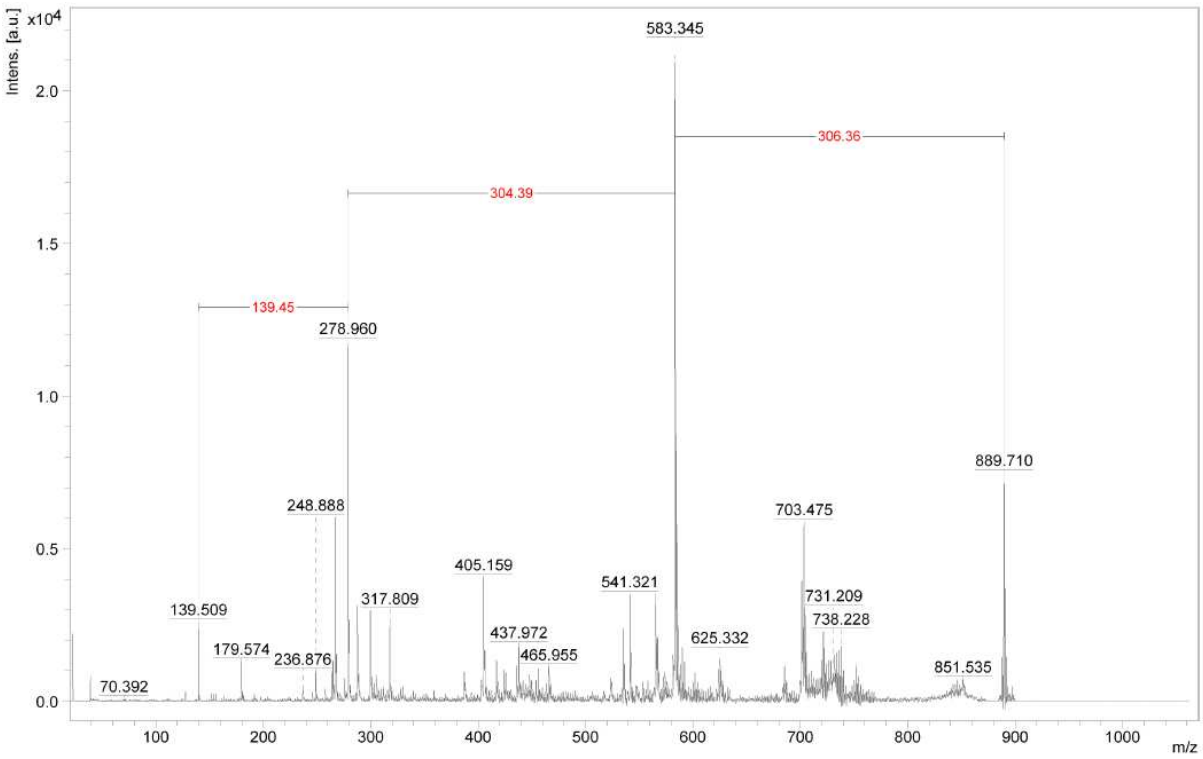

Fig. 10S. MS/MS spectrum of ion *m/z* 889 from F5 fraction series 1.

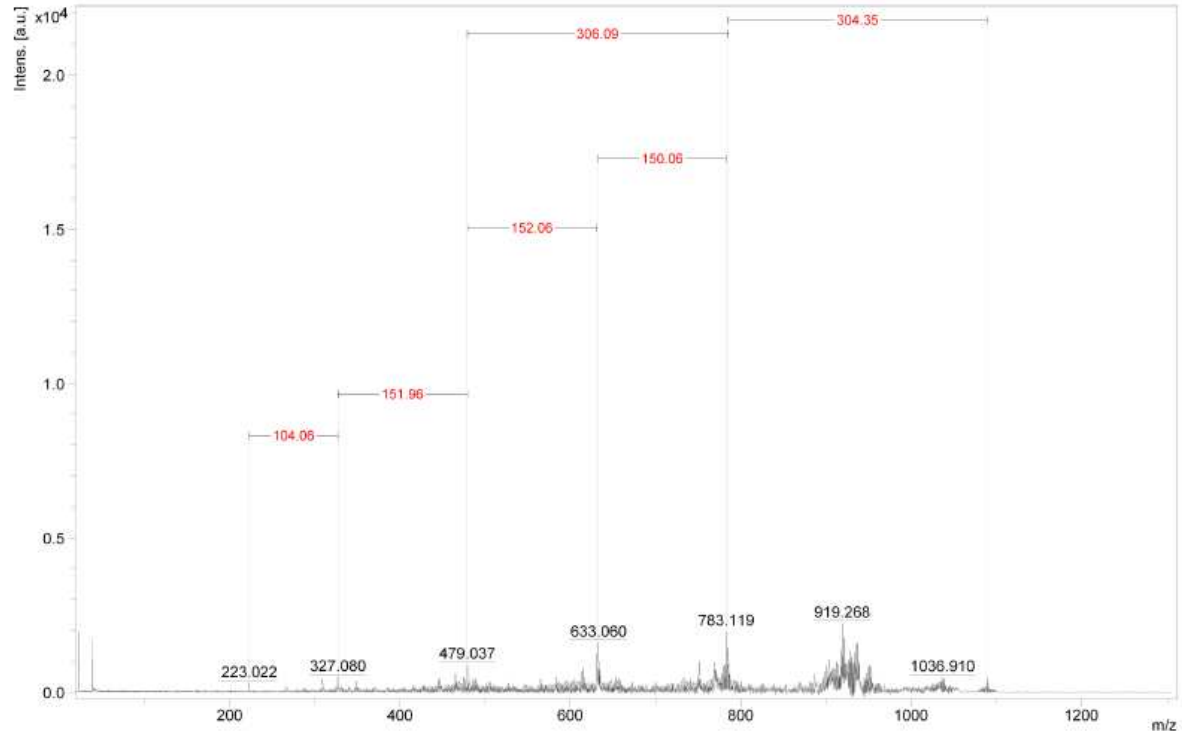

Fig. 11S. MS/MS spectrum of ion *m/z* 1089 from F6 fraction series 2.

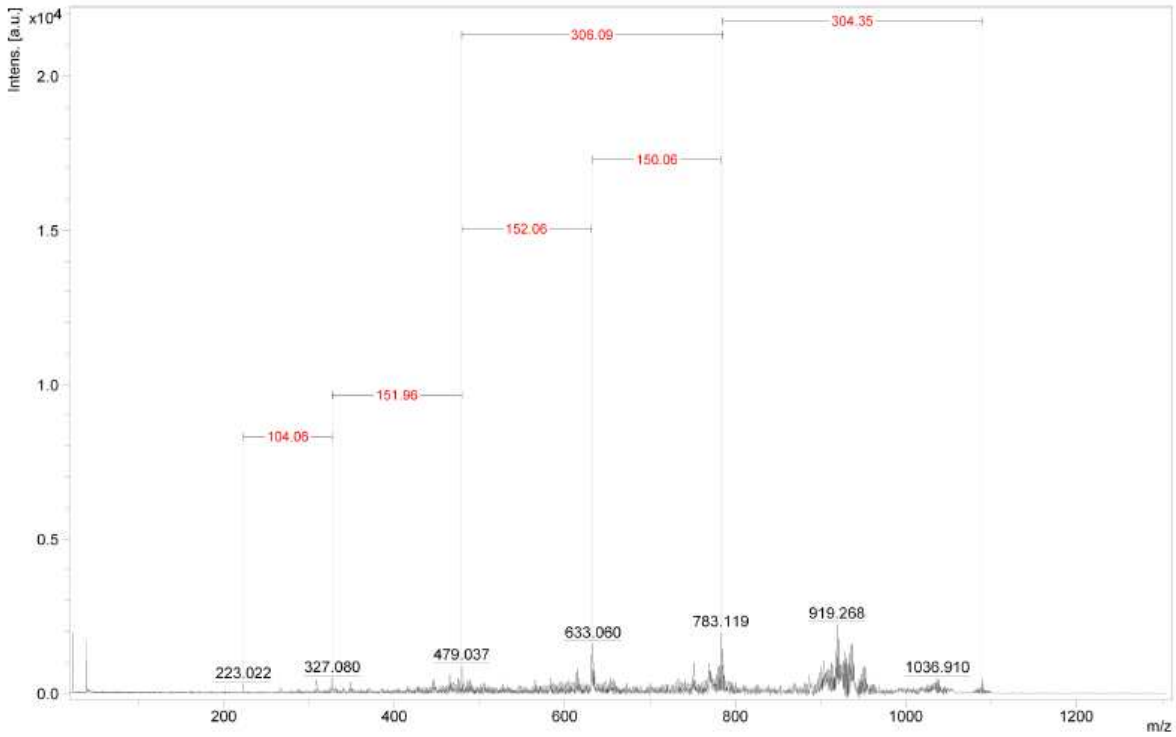

Fig. 12S. MS/MS spectrum of ion *m/z* 1089 from F7 fraction series 3.

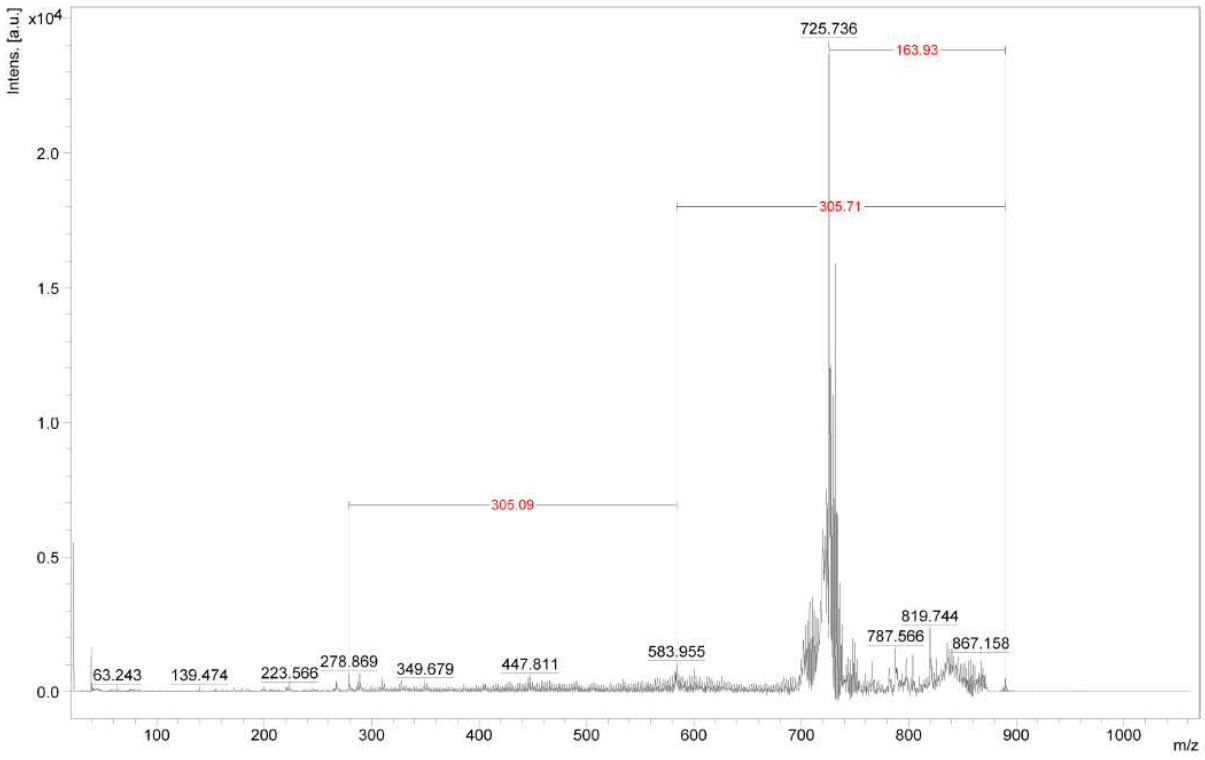

Fig. 13S. MS/MS spectrum of ion *m/z* 889 from F8 fraction series 1.

IV - CAPÍTULO 5 - Proanthocyanidins as an approach to develop anti-adherent surfaces to *Staphylococcus epidermidis* and friendly to mammalian cells

Running title: Proanthocyanidins as antibiofilm surface agent

Manuscrito a ser submetido para o periódico **Biomaterials**

Proanthocyanidins as an approach to develop anti-adherent surfaces to *Staphylococcus epidermidis* and friendly to mammalian cells

Running title: Proanthocyanidins as antibiofilm surface agent

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Abstract

Biomaterials play a fundamental role in the improvement of health care; however the microbial biofilm formation led to biomaterials-associated infection, which is now recognized as a major clinical problem. In this context, material surface modifications in order to mitigate bacterial colonization have been investigated and natural products have playing a significant role in the drug discovery. In this study, proanthocyanidins from *Pityrocarpa moniliformis* - a medicinal plant in semi-arid Brazil - characterized by B-type linkage and by richness of prodelphinidin units had their anti-adhesion effect evaluated. Prodelphinidin-riched proanthocyanidins completely prevented *Staphylococcus epidermidis* adhesion up to 0.125 mg mL^{-1} by a non-biocidal way, becoming the bacterial surface hydrophilic and covering model substratum surfaces. Interestingly, iron deprivation may not be related to the biological findings and proanthocyanidins presented a non-biological pathway of action. When the prodelphinidin-riched proanthocyanidins were spin coated onto surfaces, as demonstrated by XPS analysis and by their new hydrophilic character, surfaces were repellent to *S. epidermidis* but friendly to mammalian cells. These findings have important technological implications for a broad range of implant materials, which must promote tissue integration necessary for healing while simultaneously reduce the bacterial colonization. It evidences the potential applicability of the proanthocyanidins as coating for biomaterial surfaces.

Key-words: *Staphylococcus epidermidis*; bacterial adhesion; proanthocyanidin; coating; cell culture; XPS X-ray spectroscopy

1. Introduction

Biomaterials play a fundamental role in disease management and the improvement of health care. As the population ages, there is a growing need to sustain functions and physiological process critical to life, to restore or preserve a level of activity, for diagnosis of disease, treatment delivery and tissue engineering [1-3]. Over the last 4 decades, innovations in biomaterials and medical technology have had a sustainable impact on the diversity, function and number of biomaterials used worldwide. Currently, these market has already crossed U$28 billion and it is expected for the next few years an annual growth rate of 15%, reaching about of U$58.1 billion in 2014 [3]. Despite the advances, the major drawback to implanted biomaterials remains to be their susceptibility to microorganism adhesion and the subsequent biomaterials-associated infection that is recognized as a major clinical problem [4,5]. Importantly, bacterial attachment to a surface and further biofilm formation promotes metabolic, phenotypic and genotypic changes that make their eradication extremely difficult [6]. Bacterial biofilms constitute a structured consortium of bacteria encased in a self-produced matrix, been very resistant to antimicrobials and able to evade host immune system [7]. For these reasons, the adhesion - the first stage of biofilm formation - can be considered an attractive target to restrict or to control biofilm infection.

Surface modifications in order to mitigate bacterial colonization have been extensively investigated, focusing mainly (i) the modification of the surface itself via surface engineering methods to produce antifouling materials [8-11] or (ii) by the incorporation of antimicrobials compounds onto surfaces [12-15]. Although promising results have been showed using this second approach, the continuous use of antimicrobials agents is controversial since it can induce selective pressure for bacterial resistance and also due to the concomitant decline in the development of new antimicrobials [16,17].

While *Staphylococcus epidermidis* was for a long time regarded as innocuous, recently it has been recognized as reservoirs of genes that facilitate methicillin-resistant *S. aureus* colonization [18] and as the most frequent cause of indwelling medical devices infections, which characteristically involve biofilms [19,20]. This

probably stems from the fact that *S. epidermidis* is a permanent and ubiquitous colonizer of human skin, resulting in a high probability of device contamination during insertion [21].

In this context, natural products offer a diverse range of structurally distinctive bioactive molecules and they have been used as a major source of innovative and effective therapeutic agents throughout human history, playing a highly significant role in the drug discovery and development processes [22]. From the existing literature, polyphenols, terpenoids, alkaloids, lectins, polypeptides and poly-acetylenes represent phytochemicals that present antibacterial activity [23,24]. Regarding antibiofilm compounds, the ability of condensed tannins called proanthocyanidins (a subclass of polyphenol compounds), especially from North American cranberry (*Vaccinium macrocarpon*), to suppress bacterial adhesion of diverse microorganisms have been extensively reported [25-29]. We have shown that *Pityrocarpa moniliformis*, a widespread medicinal plant in dry forests of semi-arid Northeastern Brazil (Caatinga) is source of proanthocyanidins (polyflavan-3-ols structures) which differ from cranberry proanthocyanidins regarding the kind of intermolecular bonds (linear B-type interflavanyl linkages) and the pattern of hidroxilation (riched in prodelphinidin) (unpublished data). Our motivation for using *P. moniliformis*-derived proanthocyanidins in the prevention of *S. epidermidis* biofilm formation lies that its action as antiadhesive compound occurs independent of antibacterial mechanism, which may hamper the selective development of resistant strains (unpublished data).

In this study we investigate the *in vitro* effects and the pathways involved on anti-adhesion action of proanthocyanidins from *P. moniliformis* upon hydrophilic and hydrophobic model surfaces. In addition we performed proanthocyanidins-coated substrates and characterized them by physicochemical techniques. Importantly, the adhesion and spreading of epithelial mammalian cells on coated surface were preserved despite this modified surface being repellent to *S. epidermidis*.

2. Materials and Methods

2.1. Surface, bacterial strain and culture conditions

Bacterial adhesion is determined both by the type of microorganism and by the properties of the surface of the material involved [30]. In this study we performed the experiments employing hydrophobic (polystyrene and Permanox™) and hydrophilic (glass) material models. *Staphylococcus epidermidis* ATCC 35984 was grown in Mueller Hinton (MH) agar overnight, at 37 °C, and a bacterial suspension in 0.9% NaCl, corresponding to optical density at 600 nm (OD_{600}) of 0.150 (3×10^8 CFU mL^{-1}), was used in the assays.

2.2. Proanthocyanidins from Pityrocarpa moniliformis *leaves*

Proanthocyanidins from *P. moniliformis* leaves were purified, characterized and had their chemical structures elucidated in our previously work (see caption 4). In this study we used a proanthocyanidins fraction obtained by chromatographic purification with 50% acetone through Sephadex LH20. This bioactive fraction is constituted by a majority series of prodelphnidin homopolymer and by two minoritary series of prodelphinidin heteropolymer (having one unit of procyanidin or one residue of galloyl). These olygomers present linear B-type interflavanyl linkages and degree of polymerization varying from 1 to 10 (Fig. 1). Aqueous solutions of proanthocyanidins were prepared in the day of each experiment using ultrapure MilliQ water (Millipore, Bedford, USA) and were sterilized by 0.22 µm filtration. In the experiments of coating, the proanthocyanidins were dissolved in 30 or 70% aqueous acetone.

2.3. Adhesion and biofilm formation assay

Adhesion and biofilm formation by *S. epidermidis* were evaluated by the crystal violet test as previously described by Trentin et al., 2011[31]. Briefly, in the sterile 96-well polystyrene flat-bottom microtiter plates (Costar Corning 3599, USA), we added 80 µL of the bacterial suspension, 80 µL of the proanthocyanidins and 40 µL of tryptone soya broth (TSB) (Oxoid Ltd., England). Following the incubation period (37 °C for 24 h) the adherent biofilm layer formed was stained with crystal violet and the absorbance at 570 nm was measured (Spectramax M2e Multimode Microplate Reader, Molecular Devices, USA). The biofilm formation control was considered to represent 100% of biofilm formation, replacing the proanthocyanidins by water. Since there is

not a commercially available non-biocidal compound possessing antibiofilm activity, we can not apply a positive control to antibiofilm activity.

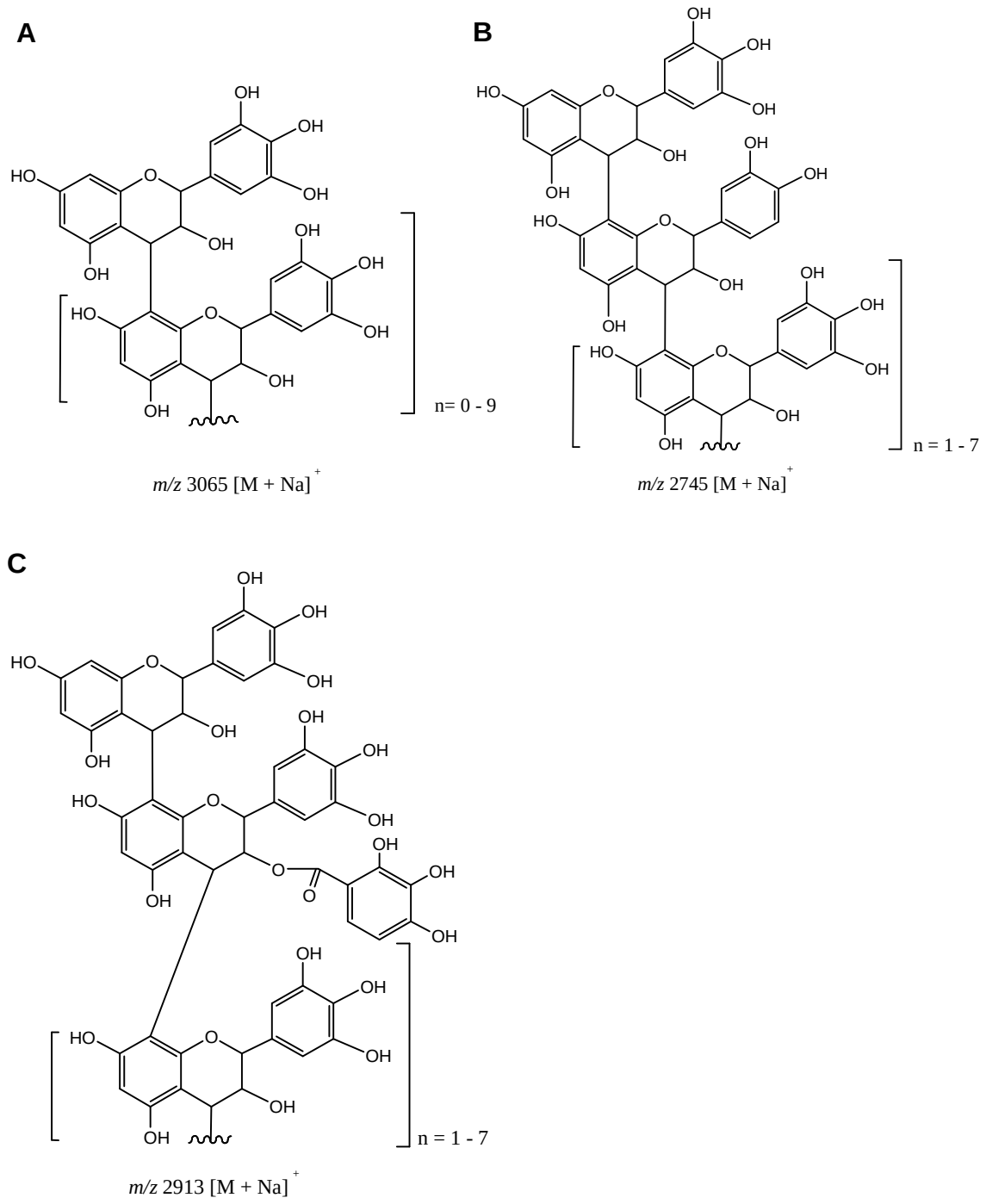

Fig. 1. Chemical structures of the proanthocyanidins from *P. moniliformis* leaves. The majority series of prodelphinidin homopolymer, reaching up to *m/z* 3065 [M+Na] (A) and the minority series having one procyanidin (B) or one galloyl (C) unit, reaching up to *m/z* 2745 and 2913 [M+Na], respectively. The monomeric and dimeric prodelphinidin and the dimer linked to the galloyl as well as the dimer prodelphinidin-procyanidin also were detected.

A set of experiments were performed by pre-treating the bacterial suspension (2 mL), proanthocyanidins (2 mL) and TSB (1 mL) in plastic tubes, respecting the proportions used in the microplates, during 24 h at 37 °C, and after evaluating its ability to adhere and to form biofilm. As control, proanthocyanidins were replaced by water. Afterwards bacterial cells were washed three times with sterile 0.9% NaCl solution and harvested by centrifugation in order to obtain a proanthocyanidin-treated bacterial pellet. Each inoculum was adjusted with sterile 0.9% NaCl solution (optical density of 0.150 at 600 nm). These pre-treated bacterial cells (80 µL), water (80 µL) and TSB (40 µL) were placed in the 96-well microtiter plates and incubated during 24 h at 37 °C. The biofilm formation was evaluated by the crystal violet assay and the bacterial viability by counting of CFU mL^{-1}, as described below.

2.4. Bacterial growth and viability

Bacterial growth was evaluated in the microplates by measuring the difference between the arithmetic means of the OD_{600} lectures after 24 h (incubation time) of wells having the proanthocyanidins, TSB and bacterial suspension, from the wells possessing the proanthocyanidins, TSB and sterile saline (without inoculum). By this way, the interference of the samples color in all the results obtained by OD evaluations was avoided. As control for bacterial growth, the proanthocyanidins were replaced by 80 µL of water, representing 100% of growth, or by rifampicin at 8 µg mL^{-1} (Sigma-Aldrich Co., USA), representing the positive control for the inhibition of bacterial growth. To verify the viability of bacterial cells, serial dilutions of the wells were performed and were spread on MH agar plates. After overnight incubation at 37 °C, the number of colony-forming units (CFU) was determined and expressed as log CFU mL^{-1}.

2.5. Scanning Electron Microscopy (SEM)

Biofilms of *S. epidermidis* were grown in 96-well microtiter plates, as described in Section 2.3, with a piece of Permanox™ slide or a glass coverslip in each well. After 24 h of incubation at 37 °C, the samples were fixed in 2.5% glutaraldehyde, washed with 100 mM cacodylate buffer pH 7.2 and dehydrated in increasing

concentrations of acetone. The samples were dried by the CO_2 critical point technique and examined in a scanning electron microscope.

2.6. Confocal Laser Scanning Microscopy (CLSM) examination of biofilm structure

Using sterile polystyrene and coverglass-bottom dishes, 800 µL of the bacterial suspension, 800 µL of the proanthocyanidins and 400 µL of TSB were incubated (24 h at 37 °C). After, the samples were stained with 4 µL of the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, USA) during 30 minutes at room temperature in darkness, and the dishes content was gently replaced by water. Finally, samples were directly observed with Confocal Laser Scanning Microscope (Leica DM 6000 CS) using a 63X upright objective. To each sample, a sequential scan in two channels was carried out of each corresponding xy optical section and 4 random images were acquired. Overlapping images and orthogonal cuts were obtained by using the MetaMorph and the Imaris x64 softwares.

2.7. Iron chelation assessment

Ferrozine is a specific reagent which forms a magenta colored complex with ferrous ions. A standard curve was established to determine the Fe^{2+} concentration to be used in the ferrozine assay as described elsewhere [32]. After, 50 µL of proanthocyanidins solution mixed with 50 µL of 200 µM Fe^{+2} ($FeSO_4$. $7H_2O$, Sigma-Aldrich Co., USA) in phosphate-buffered saline (PBS) pH 7.2 and with 50 µL of a 10 mM hydroxylamine aqueous solution, were incubated for 5 minutes at room temperature. Oxidation of ferrous iron was prevented by adding, firstly, the hydroxylamine aqueous solution to the reaction mixture. Following the incubation, 50 µL of 10 mM ferrozine aqueous solution was added to the wells. Formation of the iron-ferrozine complex was then determined at 562 nm to assess the amount of iron chelated by proanthocyanidins. For comparison of iron-chelating activity, 2,2-bipyridyl (Sigma-Aldrich Co., USA) was used as a standard iron chelator.

2.8. Bacterial surface hydrophobicity index

Surface hydrophobicity of *S. epidermidis* was determined using the microbial adhesion to hydrocarbon (MATH) test [33]. The samples were prepared in plastic tubes according to the Section 2.3 (pre-treating of samples) and washed cells were harvested by centrifugation. The bacterial suspensions were adjusted to an initial OD_{600} (OD_i) of about 0.3. Toluene (200 µL) was added to 1 mL of each adjusted bacterial suspension and mixed. The final absorbances of aqueous phase (OD_f) were measured after phase separation. The hydrophobicity index (HPBI) was expressed as: (OD_i - OD_f) / OD_i x 100%. Values of HPBI greater than 70% indicated hydrophobic bacterial surface and lesser than 70% indicated hydrophilic bacterial surface.

2.9. Nonbiological model particles adhesion experiments

Additional experiments were conducted using red fluorescent FluoSpheres™ beads (Life Technologies, USA) using a protocol modified from Eydelnant and Tufenkji (2008) [27]. The microspheres were selected to be similar in size to the *S. epidermidis* (1.0 µm diameter) and were used in the same concentration of the bacterial suspension (3×10^8 particles mL^{-1}). These experiments were performed using sterile polystyrene and coverglass-bottom dishes according Section 2.6. The dishes also were incubated at 37 °C during 24 h and afterwards, they were gently washed with sterile 0.9% NaCl and immediately visualized using an Olympus IX71 fluorescence microscope.

2.10. Proanthocyanidins-coated surfaces

Precisely 300 µL of a 4.0 mg mL^{-1} proanthocyanidins solution in 30 or 70% aqueous acetone (Merck, Germany) was spin coated onto a Permanox slide (30 x 25 mm^2) during a cycle of 500 rpm (5 seconds) and then accelerated to 5000 rpm (40 seconds) in the spin coater Laurell Model WS-650MZ-23NPP/LITE. After coating, the substrates were heat-treated (2 h at 80 °C) to allow the film annealing and to remove any excess of solvent. Samples were sterilized by UV light during 20 minutes and then were cut in three parts to produce 10 x 25 mm^2 coated-substrates. As control, samples spin coated with 300 µL of the 30 or 70% aqueous acetone solution and samples

heated at 80 °C and UV-treated but without proanthocyanidins, were included. Following preparation, the samples were investigated regarding *S. epidermidis* adhesion and biofilm formation, mammalian cells adhesion and proliferation and surface physicochemical characterization.

2.11. Surface characterization

Permanox samples were characterized before and after the coating with proanthocyanidins as well as after washing protocols using X-ray photoelectron spectroscopy (XPS) and water contact angle (WCA) measurements. Chemical speciation of the C 1s signal was achieved with an Omicron SPHERA spectrometer at a pass energy of 5 eV using Mg Kα radiation (1253.6 eV). Spectral fitting was performed using CASA XPS software. Contact angle measurements were carried out using the sessile drop technique and doubly deionized water. The drop was observed directly with an Olympus BX-41 microscope objective lens and images were digitally captured using a 1.4 megapixel CCD camera. The reported water contact angles are means of more than five measurements performed in different areas of each sample surface.

2.12. Cell culture and cytotoxicity of free- and coated- proanthocyanidins

The mammalian epithelial Vero cell line (purchased from Banco de Células do Rio de Janeiro, Brazil) was used to determine the cytotoxicity of the free- and coated-proanthocyanidins. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin and were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Once the cells reached confluence, they were sub-cultured by treating them with 0.05% trypsin-EDTA (Life Technologies, USA).

The cytotoxicity of free form-proanthocyanidins was investigated using the MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, USA) assay [34]. Exponentially growing Vero cells were washed and seeded at 1.5 x 10^4 cells/well in 96 well microplates. When a cell monolayer was formed (24 h, 5% CO_2 at 37 °C), in each well the medium was replaced by 120 μL of the proanthocyanidins dissolved in

supplemented DMEM and re-incubated for 24 h. The wells content was removed and the cells were washed with 1X PBS. A solution of 120 µL of MTT (0.5 mg mL^{-1}) was added in each well and the plates were incubated in 37 °C for 1h. After, the cells were washed with 1X PBS buffer and blue crystals were resuspended with DMSO (Sigma-Aldrich Co., USA). Reduced MTT was measured at 570 nm. In untreated cells, the proanthocyanidins were replaced by water (100% of viability) while 1% triton X-100 solution was used as positive control.

To evaluate the biocompatibility of proanthocyanidins-coated surfaces, segments of the proanthocyanidins-coated Permanox and acetone-coated Permanox were maintained onto the bottom of 24-wells tissue culture plates (TPP Techno Plastic Products, Switzerland). Exponentially growing Vero cells were washed and seeded at 5 x 10^4 cells/well. After incubation (24 h, 5% CO_2 at 37 °C), the segments were removed and washed with 1X PBS. For microscopy imaging, Vero cells that grown on the coated Permanox were mildly fixed with 0.2% glutaraldehyde in PEM buffer (100 mM pipes, 1 mM EGTA, 2 mM $MgCl_2$, pH 6.8) at room temperature for 15 minutes, and then in 2 mg mL^{-1} $NaBH_4$ to reduce glutaraldehyde autofluorescence. Segments were washed with 1X PBS and the adherent cells had the microtubules labelled with 1µM fluorescent taxoid FLUTAX-2 [35,36] (150 µL at 37 °C for 30 minutes), which was kindly provided by Dr. André A. Souto (Faculdade de Química, PUCRS, Brazil) and the DNA stained with 10 µg mL^{-1} of 4',6-diamino-2-phenylindole (Sigma-Aldrich Co., USA) (150 µL for 15 minutes). Images were obtained using an Olympus IX81 confocal microscope and UPLSAPO 60X W NA:1.20 objective and overlaid using the Olympus FV 1000 software.

2.13. Statistical analysis

The data presented are expressed as mean or percentual mean ± standard deviation (SD). Results concerning on bacterial viability are presented as mean ± SD of logarithmic CFU mL^{-1}. Each experiment was repeated three times. Statistical differences were determined by Student's t test and considered statistically significant at p value ≤ 0.05.

3. Results

3.1. Non-biocidal antibiofilm action of proanthocyanidins dose-response curve

The dose-response curve, testing 7 concentrations of proanthocyanidins showed that biofilm formation by *S. epidermidis* is completely suppressed from 4.0 up to 0.125 mg mL^{-1} without growth inhibiting association (Fig. 2A). When tested against the epithelial mammalian cell line, the proanthocyanidins did not demonstrate cytotoxicity at 0.125 mg mL^{-1}, the lesser concentration that prevented biofilm formation (Fig. 2B). These results were complemented with CLSM analysis using LIVE/DEAD staining, which demonstrated the architectural features of the untreated *S. epidermidis* biofilm in both, polystyrene and glass, surfaces (Fig. 3 A1 and B1). The images corroborated with the dose-response curve, showing that (i) at 0.125 mg mL^{-1} the proanthocyanidins inhibited biofilm formation, keeping most of cells in the planktonic state and thereby most of them were removed during the washing procedure (Fig. 3 A2 and B2) and that (ii) at 0.0625 mg mL^{-1}, *S. epidermidis* were able to form biofilm displaying to restore the typical topography of *S. epidermidis* on hydrophobic as well as on hydrophilic model surfaces (Fig. 3 A3 and B3). When bacterial cells were 24 h previously treated with proanthocyanidins and then washed to remove the exposure to this agent, *S. epidermidis* cells demonstrated to remain viable and to recover about 50% of biofilm formation ability on polystyrene (Fig. 4).

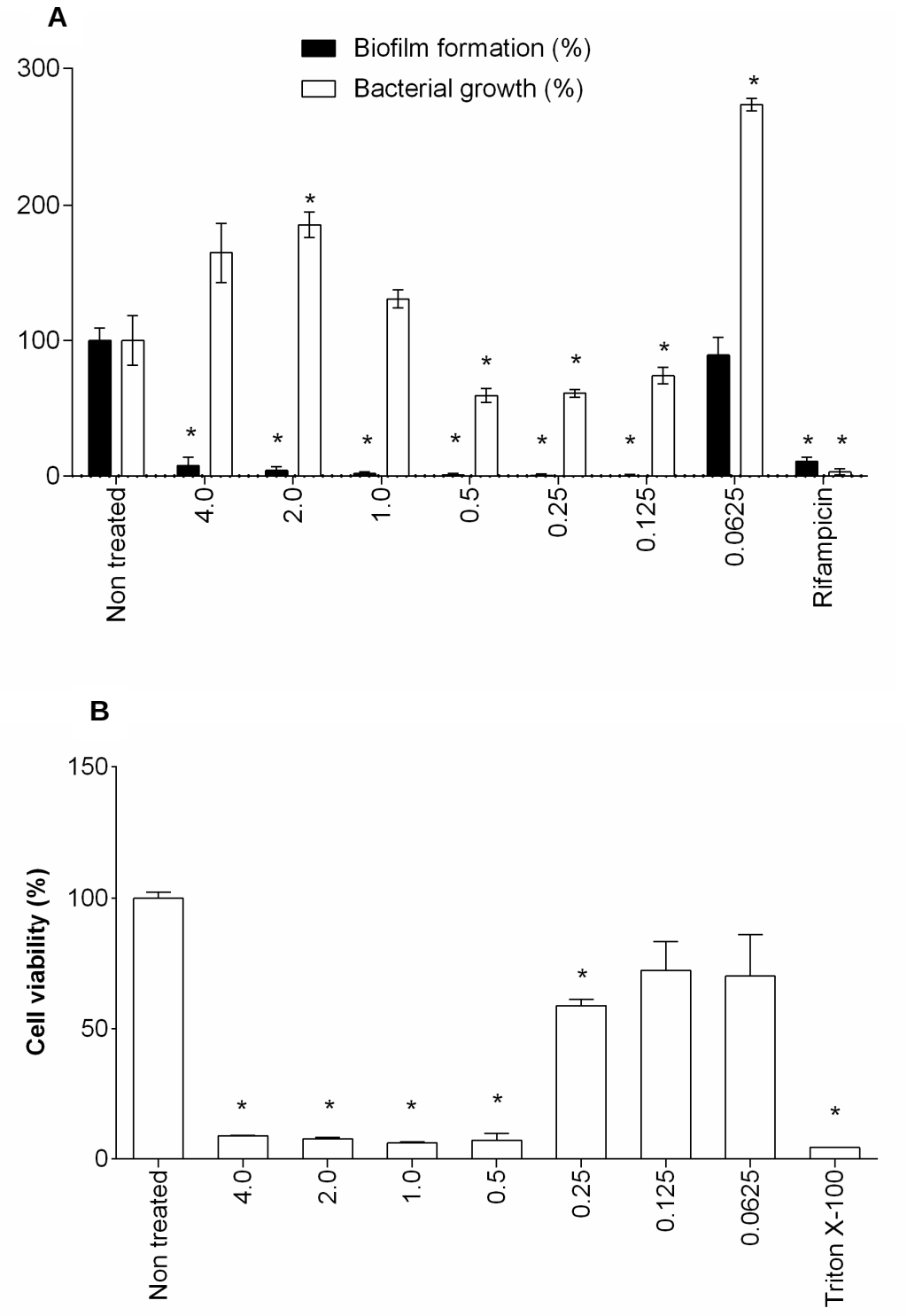

Fig. 2. Proanthocyanidins dose-response curve (mg mL^{-1}). The effect of different concentrations of proanthocyanidins on *S. epidermidis* biofilm formation and growth (A) and on epithelial mammalian cells viability (B). * represents statistical difference in relation to the non treated samples.

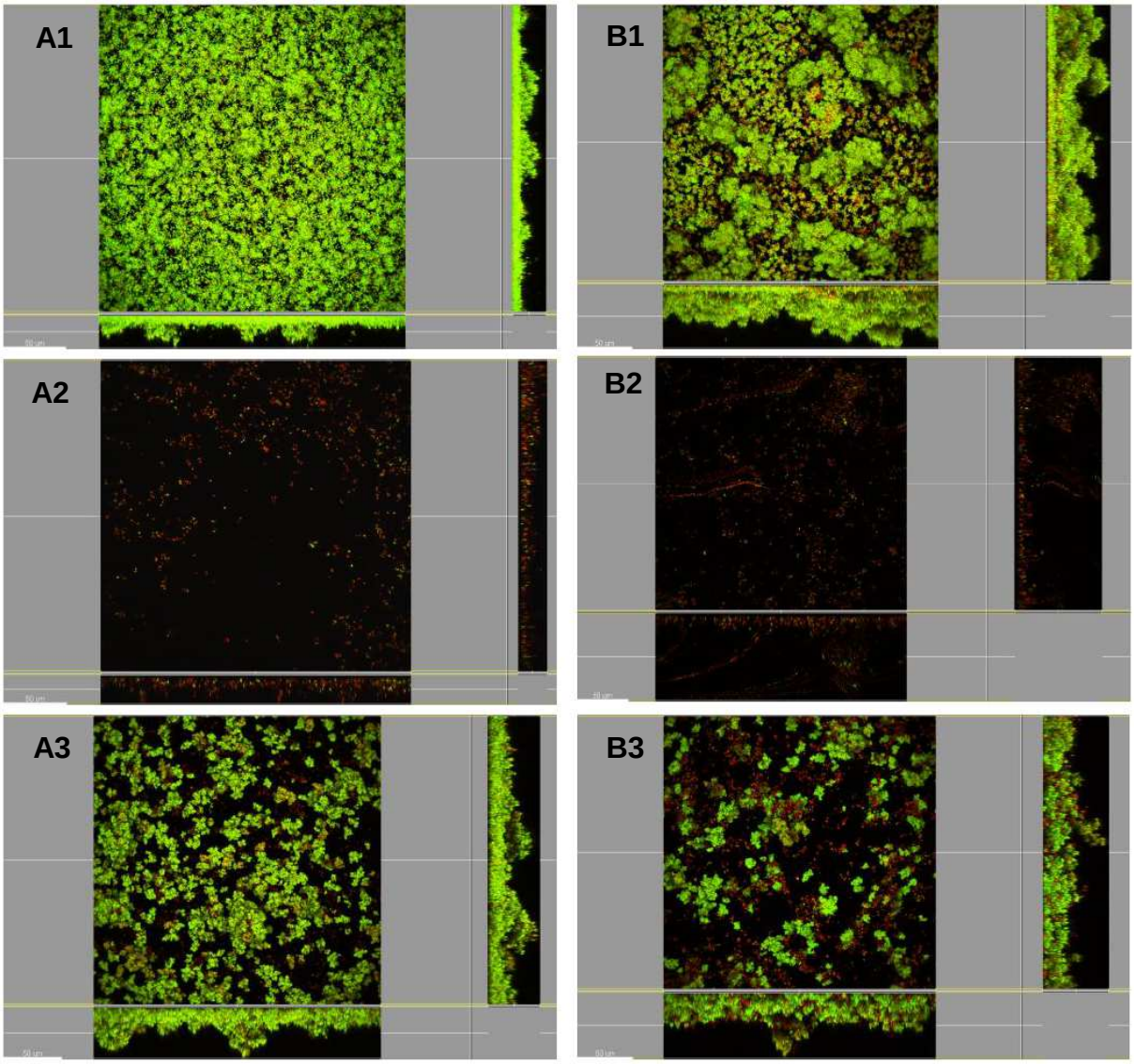

Fig. 3. CLSM analysis of biofilm topography on polystyrene (A) and on glass (B) surfaces of non-treated *S. epidermidis* (1), and of proanthocyanidins-treated bacteria at 0.125 mg mL^{-1} (2) and 0.0625 mg mL^{-1} (3).

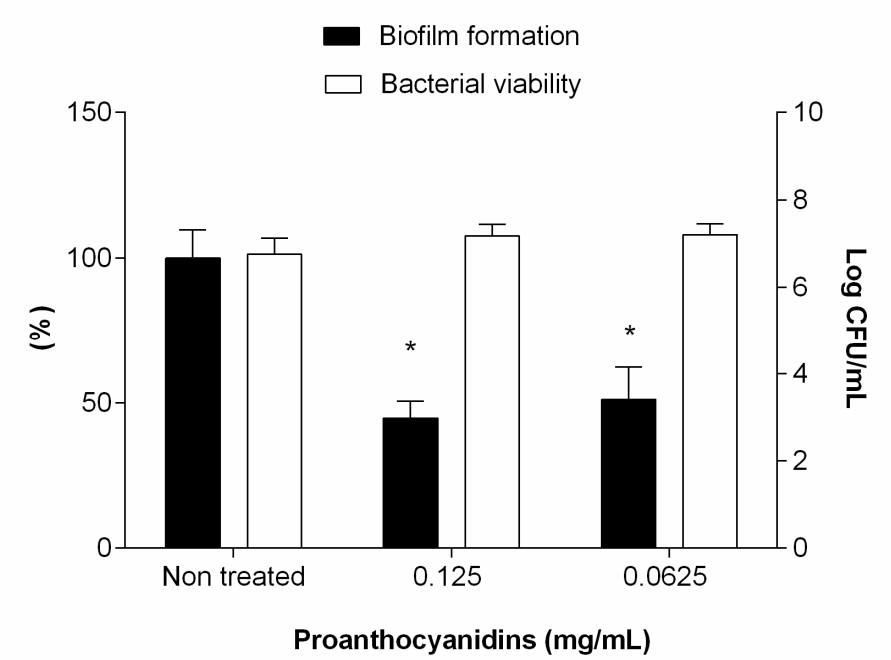

Fig. 4. The initial recovery in ability to form biofilm by *S. epidermidis* and their cell viability after the bacterial suspension be exposure to proanthocyanidins during 24 h and washed three times with sterile 0.9% NaCl solution. * represents statistical difference in relation to the non treated samples.

3.2. Iron chelating is not involved in the proanthocyanidins antibiofilm action

To evaluate the possible effect of proanthocyanidins iron chelation, the concentration of 50 µM of Fe^{+2} demonstrated to be appropriated to the ferrozine assay, according a standard curve that was established (Fig. 5A). Using the 2,2-bypiridyl, a compound that is commonly used to deplete free iron in solutions, it was possible to observe an gradual decreasing of the Fe^{+2} concentration in the mixture reaction due to its iron chelating capacity (Fig. 5B); however, using the proanthocyanidins solution, even using concentration varying from 0.0625 up to 4.0 mg mL^{-1}, the complex ferrozine-Fe^{+2} was kept constant (Fig. 5C), indicating a absence of competition between proanthocyanidins and ferrozine by the free ferrous iron.

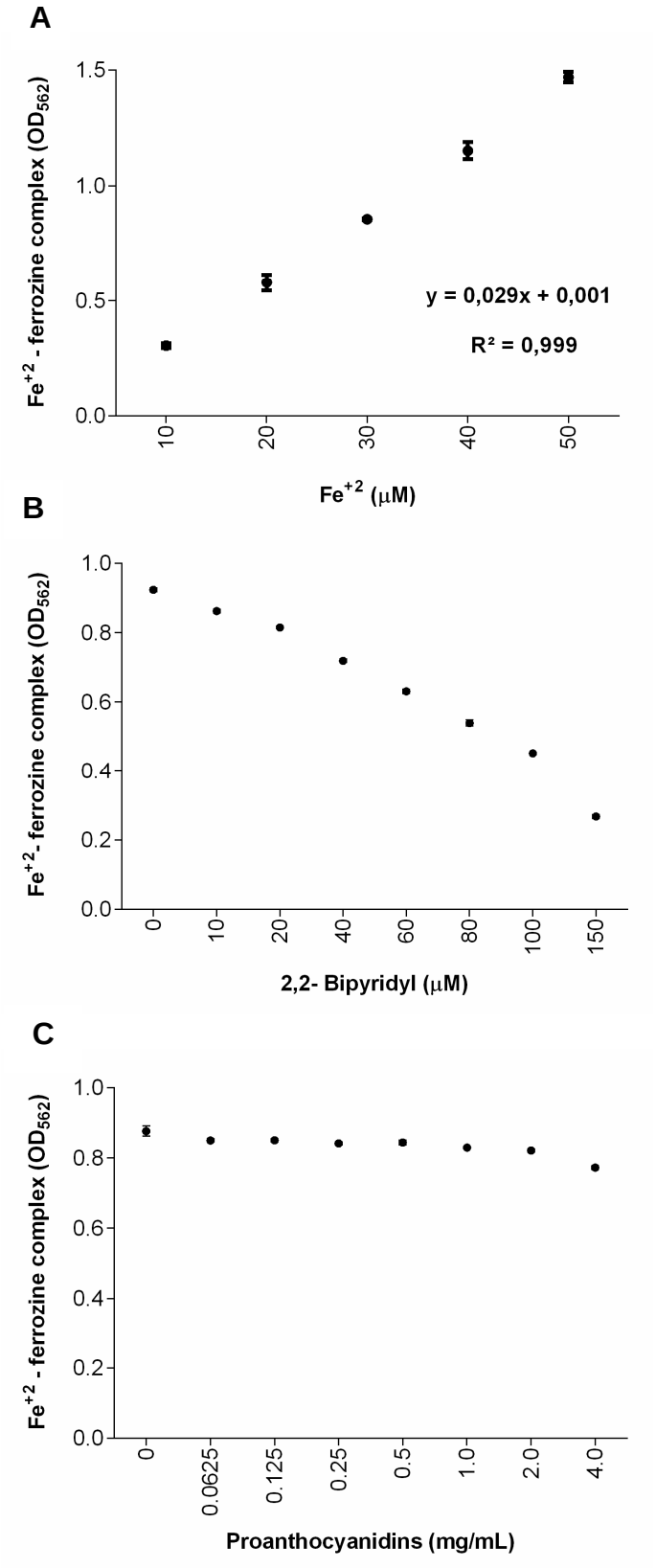

Fig. 5. Ferrous iron chelating assay. A standard curve was established to determine the Fe^{2+} concentration in a ferrozine complex (A). Ferrozine-Fe^{2+} complex quantified in the presence of positive-chelator 2,2- bipyridyl (B) and of proanthocyanidins (C).

3.3. Effect of proanthocyanidins on bacterial surface hydrophobicity

The hydrophobicity index of bacterial surface was determined, as shown in the Fig 6. Untreated- *S. epidermidis*, likewise that *S. epidermidis* treated with 0.0625 mg mL^{-1} of proanthocyanidins demonstrated a hydrophobic surface, presenting a HPBI index greater than 70%. However, at concentration of 0.125, 1.0 and 4.0 mg mL^{-1}, proanthocyanidins progressively altered the hydrophobic character of the bacterial surface making it hydrophilic (HPBI index lower than 70%).

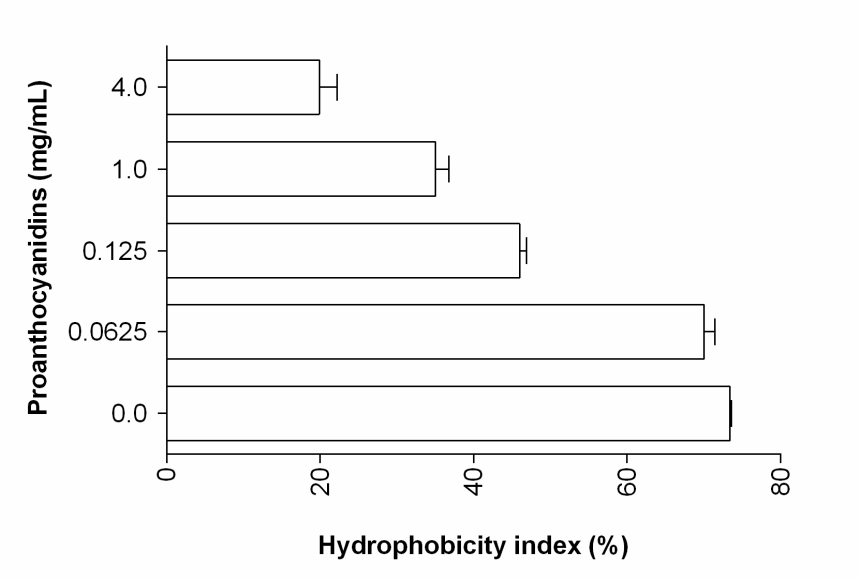

Fig. 6. *Staphylococcus epidermidis* surface hydrophobicity index according to different concentrations of proanthocyanidins.

3.4. SEM: visualization of biofilm and the proanthocyanidins self-adherence ability

The SEM analysis showed that *S. epidermidis* attached to the hydrophobic and hydrophilic surface models, produces a biofilm structured with numerous microcolonies involved by the extracellular matrix (Fig. 7 A1 and B1 and supplementary data S1 and S9). In contrast, an ausence of bacteria was demonstrated in the samples treated with all concentrations of proanthocyanidins investigated in this study, except at 0.0625 mg mL^{-1} in which *S. epidermidis* was able to develop a biofilm (Fig. 7, S2-S8 and S10-S16). Interestingly, during SEM observation it was possible to check visually that proanthocyanidins self-adhered on both surfaces models forming, spontaneously, films over the surfaces in which bacteria could not be able to adhere (Fig. 7 A2 and B2, S2-S7 and S10-S15).

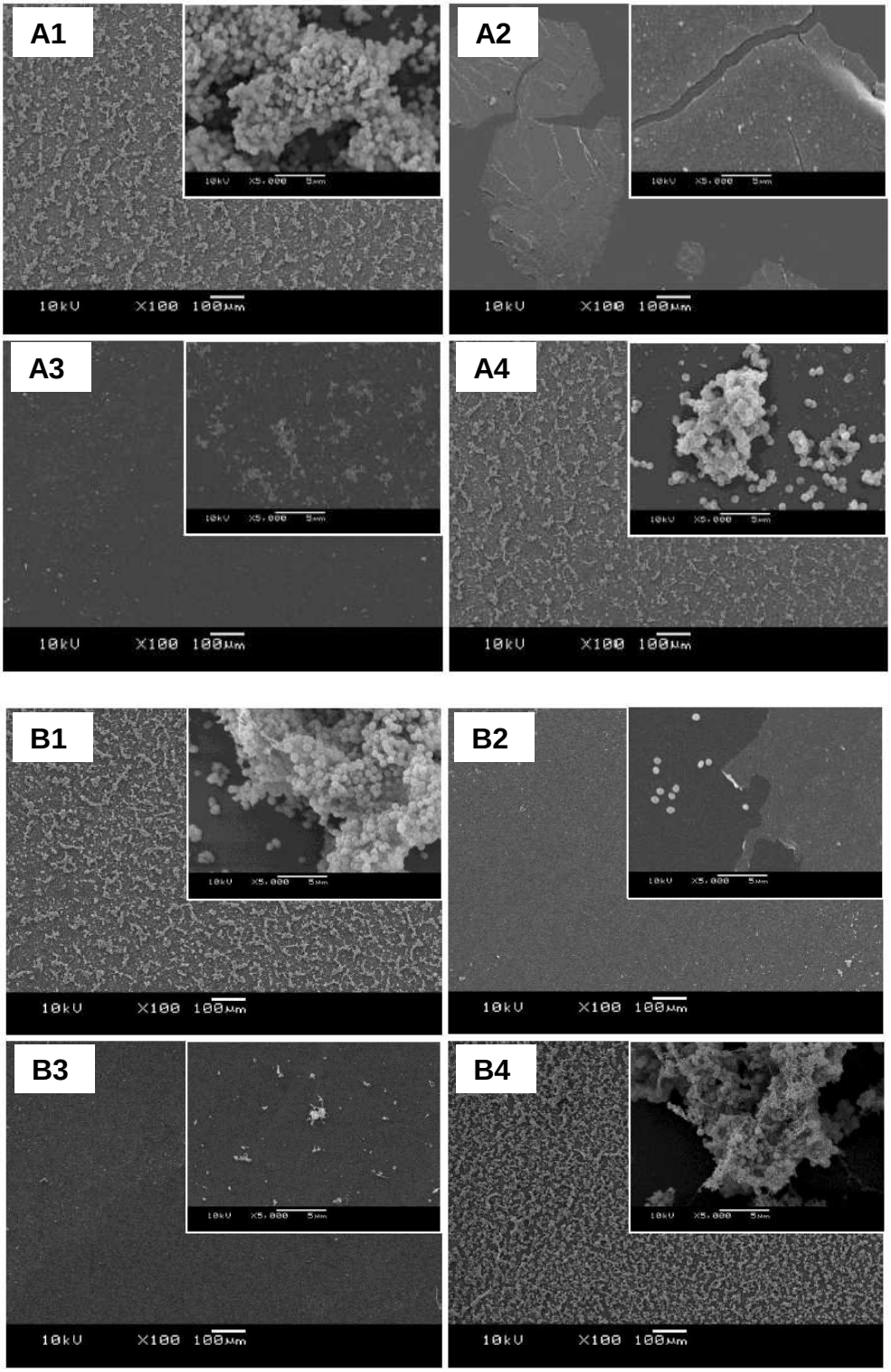

Fig. 7. Effect of proanthocyanidins free-form on the bacterial adhesion and biofilm formation by SEM upon Permanox (A) and glass (B) surfaces. Non-treated bacteria (1) and treated with proanthocyanidins at 4.0 mg mL^{-1} (2), 0.125 mg mL^{-1} (3) and 0.0625 mg mL^{-1} (4). Note the proanthocyanidins self-adherence in the images A2 and B2.

3.5. Non-biological particle model testing

An additional set of experiments was conducted to verify the effect of proanthocyanidins on the adhesion of non-biological particle model, replacing *S. epidermidis* by red fluorescent microspheres. As shown in the Fig. 8, proanthocyanidins at 0.125 mg mL^{-1} demonstrated an important inhibition of the beads adhesion, on polystyrene and on glass surfaces; otherwise, non-treated and 0.0625 mg mL^{-1} – treated samples displayed a high number of adherent beads on both surfaces.

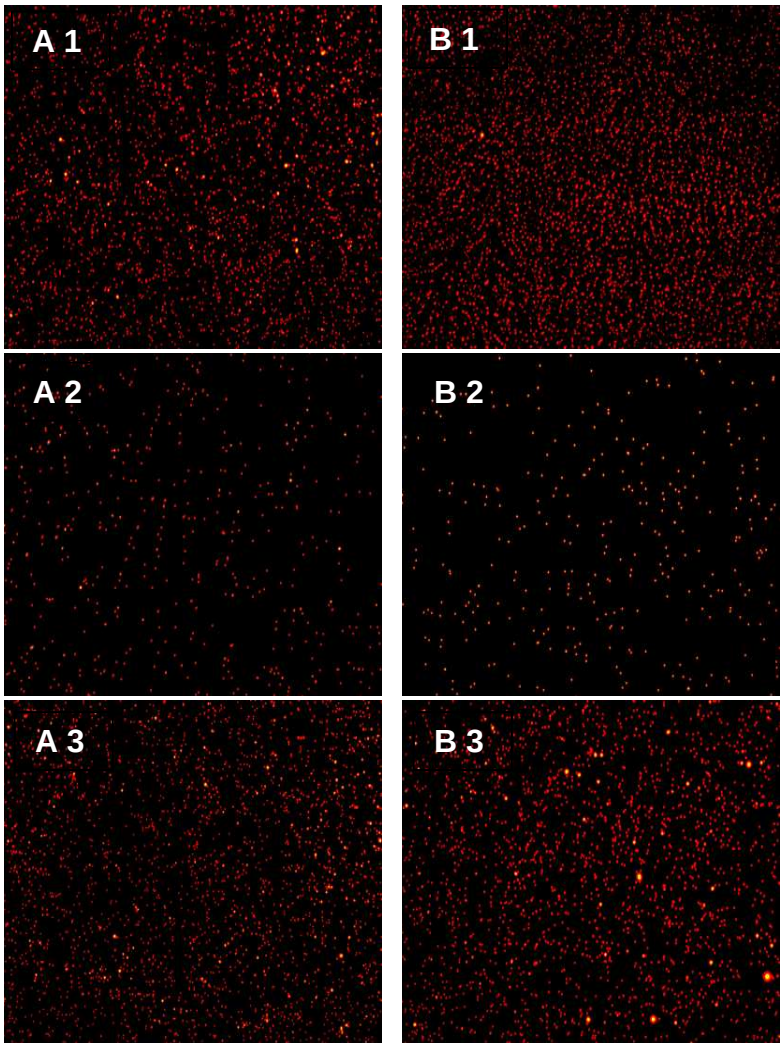

Fig. 8. Fluorescence microscopy images (10× magnification) of experiments conducted with fluorescent microspheres on polystyrene (A) and glass (B) surfaces. Non-treated microspheres (1) and treated with 0.125 mg mL^{-1} (2) and 0.0625 mg mL^{-1} (3) of proanthocyanidins.

3.6. Surfaces characterization

Permanox slides were surface-modified with proanthocyanidins solution (dissolved in 30% acetone or 70% acetone) using the spin coating technique as described previously. The proanthocyanidins-coated surfaces possess a hydrophilic character (WCA of about 40º and 20º, using 30% and 70% acetone, respectively), while the non-treated and the acetone treated-surfaces are hydrophobic (WCA of 100º and 85º, respectively) (Fig. 9). The XPS analyses for non-treated, 30% and 70% acetone samples presents very similar spectra, with one component at 284.9 eV attributed to C-C bonds in the polymer and some C-O bonds (286.5 eV) attributed to contamination and/or acetone exposure. After samples coating with proanthocyanidins films, we observe an increase in the relative concentration of the C-O component when compared to the non-treated samples. This result indicates the presence of the proanthocyanidins film since it presents a high number of C-O bonds in the structure. This is also corroborated by the WCA results where a strong reduction in the contact angle is observed after the coating of the Permanox.

3.7. Proanthocyanidins-coated surface: studies about bacterial and mammalian adhesion

Examination of the coated Permanox segments demonstrated that *S. epidermidis* cells adhered and accumulated to form a densely biofilm on the non-treated (Fig. 10E and S17) and on the acetone–coated surfaces (Fig. 10A and C, S18 and S19). Whereas, proanthocyanidins-coated surfaces displayed resistance to bacterial adhesion presenting attached on these surfaces just sparse cell clusters or even single cells (Fig. 10B and D, S20 and S21). These non-adherent cells (planktonic cells) demonstrated to be viable according CFU counting assay, as showed in Fig. 10E. Although both coatings (produced by dissolving proanthocyanidins in 30% acetone or in 70% acetone) exhibited a strong reduction in the biofilm formation, the films prepared with 70% acetone demonstrated an enhanced anti-adhesion effect (Fig. 10B and D). Therefore proanthocyanidins-coated Permanox (obtained by dissolving in 70% acetone) were challenged to adhesion of epithelial mammalian cells. These modified surfaces that are antibiofilm to *S. epidermidis* allowed the adhesion and spreading of

mammalian cells, presenting no differences when compared to non-treated and 70% acetone-coated Permanox (Fig. 11).

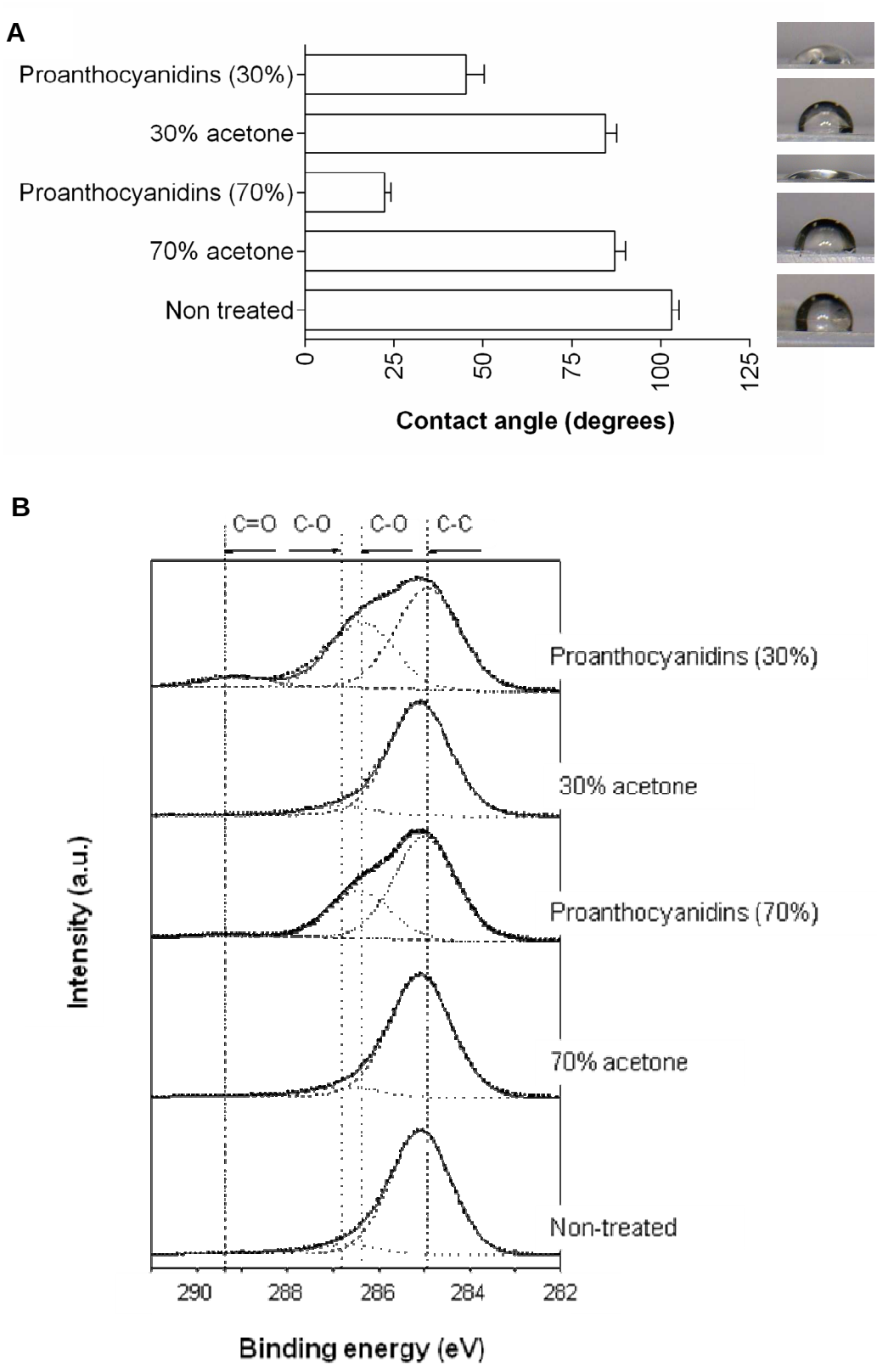

Fig. 9. Surface analysis of Permanox samples coated with proanthocyanidins or acetone. The WCA measurements and respective photographs (A) and XPS analysis (B).

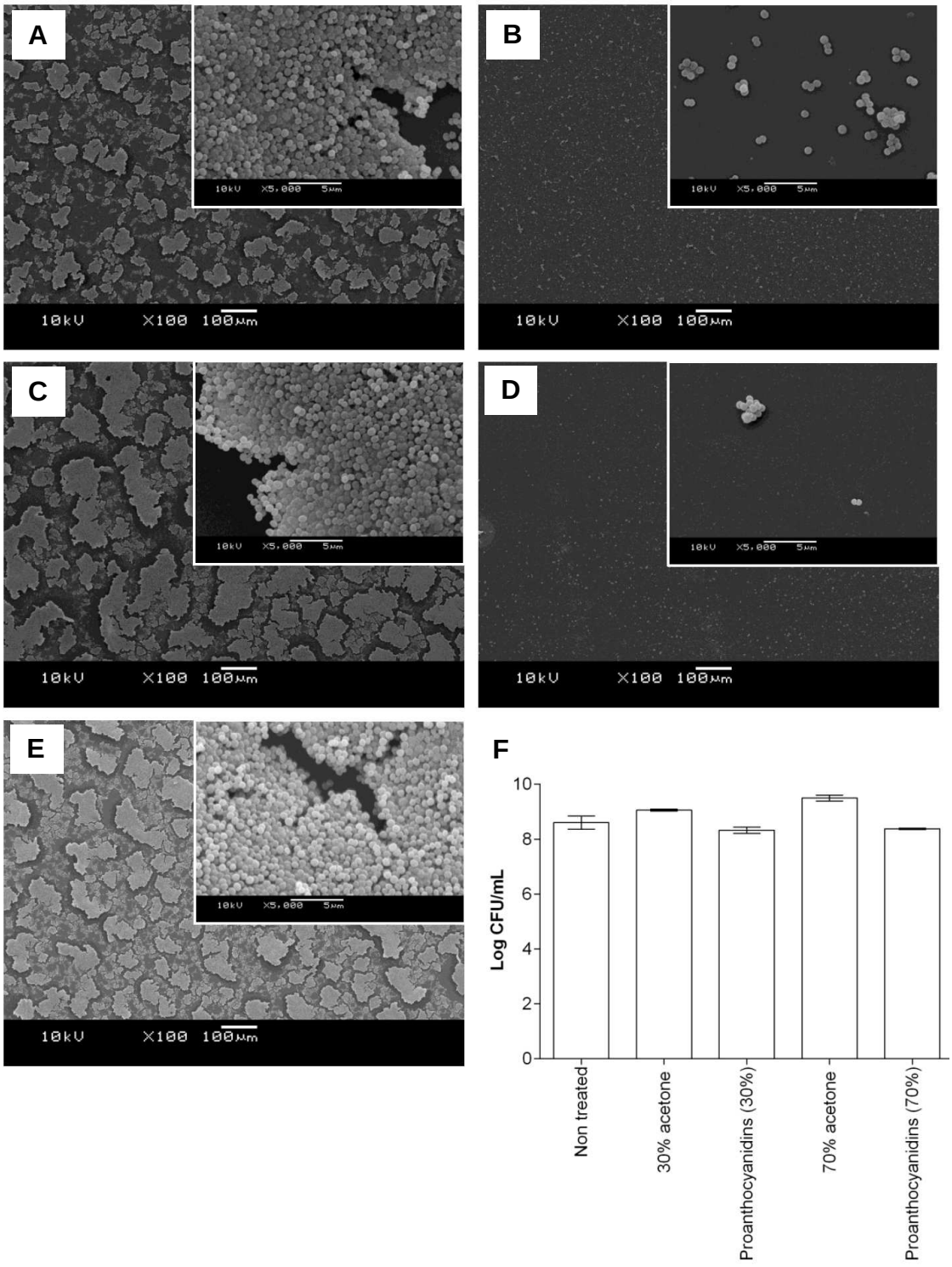

Fig. 10. Adhesion and biofilm formation by *S. epidermidis* on Permanox surfaces coated with 30%-acetone (A), proanthocyanidins in 30%-acetone (B), 70%-acetone (C), proanthocyanidins in 70%-acetone (D) and non-coated Permanox surfaces (E). The magnification of 100 x and in the inserts of 5000 x. The viability of non-adherent planktonic bacterial cells is shown in (D).

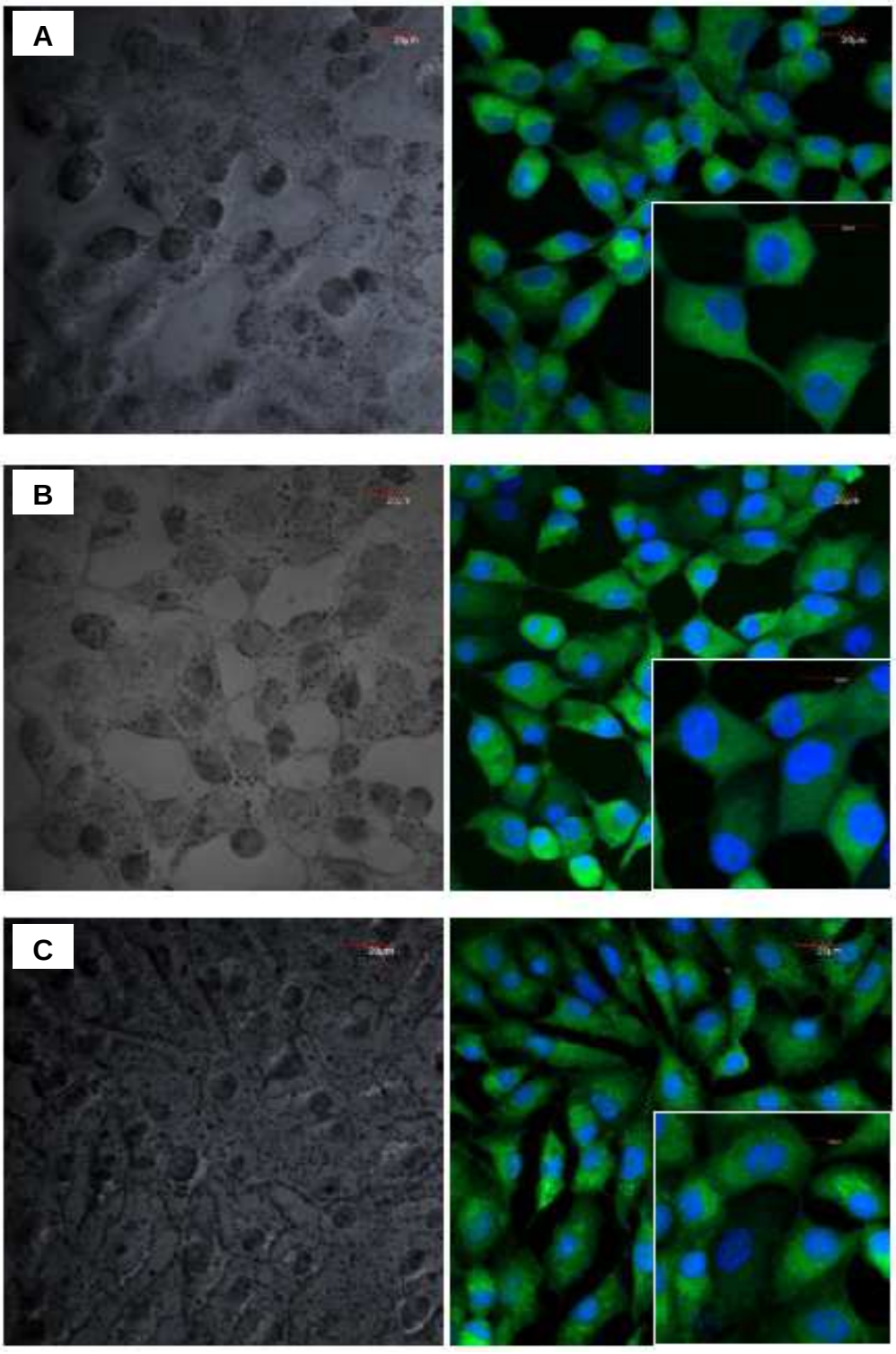

Fig. 11. Adhesion and spreading of epithelial mammalian Vero cells on non-coated (A), 70%-acetone-coated (B) and proanthocyanidins in 70% acetone-coated Permanox surfaces (C), by differential interference contrast (DIC) and by 60x CLSM imaging. Inserts are 60x plus zoom of 3x. Bars in all the images indicate 20µm.

4. Discussion

Natural products offer a rich source of structurally diverse substances with a wide range of biological activities which could be useful for the development of alternative or adjunctive antibiofilm therapies [24]. Herein, we demonstrated the ability of proanthocyanidins derived from *P. moniliformis*, presenting all type-B linkage and rich in prodelphinidin units, to mitigate, by a non-biocidal way, the *S. epidermidis* adhesion on hydrophobic and hydrophilic surface models.

According to the proanthocyanidins curve-response assay, employing polystyrene surfaces, it was possible to observe that *S. epidermidis* biofilm formation was completely prevented, without association with growth inhibition, up to 0.125 mg mL^{-1} (Fig.2A). These results were confirmed by CLSM (Fig. 3) and by SEM analysis (Fig. 7). Both microscopic techniques demonstrated that the strong prevention of bacterial adhesion by proanthocyanidins occurred in the same magnitude even when using two materials varying in chemical composition and hydrophobicity (glass and Permanox). The CLSM images, conducted using the LIVE/DEAD staining, showed the typical architecture of non-treated *S. epidermidis* biofilms, forming mushroom- or pillar-like structures according the orthogonal cuts. Likewise, the *S. epidermidis* treated with 0.0625 mg mL^{-1} also presented this biofilm phenotype (Fig. 3A1-B1 and A3-B3). However, bacteria treated with 0.125 mg mL^{-1} of proanthocyanidins could not attach to the surfaces and thereby most of cells were removed during the washing procedures (Fig. 3A2 and B2). In all samples, it was possible to observe the presence of bacterial cells stained with green, indicative of live cells, but also cells stained with red, indicative of dead cells. As reported by Harrison et al. 2006 [37], dead cells are a normal component of late logarithmic and stationary phase planktonic cell of culture suspensions, and thus part of this dead biomass may be incorporated into the biofilm during growth.

Subsequently, a set of experiments was developed in order to investigate the viability of *S. epidermidis* cells and their ability of form biofilm after a previous treatment, during 24 h, using proanthocyanidins. These treated and washed cells demonstrated the same viability as the control cells, despite of the biofilm formation

had not been completely restored as the control cells, being reduced in about 50% with the treatments of the 0.125 and 0.0625 mg mL^{-1} proanthocyanidins (Fig. 4).

Some works have evidencing that iron regulates the biofilm formation of *Staphylococcus* spp. Even though Deighton and Borland (1993) [38] had suggested that the progressive depletion of iron from culture medium results in increasing levels of *S. epidermidis* biofilm production, more recent studies had demonstrated that iron positively regulates biofilm formation by *Staphylococcus*. For instance, catecholamine inotropes, which remove iron from plasma iron-binding proteins, facilitate the bacterium iron acquisition and stimulate *S. epidermidis* growth as biofilms, suggesting that *S. epidermidis* requires iron to adhere and form biofilms onto surfaces [39]. In addition, Lin and co-works (2012) [40] revealed that the pentagalloylglucose compound impairs *S. aureus* biofilm formation through their iron chelation activity and, that the iron supplementation compensates for the effect of pentagalloylglucose and restores the biofilm formation, evidencing the essential role of iron for *S. aureus* biofilm formation. Therefore, we investigated the iron chelating activity of proanthocyanidins and we found that in all tested concentrations, proanthocyanidins not cause a significant alteration in the medium Fe^{+2} concentration (Fig. 5C), differently of the iron-chelator 2,2-bypiridyl that reduced ferrous iron in a dose responsive manner (Fig. 5B). These findings suggested that the deprivation of free iron could not be related to the biological findings.

The initial bacterial adhesion to a substratum is determined by physicochemical properties of nutrients and of both material and bacterium surfaces, besides depends of long-range bacteria-material surface interactions, including mainly electrostatic interactions, van der Waals forces and hydrophobic interactions [30,41,42]. An increase in cell surface hydrophobicity has often been associated with increased bacterial adhesion to both hydrophobic and hydrophilic surfaces; in this sense, the bacterial surface hydrophobicity seems to be the main parameter governing the bacterial attachment to abiotic surfaces [19, 42-45]. These observations agree with the results shown at Fig. 6, which indicates that proanthocyanidins modified the hydrophobic surface character of the non-treated *S. epidermidis* to a hydrophilic surface, in all concentrations that they prevented bacterium adherence (HPBI index

below 70%). In addition, when bacteria were treated with 0.0625 mg mL^{-1} of proanthocyanidins, the concentration not active against the biofilm formation, the bacterial surface remained hydrophobic (HPBI index above 70%). Regarding the SEM findings, as important as corroborate with results concerning on S. *epidermidis* biofilm formation inhibition by proanthocyanidins, was to verify the ability of self-adherence of these natural products on polystyrene and on glass (Fig. 7 and Supplementary data S1-S16), indicating, together with HPBI index measurements, that proanthocyanidins possibly cover both surfaces, the bacterium and the material. To better understand how proanthocyanidins influences the initial adhesion of *S. epidermidis*, the bacterial suspension was replaced by fluorescent microspheres and comparable extents of adhesion inhibition were verified (Fig. 8), supporting their non-biological pathway of action, similarly as demonstrated by cranberry proanthocyanidins [27].

The potential of proanthocyanidins as surface coating agent against bacterial colonization, as suggested by SEM images, led us to perform the coating of Permanox surfaces by spin coating technique. These surfaces, as well as demonstrated to bacterial cells, had their hydrophobic surface character altered to hydrophilic when were coated with proanthocyanidins, especially the coated produced with proanthocyanidins dissolved in 70% acetone, which presented WCA of about 22º (Fig. 9A). The XPS analyses evidenced the presence of a new high energetic component related to the carbon-oxygen binding due to the presence of the prodelphinidin oligomers coating (Fig. 9B). Concerning on the biological findings, the proanthocyanidins-coated surfaces demonstrated the same ability to prevent *S. epidermidis* biofilm formation as the free form-proanthocyanidins and it did not hinder planktonic bacterial growth (Fig. 10). Likewise, adsorption of cranberry proanthocyanindins to polyvinyl chloride and polytetrafluoroethylene surfaces modified its hydrophobicity and thereby, interfered with the microbial adhesion processes [27].

In contrast to classical antibiotics that generally kill bacteria, our results show that *P. moniliformis*-derived proanthocyanidins (characterized by the B-type linkage and by richness of the prodelphinidins) modify the behavior of bacteria and the characteristics of surfaces involved, preventing bacterial adhesion when it is in

solution or adsorbed onto surfaces. Antiadhesive therapies are likely to develop less issues of resistance, as blocking adhesion is not likely to be as compelling a selective pressure, since it is non-lethal and multiple adhesion mechanisms exist. By reducing the bacterial adhesion, a surface can both minimize biofilm formation and force bacteria to reside planktonically where they can be effectively cleared by the innate immune system.

The proanthocyanidins-coated surface obtained by dissolving in 70% acetone, demonstrated an improved bacterial anti-adherence property when compared to 30% acetone, possibly due to the chemical etching caused by acetone that facilitate the proanthocyanidins and polymer interactions and promoted a higher surface hydrophilicity (Fig. 9A). Even through the mammalian cells had presented viability only at the lower active concentration of the free form- proanthocyanidins (Fig. 2B), their adhesion and spread on the proanthocyanidins modified-surface was very similar than on untreated- and 70% acetone treated-surfaces (Fig. 11), indicating its compatibility with epithelial mammalian cells. This dual-function presented by the proanthocyanidins-coated surfaces, (i) providing a strong mitigation of the *S. epidermidis* adhesion and biofilm formation and (ii) allowing the appropriated adhesion and spread of mammalian cells, congregates features very required and desired in the biomaterials field. Once the dual-functional coating favors the host cells to overcome bacteria in the colonization of the surface, the risk of the biomaterial infection decrease more extensively due to the host tissue integration [5].

The fundamental behavior of living cells, including their adherence to biomaterials, have been found to be intimately linked to the surface of such a material and the characteristics of the medium that separates the cells and the substrate surface [2]. The fact that bacterium adhesion is suppressed when Permanox is coated with proanthocyanidins might be explained by a combination of factors: (i) in terms of electrostatic repulsion; since *S. epidermidis* acquire an anionic bacterial surface in aqueous environment due to the ionization of phosphoryl groups from teichoic and lipoteichoic acids and the proanthocyanidins-coated surface has a negative polarization (a schematic representation of the above argument is shown in Fig. 12); (ii) due to the high hydrophilic surface character obtained with the coated material, in which a

reduced bacterial adhesion is expected and; (iii) we can not exclude the possibility of steric forces action, that blocks the bacterium- substratum interactions, provided by the proanthocyanidins film. We attribute the differential response exhibited by the *S. epidermidis* and mammalian cells to the proanthocyanidins-coated surfaces to the differences in the size and the adhesion mechanisms that they display. Due to the inherent instability of physically adsorbed coatings, we can suggest that short-term materials are appropriate; however, the covalent attachment of the proanthocyanidins to the surface could extend the lifetime. In addition, these materials can act as devices for controlling drug delivery, such that these proanthocyanidins are also active when in solution.

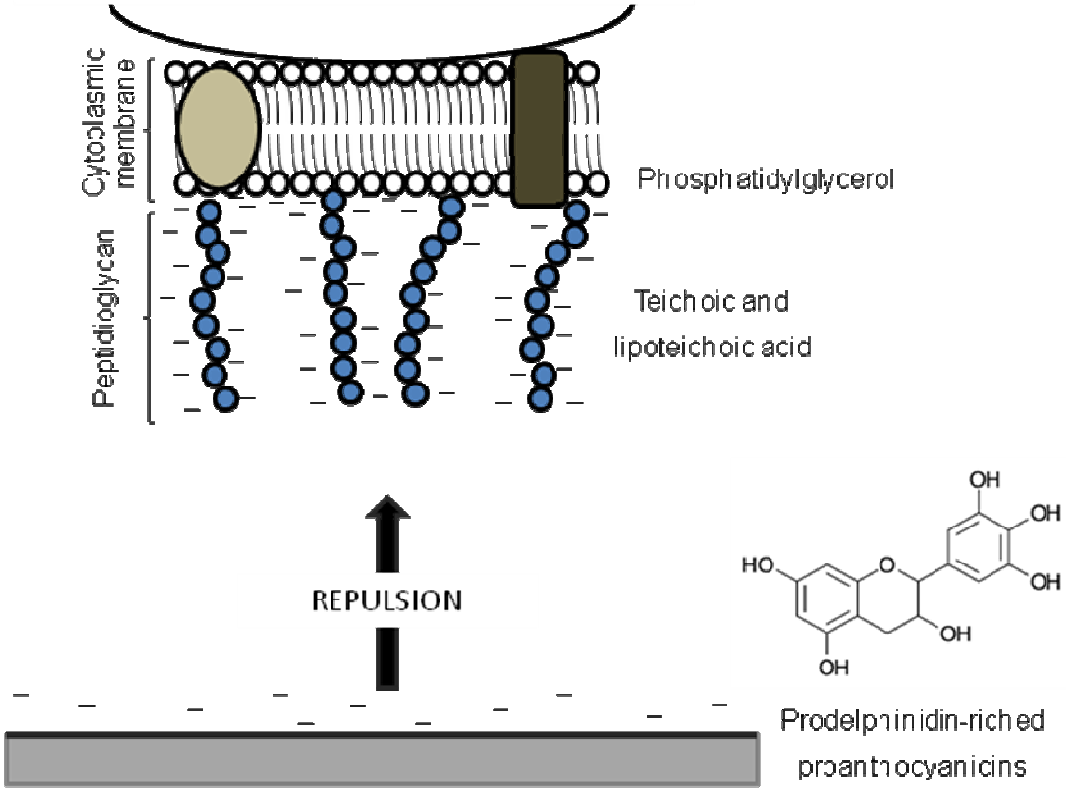

Fig. 12. Schematic illustration of the inhibition of bacterial adhesion and further biofilm formation on proanthocyanidins-coated surfaces based on repulsive forces model between the anionic *S. epidermidis* surface and the negatively polarized material surface after coating with proanthocyanidins.

Conclusions

P. moniliformis-derived proanthocyanidins, characterized by the B-type linkage and by richness of the prodelphinidins, being structurally distinct from cranberry-

derived proanthocyanidins was used in this study. Herein, we reported that these proanthocyanidins has *S. epidermidis* antibiofilm action and that, in contrast to classical antibiotics that generally kill bacteria, these natural products modify the behavior of the bacteria. Prodelphinidin-riched proanthocyanidins presented anti-adhesion activity by becoming the bacterial surface hydrophilic and by freely cover both hydrophobic and hydrophilic surfaces, presenting a non-biological mechanism of action. In addition, the proanthocyanidins did not presented iron-chelating activity, excluding the involvement of iron removal in the biological findings. When proanthocyanidins were coated onto surfaces, as demonstrated by XPS analysis and by their new hydrophilic character, they were repellent to *S. epidermdis* but friendly to mammalian cells. In the particular case of epithelial mammalian cells and *S. epidermidis*, this finding has important technological implications for a broad range of implant materials, which must promote tissue integration necessary for healing while simultaneously reduce the bacterial colonization that leads to infection, evidencing the potential applicability of the proanthocyanidins to coat biomaterial surfaces.

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Supplementary data

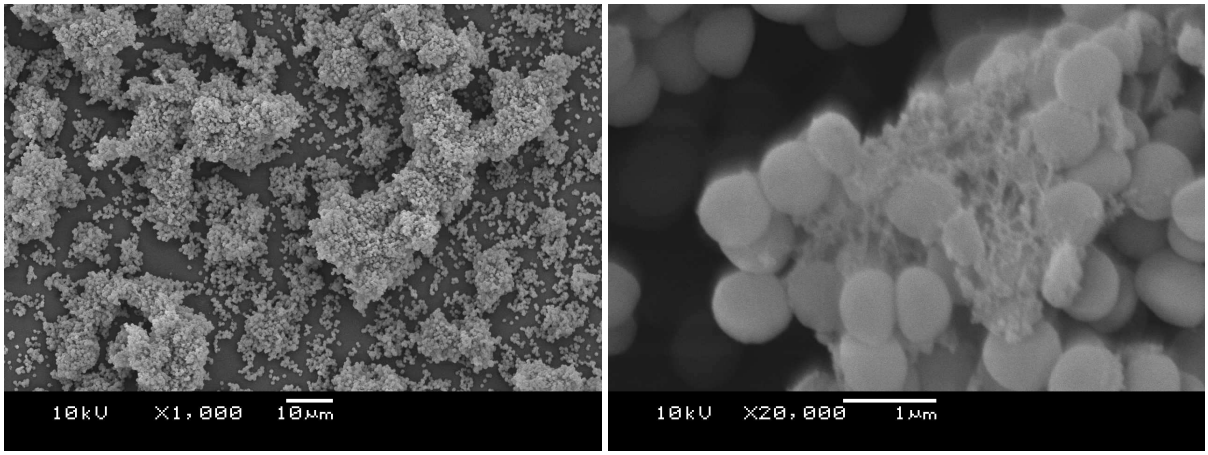

Fig. S1. SEM of *S. epidermidis* adhesion and biofilm formation on the non-treated Permanox surface. Magnification of 1000 and 20 000 x.

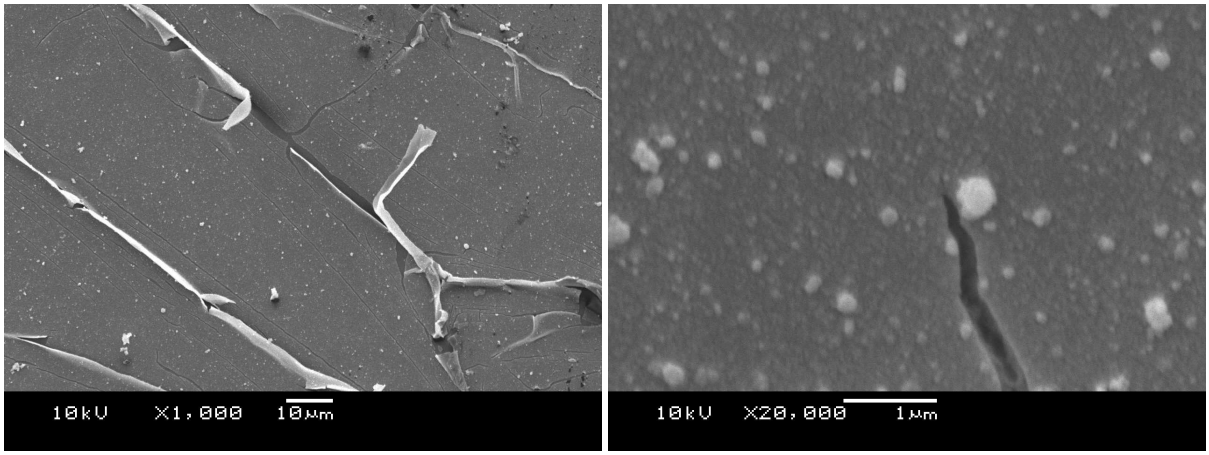

Fig. S2. Effect of proanthocyanidins free-form at 4.0 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon Permanox surface. Magnification of 1000 and 20 000 x.

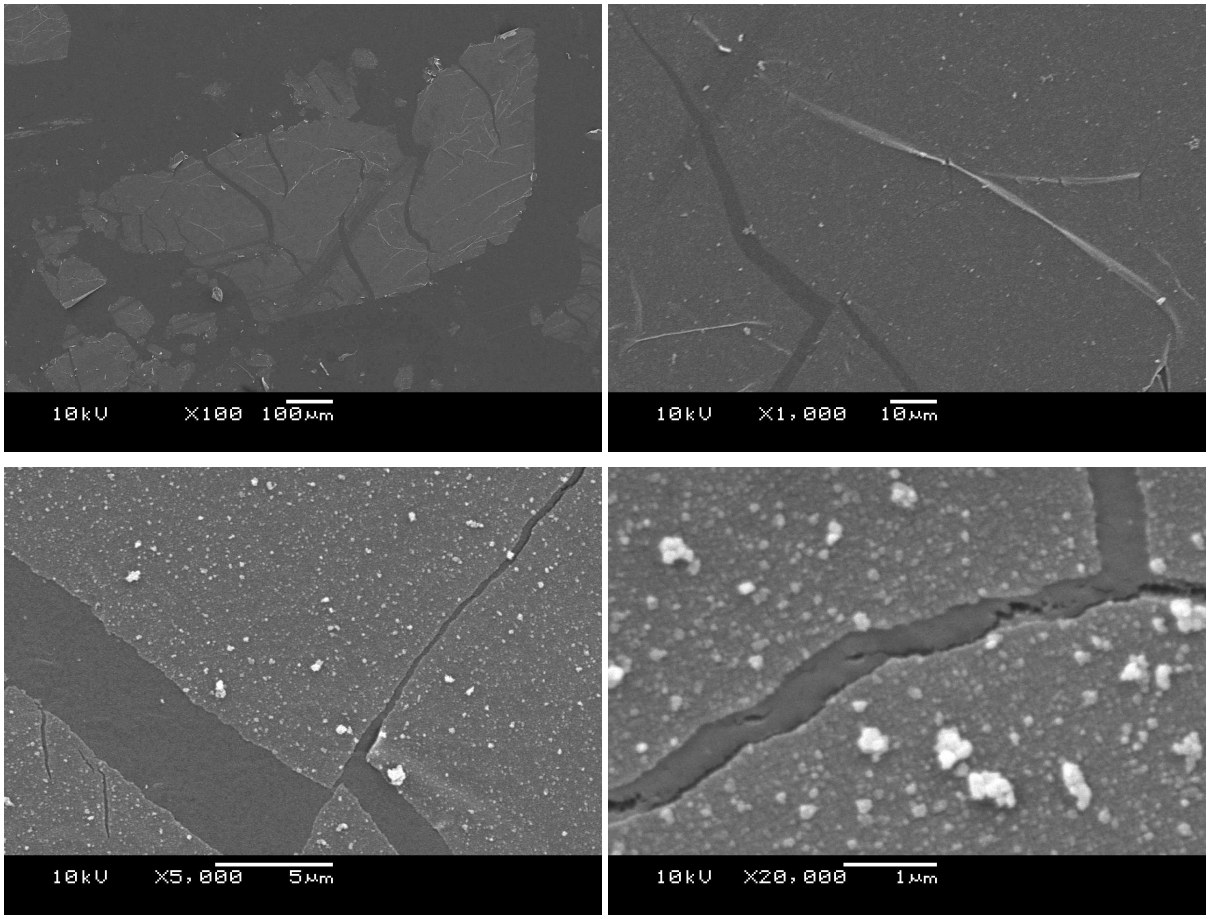

Fig. S3. Effect of proanthocyanidins free-form at 2.0 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon Permanox surface. Magnification of 100, 1000, 5000 and 20 000 x.

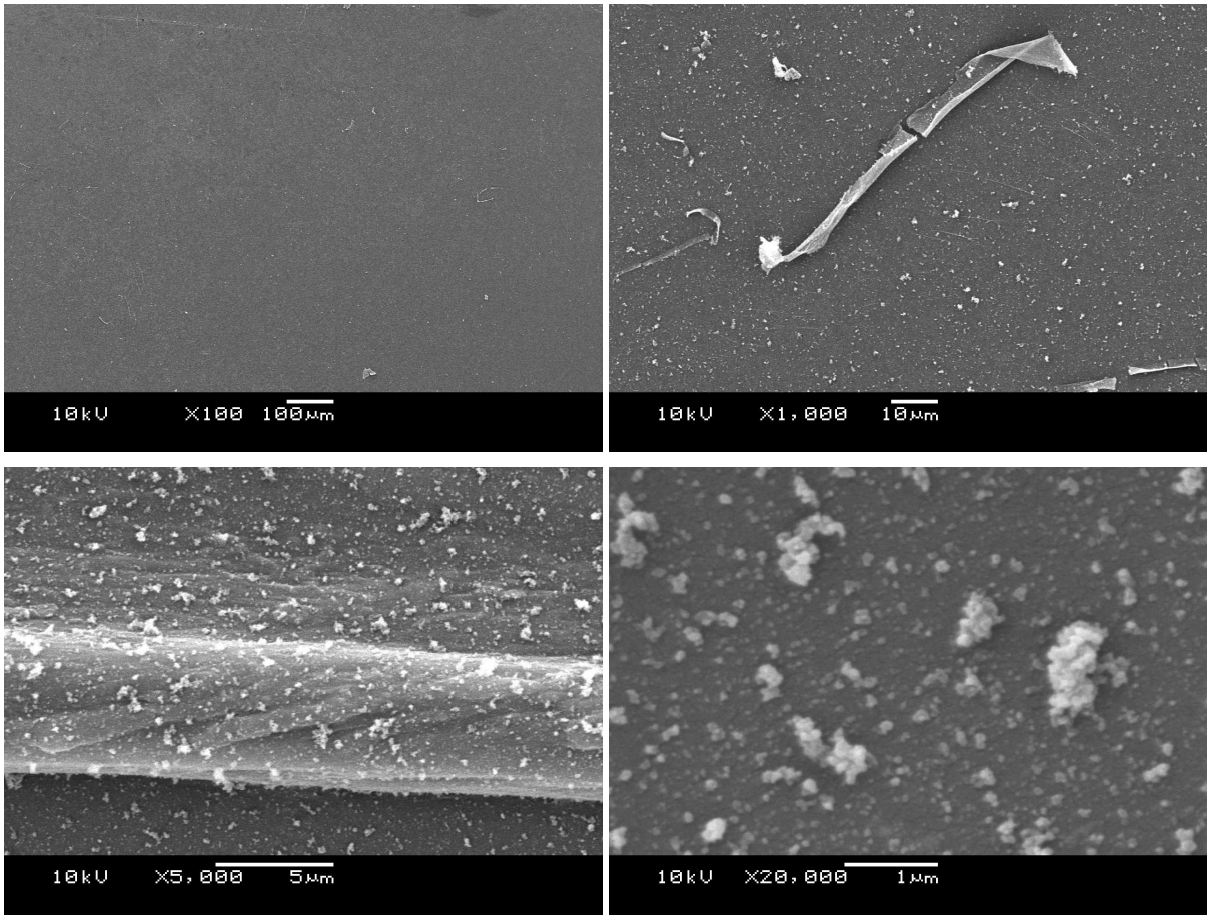

Fig. S4. Effect of proanthocyanidins free-form at 1.0 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon Permanox surface. Magnification of 100, 1000, 5000 and 20 000 x.

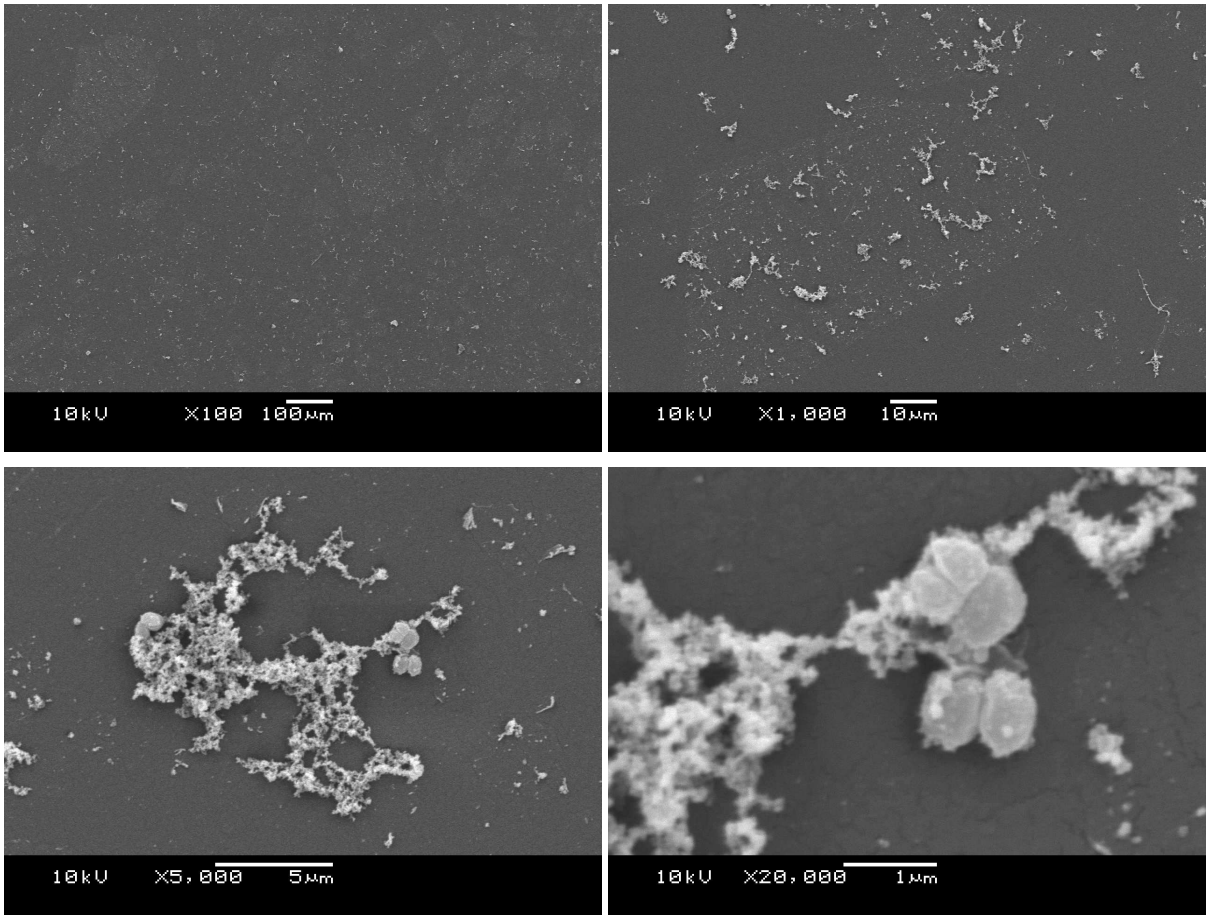

Fig. S5. Effect of proanthocyanidins free-form at 0.5 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon Permanox surface. Magnification of 100, 1000, 5000 and 20 000 x.

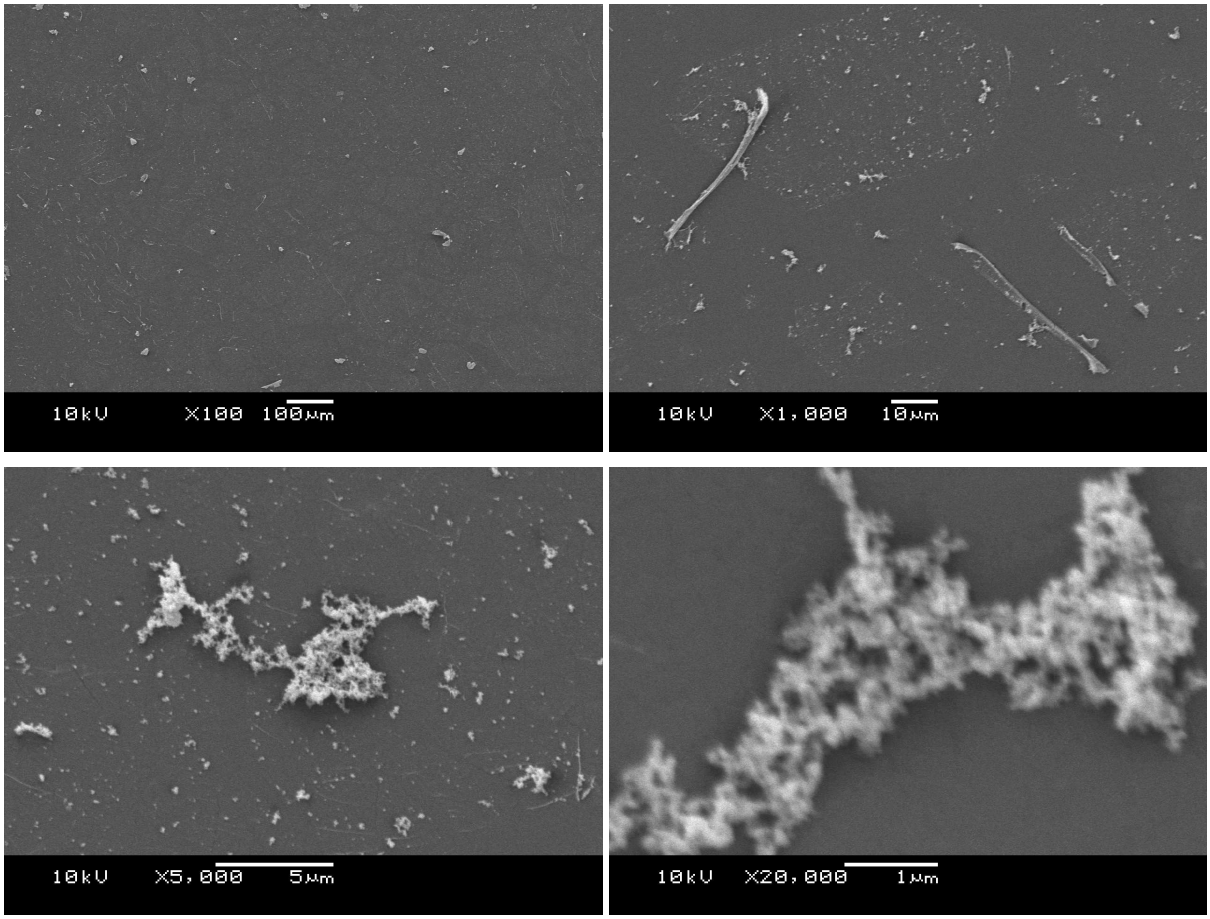

Fig. S6. Effect of proanthocyanidins free-form at 0.25 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon Permanox surface. Magnification of 100, 1000, 5000 and 20 000 x.

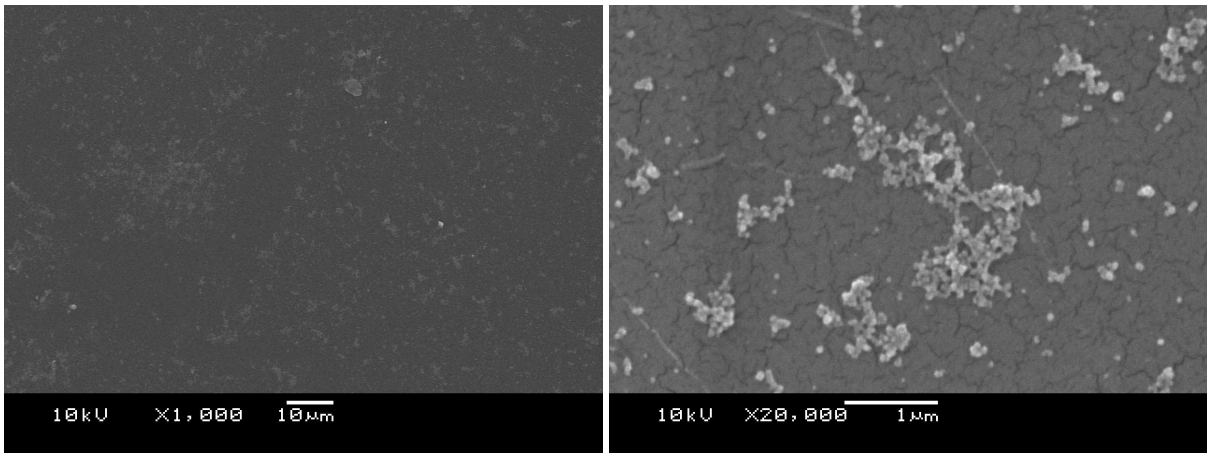

Fig. S7. Effect of proanthocyanidins free-form at 0.125 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon Permanox surface. Magnification of 1000 and 20 000 x.

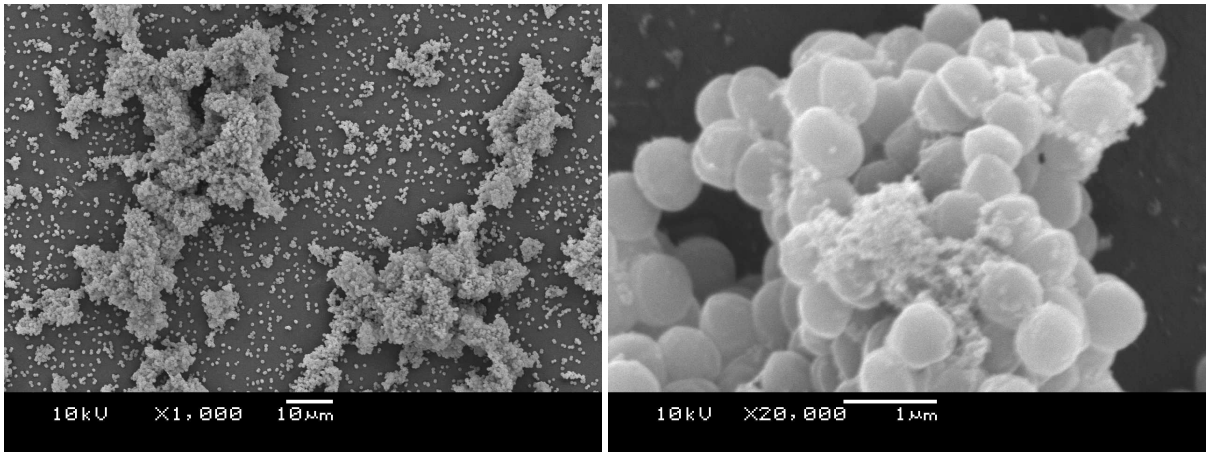

Fig. S8. Effect of proanthocyanidins free-form at 0.0625 mg mL^{-1}on the *S. epidermidis* adhesion and biofilm formation by SEM upon Permanox surface. Magnification of 1000 and 20 000 x.

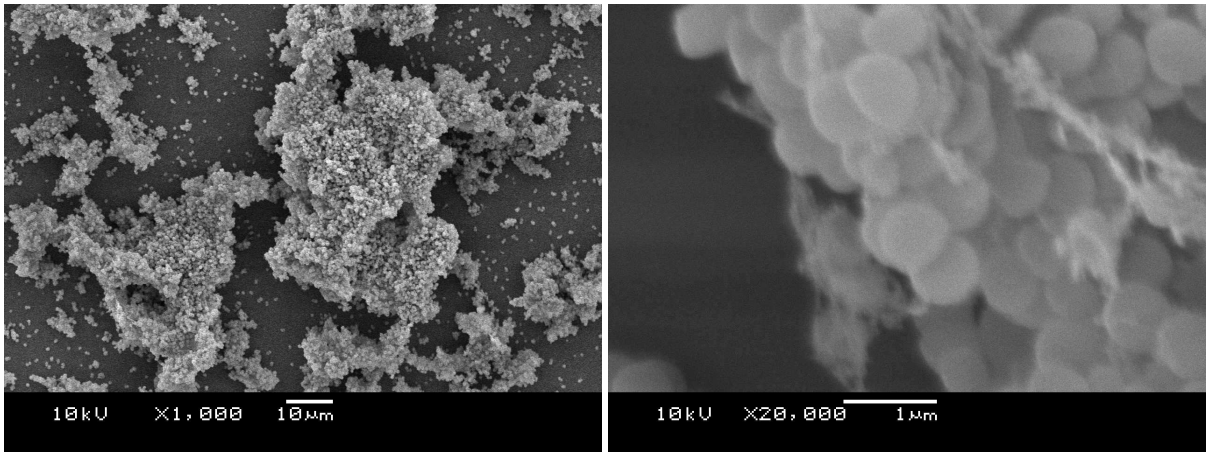

Fig. S9. SEM of *S. epidermidis* adhesion and biofilm formation on the non-treated glass surface. Magnification of 1000 and 20 000 x.

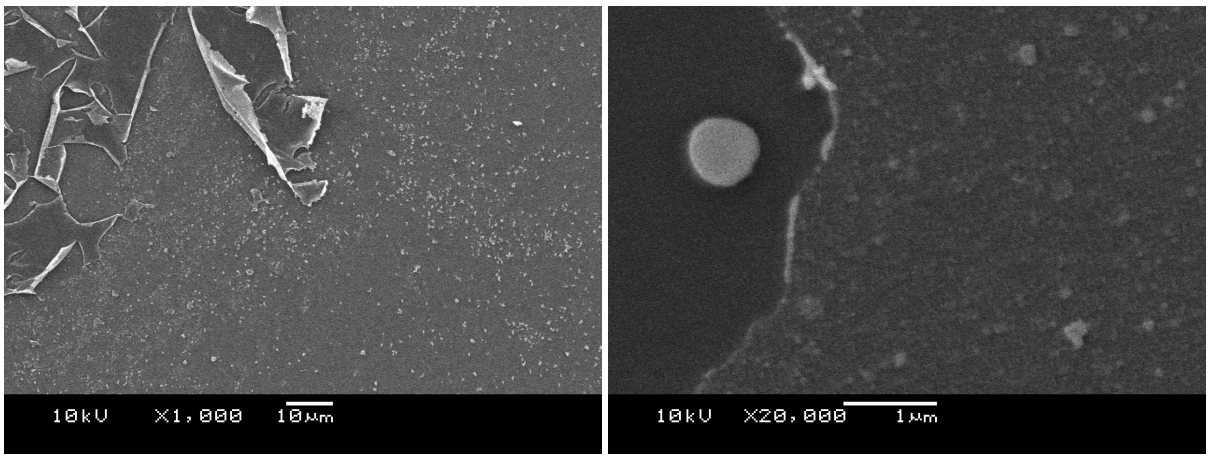

Fig. S10. Effect of proanthocyanidins free-form at 4.0 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon glass surface. Magnification of 1000 and 20 000 x.

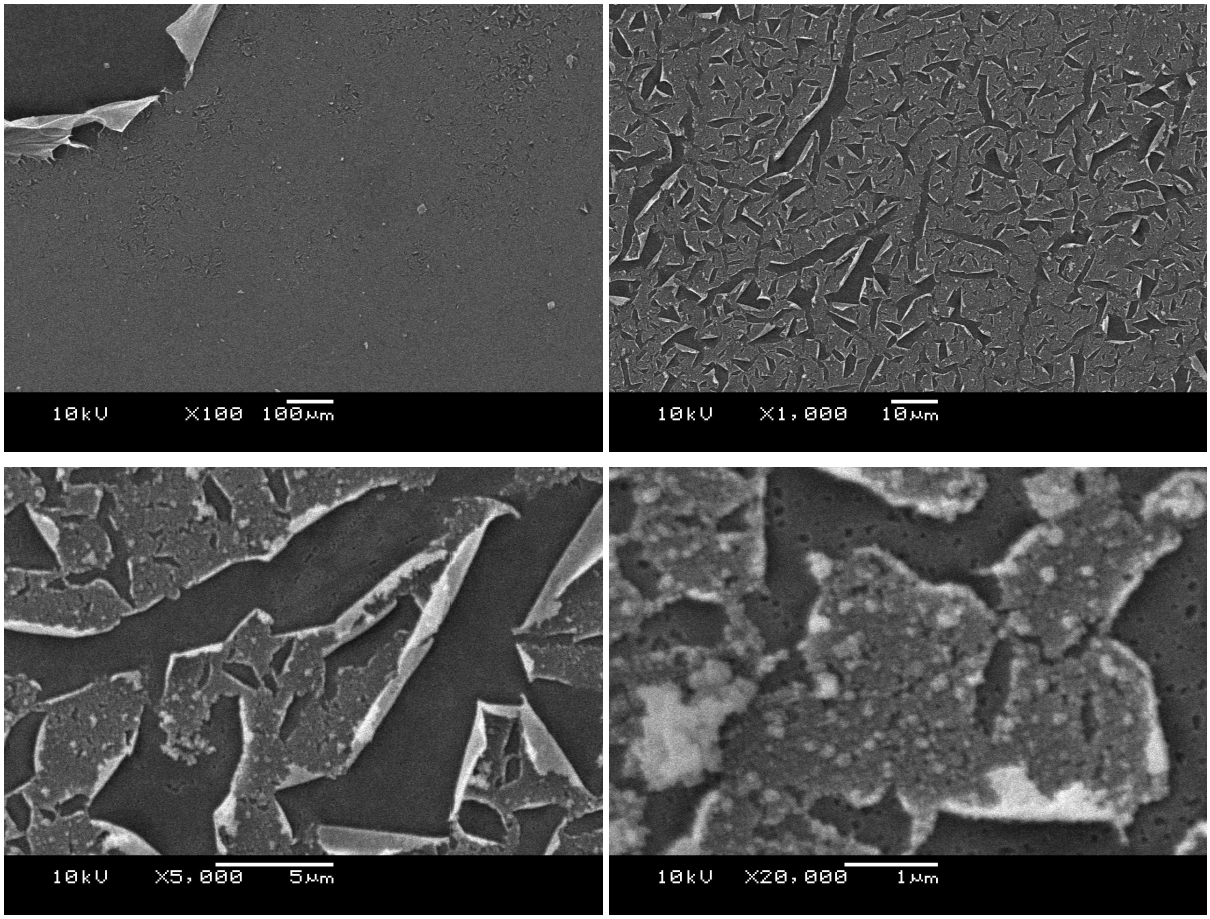

Fig. S11. Effect of proanthocyanidins free-form at 2.0 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon glass surface. Magnification of 100, 1000, 5000 and 20 000 x.

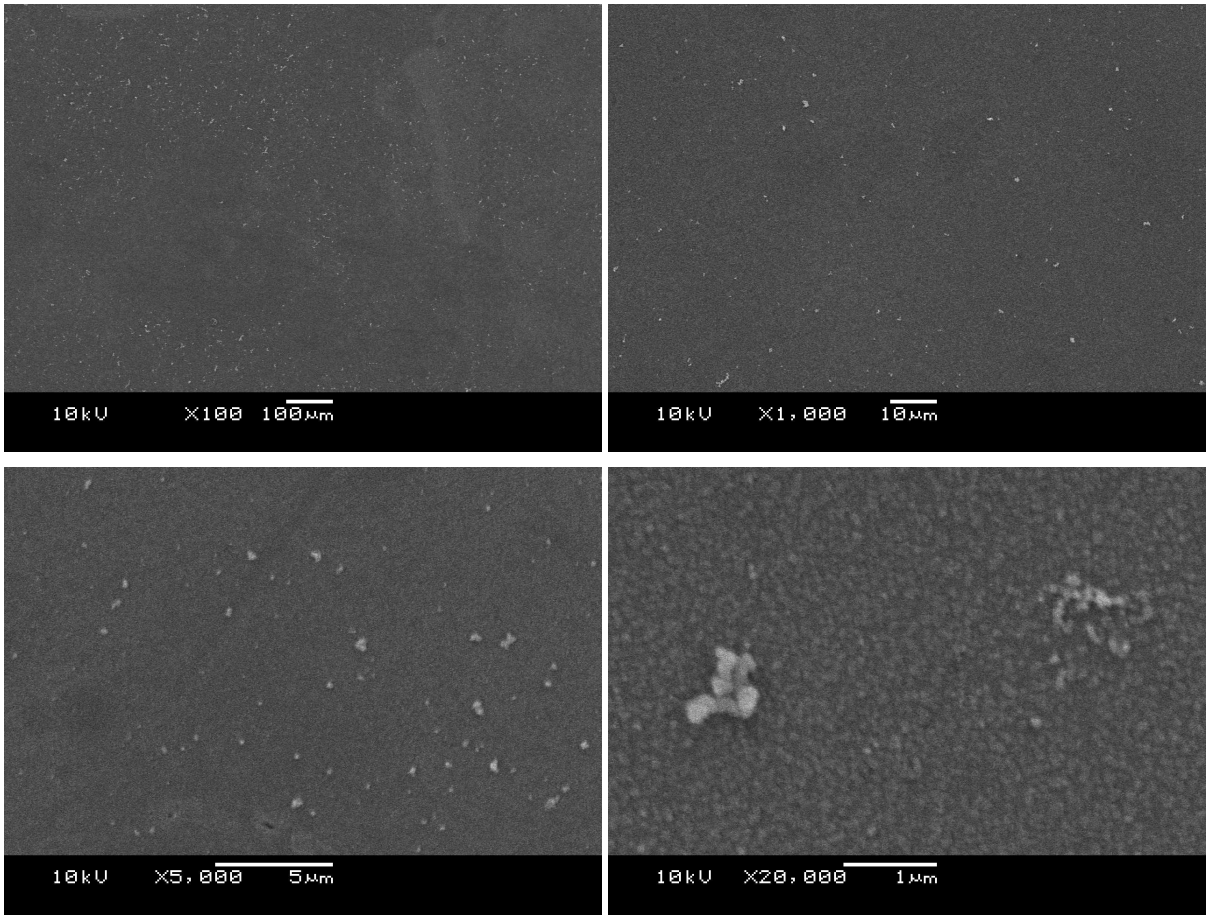

Fig. S12. Effect of proanthocyanidins free-form at 1.0 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon glass surface. Magnification of 100, 1000, 5000 and 20 000 x.

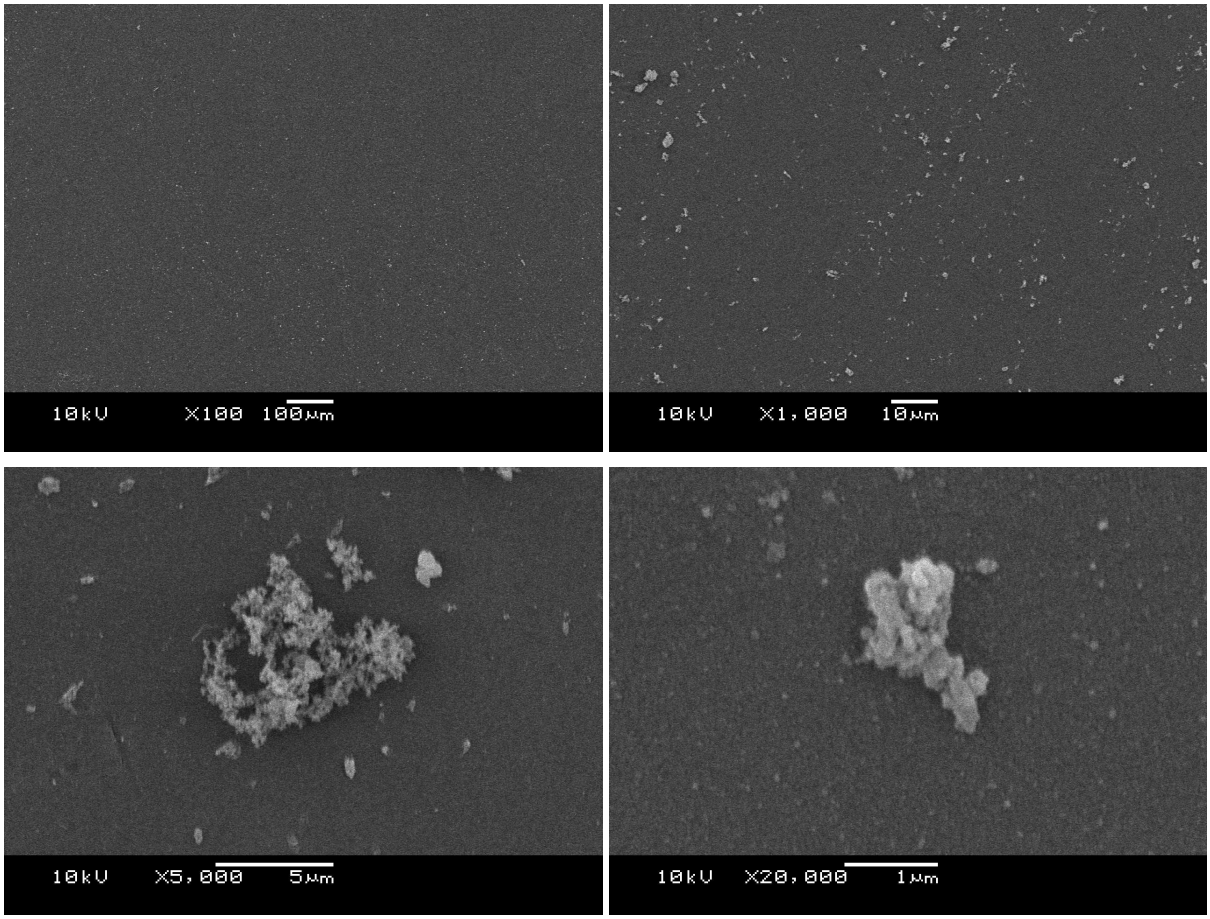

Fig. S13. Effect of proanthocyanidins free-form at 0.5 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon glass surface. Magnification of 100, 1000, 5000 and 20 000 x.

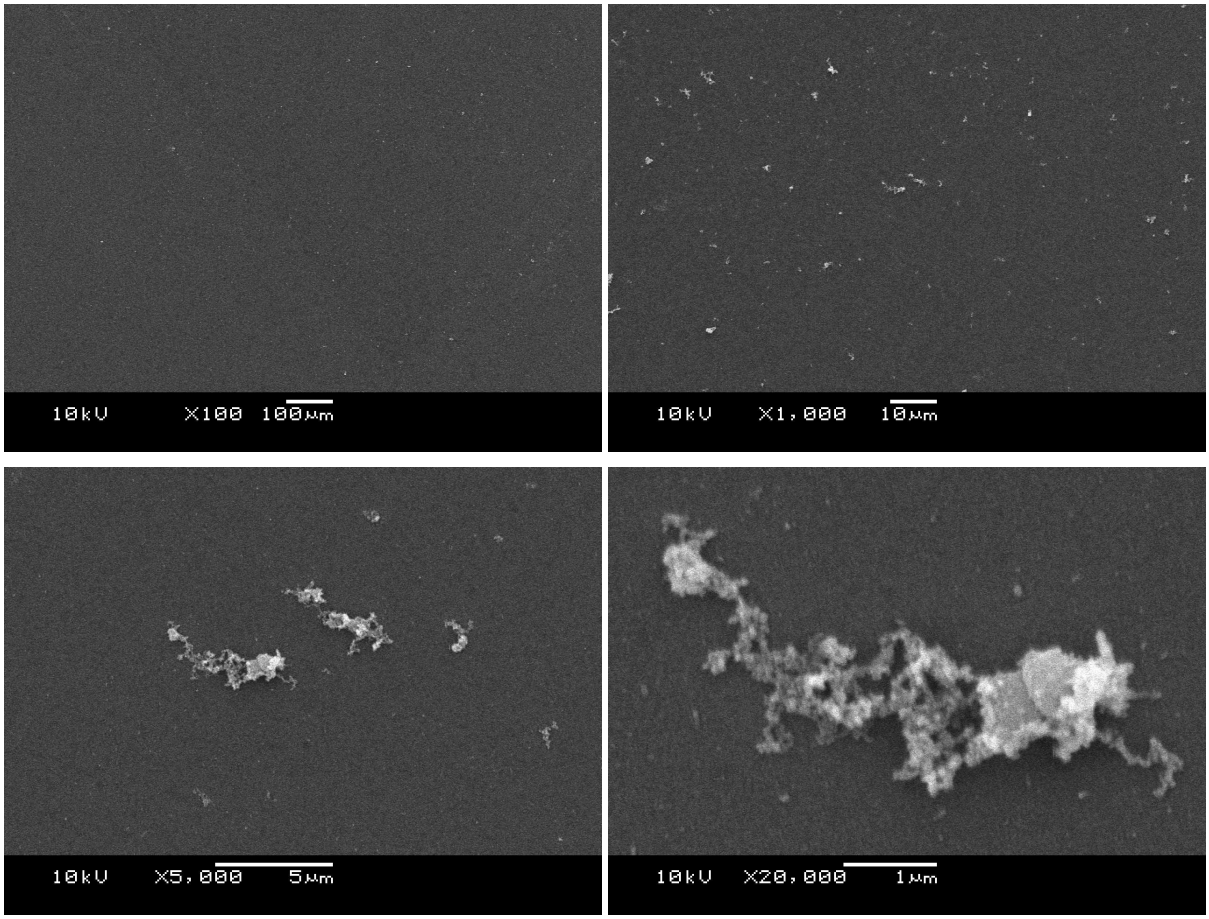

Fig. S14. Effect of proanthocyanidins free-form at 0.25 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon glass surface. Magnification of 100, 1000, 5000 and 20 000 x.

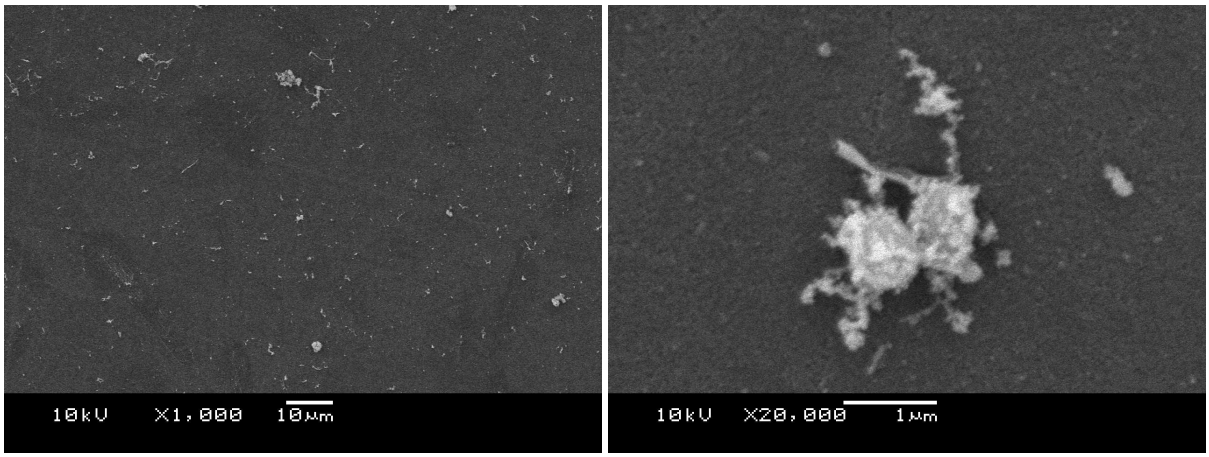

Fig. S15. Effect of proanthocyanidins free-form at 0.125 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon glass surface. Magnification of 1000 and 20 000 x.

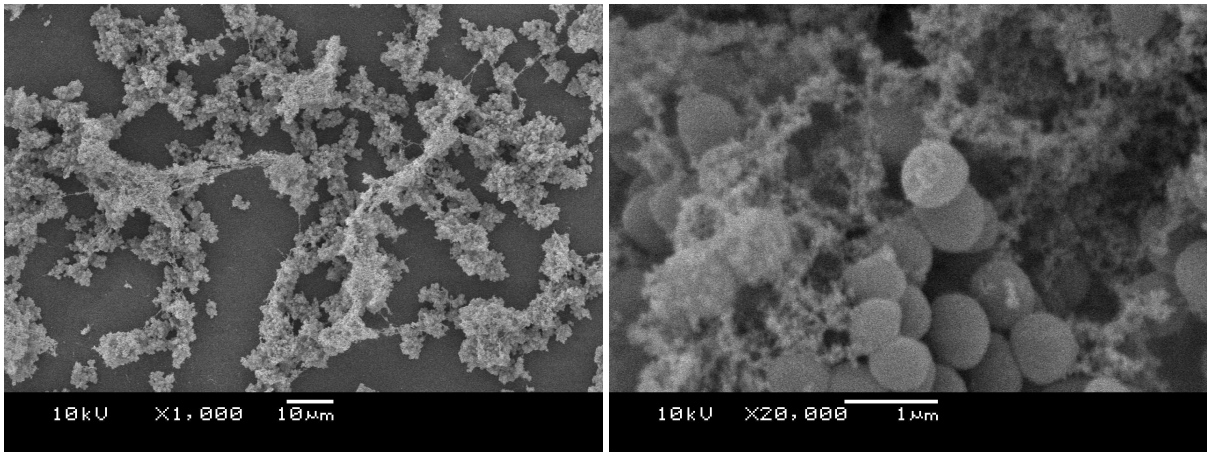

Fig. S16. Effect of proanthocyanidins free-form at 0.0625 mg mL^{-1} on the *S. epidermidis* adhesion and biofilm formation by SEM upon glass surface. Magnification of 1000 and 20 000 x.

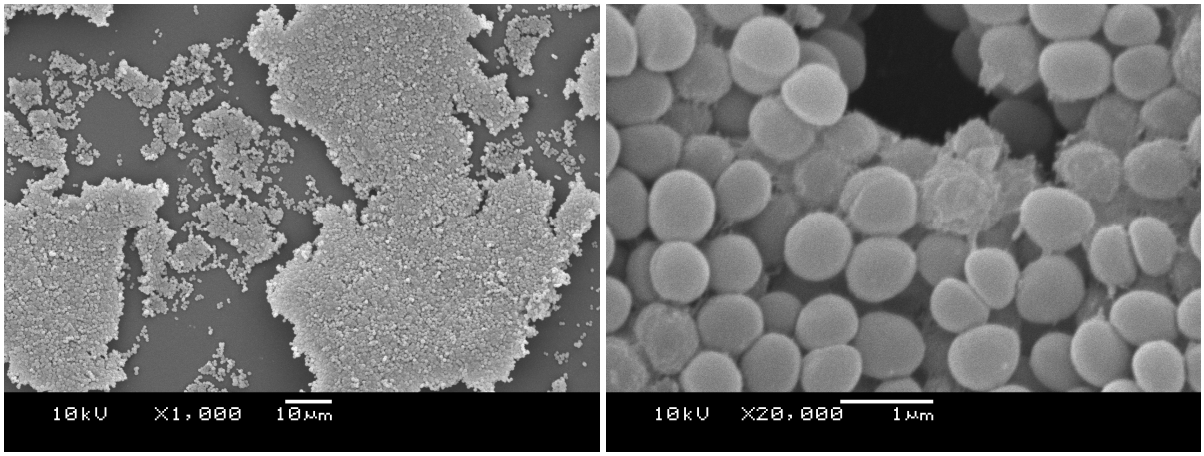

Fig. S17. SEM of *S. epidermidis* adhesion and biofilm formation on the non-coated Permanox. Magnification of 1000 and 20 000 x.

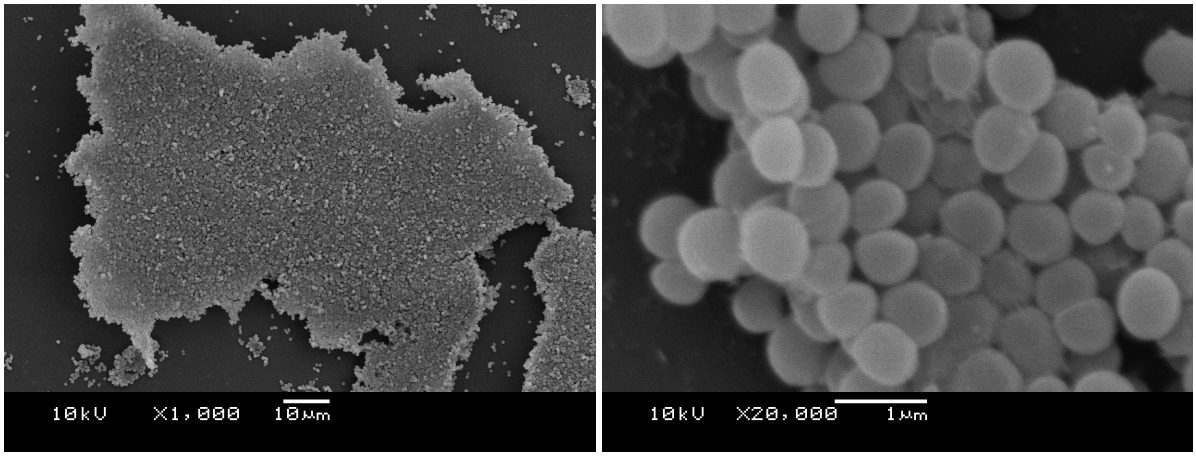

Fig. S18. SEM of *S. epidermidis* adhesion and biofilm formation on the 30% acetone-coated Permanox. Magnification of 1000 and 20 000 x.

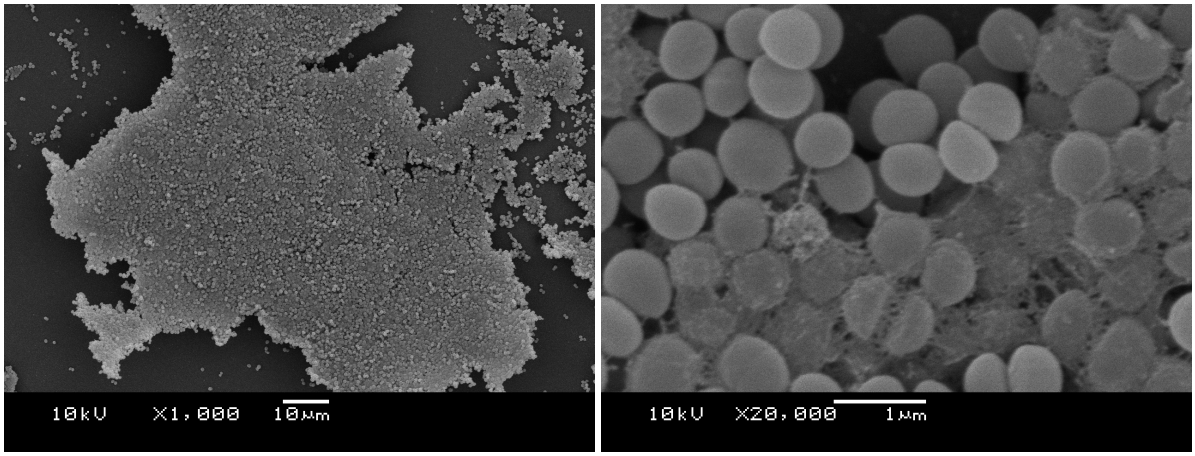

Fig. S19. SEM of *S. epidermidis* adhesion and biofilm formation on the 70% acetone-coated Permanox. Magnification of 1000 and 20 000 x.

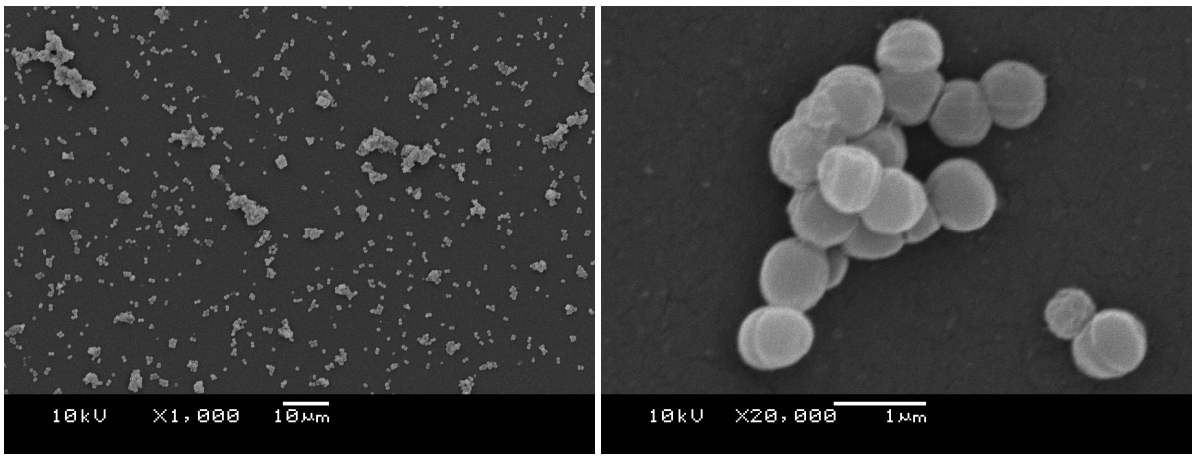

Fig. S20. SEM of *S. epidermidis* adhesion and biofilm formation on the proanthocyanidins in 30% acetone-coated Permanox. Magnification of 1000 and 20 000 x.

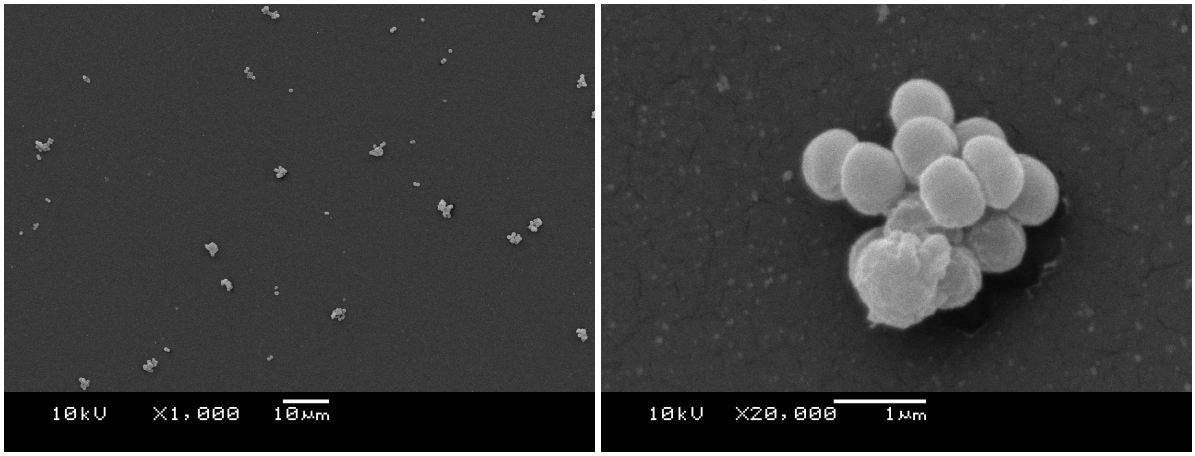

Fig. S21. SEM of *S. epidermidis* adhesion and biofilm formation on the proanthocyanidins in 70% acetone-coated Permanox. Magnification of 1000 and 20 000 x.

IV - CAPÍTULO 6 - Plasma surface modifications of polystyrene inhibit biofilm formation by multidrug resistant bacteria

Manuscrito a ser submetido para o periódico **Plasma Processes and Polymers**

N_2/H_2 Plasma Surface Modifications of Polystyrene Inhibit the Adhesion of Multidrug Resistant Bacteria

Running title: **N_2/H_2Plasma Prevents Adhesion of Hydrophilic Bacteria**

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Abstract

This work challenges multidrug resistant pathogenic emerging bacteria to adhere on polystyrene submitted to radiofrequency discharges containing N_2 and H_2. It is shown that the two employed treatments, which differ in duration and power applied to the plasma, are able to inhibit up to 83% *Klebsiella pneumonia* adhesion in the fisrt 24 h, without biocidal effect. Results from X-ray photoelectron spectroscopy and water contact angle measurements reveal correlation with biological findings and indicate that the limited adhesion of bacteria possessing hydrophilic surface onto plasma-treated surfaces may be explained in terms of electrostatic repulsion.

Introduction

Polymers are widely used as biomaterials in prosthesis, bone replacement implants, drug delivery, catheters and tissue engineering.[1,2] Among them, poly(methyl methacrylate),[3] poly(ethylene glycol),[4] polytetrafluoroethylene,[5] and polyurethane[6] are commonly used due to their biocompatibility, mechanical properties and ease of molding into desirable shapes. Either in single form or as copolymer, polystyrene (PS) has attracted considerable attention, since the polymer presents excellent mechanical properties, side by side with the wide availability of its precursor, the styrene monomer, which lowers production costs.[7-9] This polymer is widely used in cell culture applications because of its non-toxicity and high transparency, as indicated by the number of brand names available in research area for this purpose. Although the use of PS as biomaterial is uncommon in clinical practice, polystyrene is used for medical applications in artificial liver support and in controlled release devices.[10] Also its use is described in wound dressing and coatings for implantable medical devices[11] as well as in implantable medical devices for controlled delivery of therapeutic agent.[12]

Many of these materials were not originally designed for medical applications, and were selected for such uses based solely on their bulk properties, such as mechanical strength.[13] As a result, many widely used biomaterials suffer from significant drawbacks, including the proneness to bacterial infection. When a given biomaterial is implanted, it can become a site for bacterial adhesion, colonization, and further formation of a multicellular structure highly resistant to antimicrobials, named biofilm.[14,15] According to the National Institutes of Health, 80% of hospital-acquired infections worldwide are attributed to bacteria forming biofilms.[16] *Staphylococcus epidermidis* is known to be one of the most important pathogens involved in nosocomial infections on medical devices.[17] Equally important are the infections caused by *Staphylococcus aureus*, in which the resistance to methicillin has been held accountable for increased hospital stays and healthcare costs.[18] In addition, considerably important is the emergence and rapid spread of multidrug resistant bacteria, like *Klebsiella pneumoniae* Carbapenemase (KPC)-producing enterobacteriaceae, whose infections are associated with high mortality rates.[19]

Carbapenemases are bacterial enzymes able to hydrolyse all β-lactam antimicrobials,[20] including carbapenems - one of the few available treatment options in infections caused by Gram-negative bacteria. Recently, a study showed the high prevalence of KPC-producing enterobacteriaceae in patients who had been implanted with artificial devices.[19]

Since biomaterial infections develop following initial adhesion of the pathogens to the material surface, this issue evidences the need for the development of materials with antibiofilm surfaces. To avoid search for new biomaterials, management of bacterial adhesion through the control of surface properties of materials used in the manufacture of indwelling devices can be pursued. In order to obtain anti-infective properties, different approaches have been used, such as impregnation of antibacterial molecules on the polymer surface, polymer surface engineering methods, or a combination of both.[21-23] Although the use of antimicrobials leads to anti-infective surfaces for a wide range of pathogens, it requires relatively large amounts of chemicals, increasing costs, and there is the possibility of leaching. Moreover, the continuous use of antimicrobials is controversial, since it can induce the development of bacterial resistance. Surface engineering methods have been used to modify the surface properties of materials in different ways.[24] Among the available strategies, plasma surface modification (PSM) shows advantages[25,26] such as relatively low cost, environmental friendliness, applicability to diverse materials of complex shapes, changing only surface properties and preserving favorable bulk characteristics of materials.

This work intends to be a contribution to explore the potential of the polymer surface engineering by PSM methods rather than the presentation of a new modified material suitable for indwelling clinical applications. Thus, PS was modified by plasma treatments in a mixture of N_2 and H_2 gases and seven bacteria, presenting remarkable resistance to antimicrobials and distinct bacterial surface hydrophobicity index, were tested with respect to adhesion on the treated surfaces. We used two well-known reference strains, *S. epidermidis* ATCC 35984 and *S. aureus* MRSA ATCC 33591, and also fresh clinical isolates of *S. epidermidis* and KPC-producing Enterobacteriaceae in order to challenge the plasma-treated-PS to multidrug resistant

pathogenic emerging strains. The characterization of material and bacterial surfaces was performed, and findings thereof were associated with biological assays.

Experimental Section

Polystyrene substrate

Standard sterile 96-well PS flat bottom microtiter plates (Costar 3599) were purchased from Corning, Inc. (NY, USA).

Bacterial strains and culture conditions

In this study we focused on multidrug resistant bacteria, regarding the extreme clinical importance of these microorganisms. All the evaluated strains were previously identified through conventional phenotypic methodology[27,28] and further their identification were confirmed using MicroScan Walk-Away (Dade Behring, USA) or Vitek (bioMérieux, USA), as automated systems. The presence of bla_{KPC} gene, responsible for carbapenemase enzyme production, was confirmed by the gold standard polymerase chain reaction (PCR) technique using specific primers, as previously described. [29]

All clinical isolates were collected from two hospitals located in Porto Alegre, Brazil. As Gram negative bacteria, we studied *Klebsiella pneumoniae* (isolate 174), *Serratia marcescens* (isolate 177) and *Enterobacter cloacae* (isolate 182), which are KPC-producing strains (bla_{KPC} gene positive). As Gram positive bacterium, we used *S. epidermidis* (isolates 122 and 167b). Two reference strains were included: *S. epidermidis* ATCC 35984 and the methicillin-resistant *S. aureus* (MRSA) ATCC 33591. They were grown in Mueller Hinton agar (Oxoid Ltd., England) overnight at 37°C and a bacterial suspension (3×10^8 CFU mL^{-1}) in 0.9% NaCl was used in the assays. Minimum inhibitory concentration (MIC) was established according to the broth microdilution method for different drugs, in order to assess the bacterial susceptibility profile[30] (**Table 1**).

Table 1. Bacterial strains: site of isolation, ability to produce biofilm on PS surface, HPBI and antimicrobial susceptibility profile.

Isolate	Bacterial identification	Biofilm production ability	HPBI (%)	Clinical specimen	MIC(µg/mL)/susceptibility profile (CLSI 2012)[a)]											
					IMI[b)]	MER[c)]	ERT[d)]	CAZ[e)]	PIP[f)]	CIP[g)]	AK[h)]	OXA[i)]	VAN[j)]	RIF[k)]	GEN[l)]	ERI[m)]
K. pneumoniae	174 (KPC)	Moderate	24	Blood	64/R	32/R	64/R	64/R	256/R	64/R	16/S	-	-	-	-	-
S. aureus	ATCC 33591	Strong	33	Standard strain	-	-	-	-	-	-	-	128/R	0.5/S	1.125/R	0.5/S	1/I
S. marcescens	177 (KPC)	Strong	42	Respiratory tract	8/R	8/R	64/R	4/S	128/R	64/R	32/I	-	-	-	-	-
E. cloacae	182 (KPC)	Strong	55	Urine	8/R	4/R	8/R	> 256/R	128/R	64/R	2/S	-	-	-	-	-
S. epidermidis	ATCC 35984	Strong	77	Standard strain	-	-	-	-	-	-	-	≥ 128/R	1/S	0.125/S	≥ 64/R	1024/R
S. epidermidis	122	Strong	79	Central venous catheter	-	-	-	-	-	-	-	128/R	2/S	0.75/S	0.25/S	2048/R
S. epidermidis	167b	Strong	84	Central venous catheter	-	-	-	-	-	-	-	0.5/R	1/S	0.125/S	0.125/S	1024/R

[a)]Breakpoints CLSI 2012: IMI/MER (S ≤1; I =2; R ≥4); ERT (S ≤0,25; I =0,5; R ≥1); CAZ (S ≤4; I =8; R ≥16); PIP (S ≤16; I 32-64; R ≥28); CIP (S ≤1; I =2; R ≥4); AK (S ≤16; I =32; R ≥64); OXA for *S. aureus* (S ≤2; R ≥4) and for *S. epidermidis* (S ≤0.25; R ≥0.5); VAN (S ≤2; I = 4-8; R ≥16); RIF (S ≤1; I =2; R ≥4); GEN (S ≤4; I=8; R ≥16); ERI (S ≤0.5; I =1-4; R ≥8).

[b)] imipenem; [c)] meropenem; [d)]ertapenem; [e)] ceftazidime; [f)] piperacillin-tazobactam; [g)] ciprofloxacin; [h)] amikacin; [i)] oxacillin; [j)] vancomicyn; [k)] rifampicin; [l)] gentamicn; [m)] erythromycin. S=susceptible; I=intermediate; R=resistant; - = not determined

Microbial surface hydrophobicity index

Surface hydrophobicity of bacterial strains was determined using the microbial adhesion to hydrocarbon (MATH) test.[31] The bacterial suspension was cultured with tryptone soya broth (TSB) (Oxoid Ltd., England) during 24 h at 37 °C. The cultures were washed with sterile saline solution and cells were harvested by centrifugation. The suspensions were adjusted to an absorbance (A_i) of about 0.3 at 600 nm (Spectramax M2e Multimode Microplate Reader, Molecular Devices, USA). Toluene (500 µL) was added to 3 mL of each adjusted bacterial suspension and mixed. The new absorbances of aqueous phase (A_f) were measured after phase separation. The hydrophobicity index (HPBI) was expressed as: $(A_i - A_f) / A_i \times 100\%$. Bacterial strains with an HPBI greater than 70% were classified as hydrophobic while strains with an HPBI lower than 70% were classified as hydrophylic.[31]

Plasma surface modification

The 96-well PS samples were inserted in a vacuum chamber that was pumped down to a pressure of 2×10^{-2} mbar. The chamber was then pressurized to 1.3 mbar with a mixture containing 24 mol% H_2 and 76 mol% N_2 (purity > 99.999%). Pumping was maintained and the pressure was kept by flowing gas continuously into the chamber.

A radio frequency power source (13.56 MHz) was used to generate the plasma. In order to minimize thermal effects, the sample holder was monitored; it stayed below two thirds of the glass transition temperature of PS (95°C) throughout the treatment period. After the treatment, samples were removed from the vacuum chamber and immediately sealed in a sterile package that was not opened until surface characterization or biological assay (see below). The effects of PSM were investigated stipulating two different time and power conditions, namely: treatment 1 (300 s at 125 W), and 2 (60 s at 75 W), while all other parameters were kept constant. The effect of shelf life was evaluated by analyzing samples 15 and 30 days after plasma treatments, simulating the storage of the plasma-treated material. The control for plasma treatments was performed by exposing polystyrene microplates to the N_2/H_2 gas mixture without igniting the plasma.

Surface characterization

PS samples were characterized before and after the plasma treatment using X-ray photoelectron spectroscopy (XPS) and water contact angle (WCA) measurements. Surface concentrations of C, N, and O were determined by XPS in a setup calibrated using UK's National Physical Laboratory (NPL) reference materials and software. Chemical speciation of the C 1s signal was achieved with an Omicron SPHERA spectrometer at a pass energy of 5 eV using Mg Kα radiation (1253.6 eV). Spectral fitting was performed using CASA XPS software. Contact angle measurements were carried out using the sessile drop technique and doubly deionized water. The drop was observed directly with an Olympus BX-41 microscope objective lens and images were digitally captured using a 1.4 megapixel CCD camera. The reported water contact angles are means of more than five measurements performed in different areas of each sample surface. Polar and dispersive components of the surface free energy were obtained using the Owens-Wendt method[32] from contact angle measurements with water and diiodomethane. The control for plasma treatments was performed by exposure polystyrene microplates to the N_2/H_2 gases mixture without plasma discharge.

Bacterial adhesion assay

After plasma treatments, PS microtiter plates were directly used as substrates in bacterial adhesion assay following a modified described protocol.[33] The adhesion verified for each strain to the control microplate (plate that had been submitted to the gas mixture without plasma discharge) was considered 100% bacterial adhesion. In each well, 100 µL of the bacterial suspension and 100 µL TSB were added. Sterility control consisted of 200 µL of TSB. Following the incubation period (37 °C for 24 h) the content of the wells was removed and the wells were washed. The remaining attached bacteria were heat-fixed (60°C, 1 h) and stained with crystal violet. The dye was resuspended with DMSO (Sigma-Aldrich Co., USA) and absorbance was measured at 570 nm. Using this assay, each strain was categorized regarding its ability to adhere and to produce biofilm on the untreated PS, as previously described.[34]

Bacterial growth assay

To assess if the plasma treatments affect bacterial growth, two assays were performed: optical absorption at 600 nm (OD_{600}) and cell viability (resazurin indicator). OD_{600} was measured immediately before and after incubation as described in the previous section (bacterial adhesion assay). The difference between final and initial absorbance was taken as a measure of bacterial growth. To evaluate cell viability, we took the contents of wells after incubation in the bacterial adhesion assay and transferred to another standard sterile 96-well PS, to which 25 µL of a resazurin solution (0.1 mg mL^{-1}) was added. The plates were incubated (2 h at 37°C) and assessed visually: shades of blue indicated non-viable cells and shades of pink indicated viable cells.[35] The bacterial growth obtained for each strain by using the control microplate (plate that had been submitted to the gas mixture without plasma discharge) was considered 100% bacterial growth. Also, appropriate antimicrobial agents were used as positive control for bacterial growth.

Statistical analysis

Bacterial adhesion and growth by OD_{600} measurements are represented as percentage means ± standard deviation for each bacterial strain (n=3). Values higher than 100% represent a stimulation of bacterial adhesion or growth in comparison to the untreated samples. Differences were analyzed by one-way ANOVA followed by the Tukey test, and $p \leq 0.05$ was considered to be significant (SPSS 10.0 Software).

Results

Bacterial strains features

Bacterial strains used in this study presented a remarkable resistance profile to antimicrobials according to the MIC determinations (Table 1). All evaluated bacteria were able to adhere and developed biofilm on PS. *K. pneumoniae* (174) demonstrated moderate capability, while all other strains presented a strong capability to form biofilm on PS, based on the described classification scheme[34] (Table 1). Bacterial hydrophobicity (HPBI) varied significantly among strains. An HPBI higher than 70% was observed for all *S. epidermidis* strains, which were classified as hydrophobic

bacteria and an HPBI lower than 70% (ranging from 24 to 55%) was found for other strains, which were classified as hydrophilic bacteria (Table 1), according to the categorization method previously proposed.[31]

Bacterial adhesion and growth on PS surfaces

Adhesion on PS and bacterial growth as a function of N_2/H_2 plasma treatments is shown in **Figure 1** and **Figure 2**, respectively. Statistical analysis indicates that the two treatments tested are equivalent regarding bacterial adhesion and growth. The adhesion of *K. pneumoniae* 174, *S. aureus* ATCC 33591, *S. marcescens* 177 and *E. cloacae* 182 was significantly inhibited after plasma treatments when compared to untreated controls. *Klebsiella pneumoniae* was the pathogen more susceptible to plasma treatments (up to 83% adhesion inhibition), followed by *S. marcescens*, *S. aureus* and *E. cloacae* (up to 77, 65 and 48% of adhesion inhibition, respectively). These rates were maintained even after 15 days shelf life of PS samples. However, the adhesion prevention observed up to 15 days was considerably diminished after 30 days for the Gram-negative strains, in particular *K. pneumoniae* and *E. cloacae*, which reached almost 90% of their original attachment capability. Concerning *S. epidermidis* strains, neither treatment effectively prevented the bacterial adhesion.

Plasma treatments did not interfere with bacterial growth for any of the studied strains, as judged from OD_{600} measurements (Figure 2) and confirmed by the resazurin assay (data not shown).

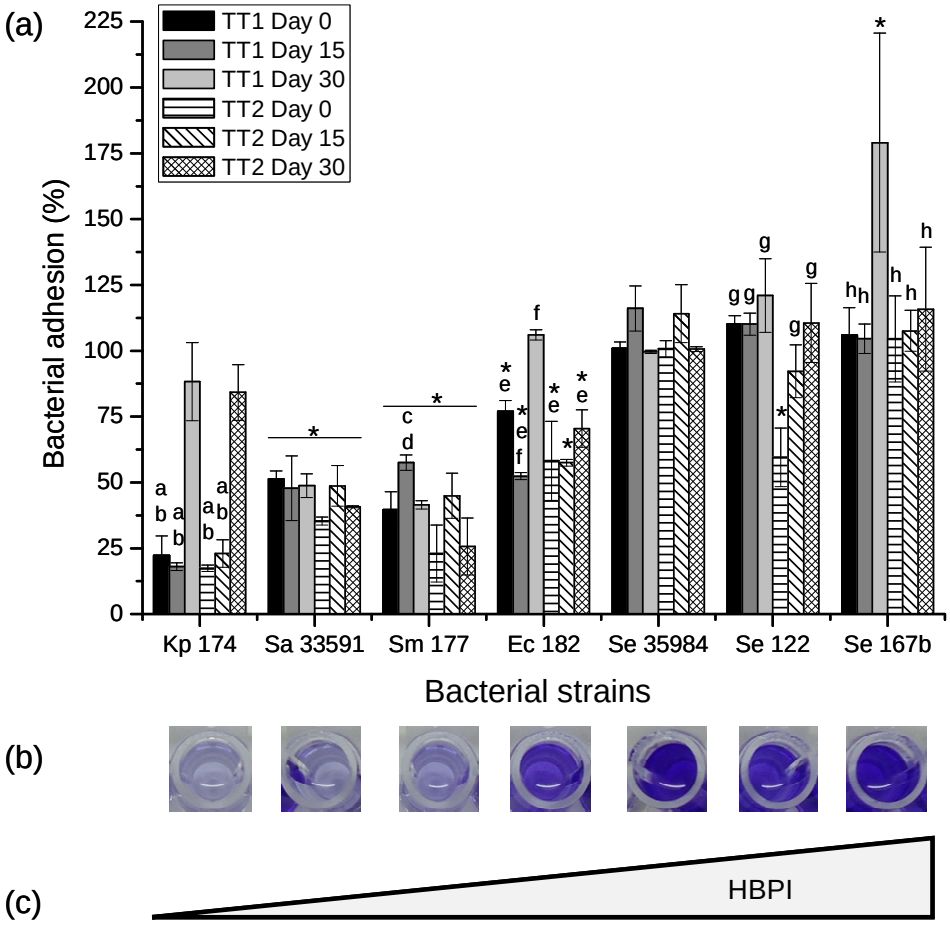

Figure 1. (a) Bacterial adhesion on plasma treated polystyrene. Bars represent percentage mean ± standard deviation of three experiments in comparison to untreated samples (100%). TT 1 and TT 2 represent treatment 1 and 2, respectively. * statistical difference in comparison to the untreated samples for each bacterium; *S. aureus*: a - statistical difference from TT 1 Day 30 and b - from TT 2 Day 30; *S. marcescens*: c - statistical difference from TT 2 Day 0 and d - from TT 2 Day 30; *E. cloacae*: e - statistical difference from TT 1 Day 30 and f – from TT 1 Day 0; *S. epidermidis* 122: g - statistical difference from TT 2 Day 0; *S. epidermidis* 167b: h - statistical difference from TT 1 Day 30. (b) Photos of crystal violet assay: bacterial adhesion on treated PS (TT 1 – day 0). (c) Schematic representation of the increasing HPBI values of the tested strains.

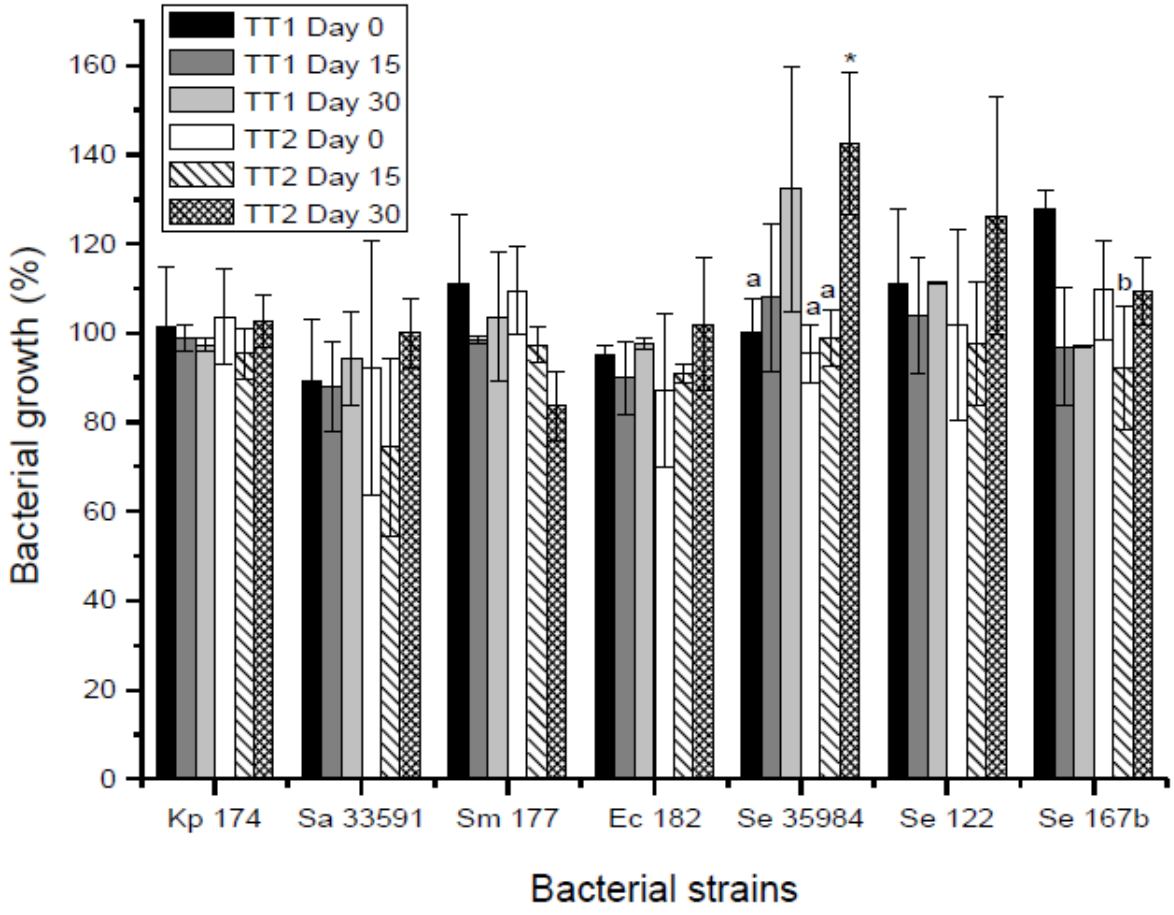

Figure 2. Bacterial growth on plasma treated polystyrene. Bars present percentage mean ± standard deviation of three experiments in comparison to untreated samples (100%). TT 1 represents treatment 1 and TT 2 represents treatment 2. *S. epidermidis* ATCC 35984: * statistical difference in comparison to the untreated samples and a - statistical difference from TT 2 Day 30; *S. epidermidis* 167b: b - statistical difference from TT 1 Day 0.

Surface composition

Figure 3 shows C 1s XPS spectra from pristine and plasma-treated PS. Spectra from the treated samples were highly asymmetric, indicating a variety of chemical moieties. Bonding configuration was evaluated by peak-fitting the C 1s envelopes. Within the resolution of the measurements, five peaks of equal width at half maximum were fitted. Based on the NIST X-ray Photoelectron Spectroscopy Database,[36] the component peaks used in this work were assigned as follows: C-C: 285 eV; C-N: +0.9 eV; C-O/C=N: +1.5 eV; C=O: +2.9 eV; and N-C=O: +4.2 eV.

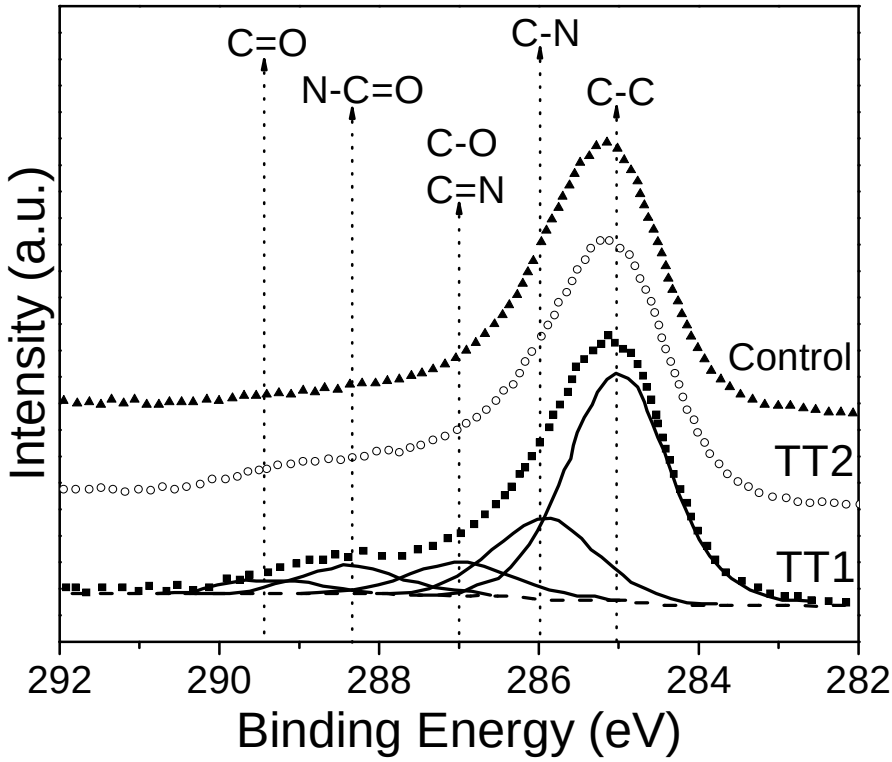

Figure 3. C 1s XPS spectra from pristine PS (Control-solid triangles) and samples submitted to plasma treatments 1 (TT 1 – solid squares) and 2 (TT 2 – open circles). Dashed line corresponds to the background and solid lines to spectral components that were used to fit experimental data. A.u stands for arbitrary units.

XPS revealed the overall surface composition of untreated PS as 87 at. % C and 13 at. % O; the technique does not detect hydrogen. The relatively high amount of oxygen can be understood based on the high surface sensitivity of XPS (analysis is restricted to less than 10 nm at the sample surface); on the absorption of oxygen resulting in surface termination such as -OH; and on the adsorption of H_2O. **Figure 4** shows that just after plasma treatments, the samples incorporated 13 and 11 at. % N, respectively for treatments 1 and 2. While significant amounts of oxygen were still detectable, its abundance fell to about half the original value. In fact, nitrogen ion bombardment during plasma treatment is expected to remove essentially all the oxygen originally at the PS surface. The oxygen detected after treatments should originate mostly from reaction of the freshly treated surface with water vapor in the atmosphere, and to a lesser extent from residual oxygen in the plasma reactor.

Plasma processing occurs far from thermodynamic equilibrium, and the results can often be explained in the light of kinetic arguments. In this work, the treatments 1

and 2 differed in duration and power applied to the plasma. The fact that they nevertheless produced similar XPS results suggests that after 60 s at 75 W a situation of dynamic equilibrium has been reached and the rate of nitrogen incorporation to the PS surface from ions in the plasma is already the same as the rate of nitrogen removal due to the associated ion bombardment effect. In this case, processing longer (and at higher power) simply results in additional erosion of the substrates without significant changes in surface composition.

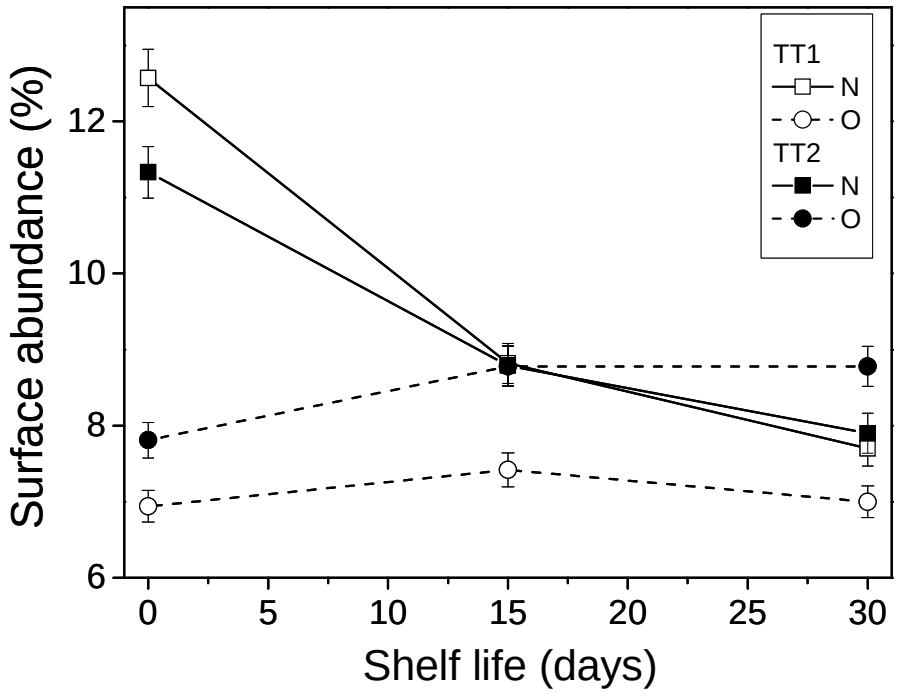

Figure 4. Nitrogen (squares) and oxygen (circles) surface abundance of plasma treated PS as a function of shelf life for samples submitted to treatment 1 (TT 1 open symbols) treatment 2 (TT 2 full symbols). Lines are only to guide the eyes.

As noted above, the surface composition of PS submitted to treatments 1 and 2 were similar; even during the shelf life experiment (Figure 4). The significant change in composition between preparation (day 0) and days 15 and 30 could be accompanied by a significant change in performance regarding bacterial adhesion. An additional physical property was searched aiming at increased correlation with the bioassays.

Effects of plasma treatment on water contact angle

The polar component of the surface energy of PS, which is essentially proportional to the abundance of polar (hydrophilic) species on the surface, such as nitrogen-

containing chemical moieties, is shown in **Figure 5**. Surface energy increased sharply with the application of plasma treatment and then declined, as samples age. Surface energies resulting from treatments 1 and 2 were similar within 5 mJ/m^2 (ranging from 17 to 21 mJ/m^2) with a minimum of 14 mJ/m^2 for treatment 1 aged 30 days. The polar component for the pristine PS substrate never exceeded 9 mJ/m^2.

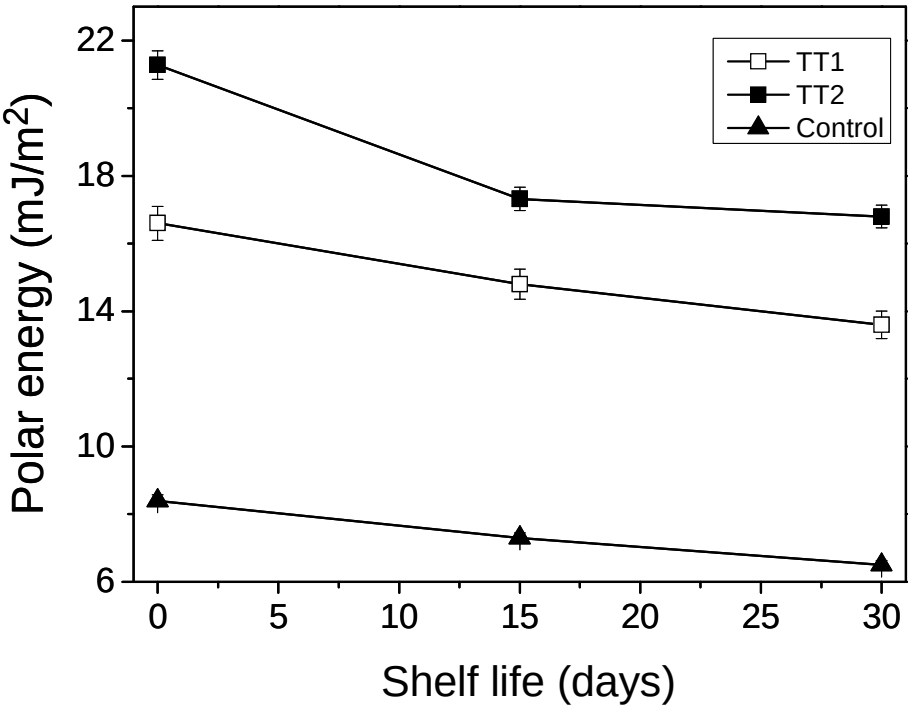

Figure 5. Polar energy of the PS surface as determined from water contact angle measurements for samples submitted to treatment 1 (TT1 - open square), treatment 2 (TT 2 -solid square) and control sample (solid triangles). Lines are shown only to facilitate visualization.

Discussion

Planktonic bacteria that attach to a surface and grow as a biofilm are protected from killing by antimicrobials, contributing to the persistence of infections such as those associated with medical implanted devices.[37] In this context, the emergence and global spread of carbapenemase-producing enterobacteriaceae, such as *Klebsiella* spp., *Enterobacter* spp., *Escherichia coli*, and *S. marcescens*, pose an immediate infection threat to vulnerable hospitalized patients and a potential threat to individuals in general. Furthermore, long-lasting concerns about *Staphylococcus* infections likewise persist, since they represent the most frequent causes of nosocomial infections and infections on indwelling medical devices.[38,17]

This study focused on seven bacterial strains which are considered highly relevant from the clinical point of view, since they presented high level of resistance profile to antimicrobials, according to the MIC determined based on a panel of antimicrobial agents (Table 1). All strains studied (*E. cloacae*, *S. marcescens*, MRSA and *S. epidermidis*), demonstrated a strong ability to form biofilm on polystyrene, except *K. pneumonia*, which was classified as a moderate biofilm producer on this surface (Table 1). The exposure of PS to N_2/H_2 plasma (treatments 1 and 2) inhibited bacterial adhesion of strains that present hydrophilic surface (HBPI below 70%), reaching up to 83% adhesion inhibition for *K. pneumoniae*, 77% for *S. marcescens*, 65% for *S. aureus* and 48% for *E. cloacae*, during the first 24 h. Oppositely, *S. epidermidis*, which present hydrophobic surfaces (HBPI above 70%), retained its ability to adhere on the treated surfaces (Figure 1). The results of OD_{600} (Figure 2) and resazurin evaluations revealed bacterial cell viability of the planktonic cells in treated samples. Therefore, plasma treatments are indeed inhibiting bacterial adhesion, not growth.

Bacterial adhesion is the first step in biofilm formation. The initial adhesion process is influenced by bacterial features, by the material surface properties and by the microenvironmental interactions.[10] In order to explain the complex processes involved in adhesion and non-adhesion mechanisms of bacteria on surfaces, some theories have been proposed. Initial bacterial adhesion is governed by non-specific interactions, and is commonly explained using the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory.[39] Briefly, the classical DLVO approach considers two additive forces: (i) an attractive van der Walls interaction and (ii) a repulsive or attractive interaction, depending on the surface charge of the substrate and on the electrostatic double layer of the bacteria cell. This model is useful to describe qualitatively the cell adhesion onto solid substrates, but its theoretical predictions not always elucidate biological phenomena observed.[40-42] The thermodynamic approach considers the Gibbs energy involved in the bacterial adhesion, but also fails to properly explain some results.[43,44] Nowadays, the most promising theory, which uses components from both models and includes distance dependent hydrophobicity/hydration effects – the extended DLVO theory – has been successfully

applied to explain some bacterial behavior regarding adhesion.[45,46] Thereby, several surface properties, including roughness, wettability, composition, electric charge, and surface free energy seem to contribute in the complex mechanism of bacterial adhesion.[10]

This work reports the composition and the polar component of surface energy for plasma-treated PS. Previous plasma treatments experiments (data not shown) indicated negligible roughness (root mean square value in the nanometer range) and an essentially constant dispersive component of surface energy. Data presented herein show that both PSM treatments introduce nitrogen-containing moieties at the PS surface (Figure 4), increasing the polar component of surface energy. **Figure 6** (a) and (b) illustrates bacterial adhesion for PS surfaces submitted to treatments 1 and 2, respectively, as a function of the bacterial hydrophobicity investigated in this work. It can be observed that the higher the bacterial surface hydrophobicity, the higher the bacterial attachment, regardless the treatment (1 or 2). The fact that adhesion of hydrophilic bacteria (HPBI below 70%) is suppressed more effectively might be explained considering that the bacterial surface and the PS substrate polarization is the same, i.e. charges due to nitrogen-containing groups on PS and bacterial surface charge are of the same type and thus lead to electrostatic repulsion. Hydrophobic bacteria (HPBI above 70%) continue to adhere to the PS surface because charges on the substrate induce opposite polarization in the bacteria, as seen for S. *epidermidis* strains (Figure 1). Such reasoning does not depend on particular properties of the biomaterial or bacteria utilized, and so it should be a general rule that PSM of biomaterials using nitrogen either inhibits or promotes the adhesion of hydrophilic bacteria (depending whether surface charges on the bacteria are of the same or different type compared to the biomaterial surface), while hydrophobic bacteria are the least affected. Interestingly, another study demonstrated that the surface hydrophobicity of *S. epidermidis* strains presents large variations, varying from 3 to 89% using the MATH test.[47] This finding could support the effectiveness of the plasma treatments herein used against hydrophilic strains of *S. epidermidis*.

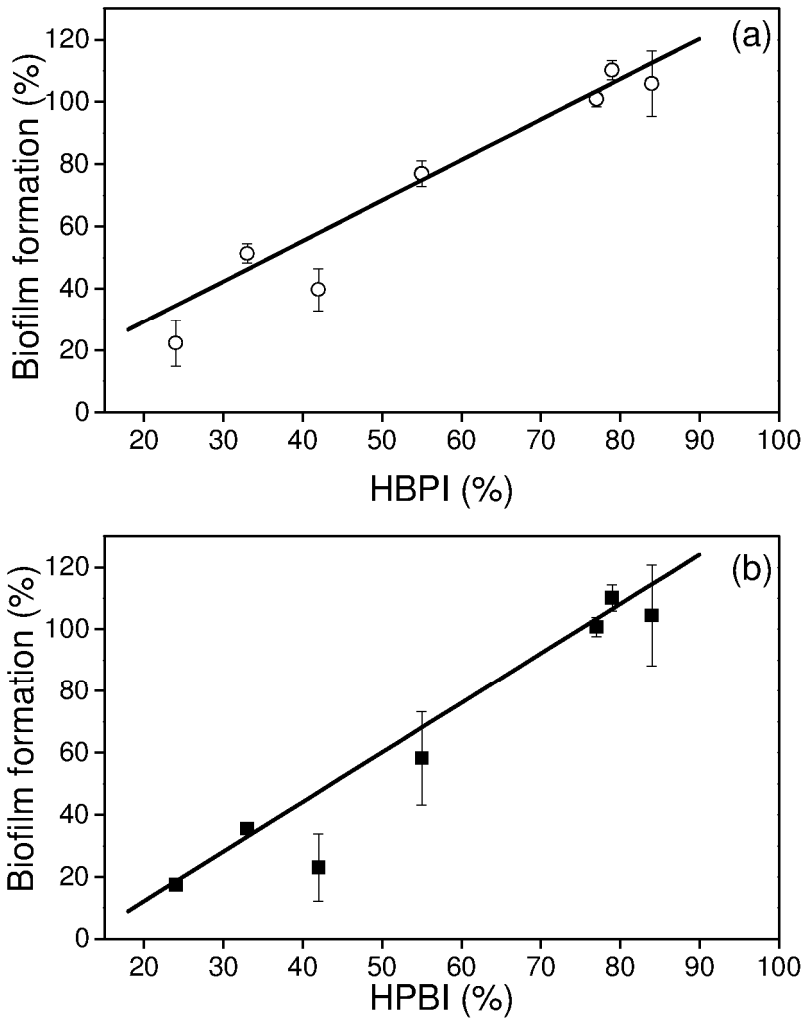

Figure 6. Bacterial adhesion as a function of bacterial hydrophobicity for bacterial strains on plasma treated PS: (a) TT 1 (open circles) and (b) TT 2 (full squares).

Beyond the qualitative discussion, we observed that surface nitrogen concentrations in excess of 8.8 % or polar component of the surface energy above 15 mJ/m^2 are sufficient to produce the beneficial effects sought. It has been reported that plasma induced modifications of polymer surfaces can undergo changes with time after treatment.[48] Lifetime will thus depend on how aggressive (chemically) is the medium in which the biomaterial will be used or stored, and that, if known in advance, could be used to select between oxygen and nitrogen plasma modification for a given application. As it can be seen, experiments of shelf life demonstrated that the surface properties of the PS start to degrade. A drop in nitrogen concentration (Figure 4) for 15

and 30 days after treatment and consequent reduction in the polar component of surface energy (Figure 5) were observed.

The control of biofilm formation is very complex, as demonstrated by the historical inability to produce clinically effective biomaterial implants resistant to infection.[49] This study focuses on the applicability of non-thermal N_2/H_2 plasma to generate antibiofilm PS surfaces, but it is important to observe that the inhibition of bacterial adhesion obtained was not 100%. In this sense some aspects should be considered: (i) in a potential future application of these surfaces in clinical settings, the insertion or incision procedures will probably be accompanied by appropriate prophylactic antibiotic therapies and; (ii) the total number of bacteria-laden particles related to possible contaminant microorganisms is much lower than the inoculum used in this study (1.5×10^8 CFU/mL in the wells). Indeed, the understanding and control of polymer-bacterium interactions may be more complex when the formation of a conditioning film is taken in account. After implantation, a conditioning film from organic matter present in the host surrounding fluid may be deposited on the biomaterial surface. The composition of this film depends both on the material surface features and the site body (tissue fluid, salive, urine, blood and serum), and comprises mostly proteins, such as albumin, immunoglobulin, fibrinogen and fibronectin.[50,51] In this sense, current findings are promising, since nitrogen-based plasma systems, such as N_2, NH_3, Ar/NH_3 and O_2/NH_3 have been used to produce hydrophilic surfaces on different polymer substrates, such as polyethersulfone and polypropylene membranes, in order to overcome protein fouling.[52-54] The performance of N_2/H_2 plasma processing-PS regarding bacterial adhesion in the presence of the conditioning film remains to be explored in a further investigation.

Summary

In conclusion, this study presents N_2/H_2 plasma treatments that produce a much less bacterial adherent and non-biocidal material, underlining plasma technical applicability as a suitable clean and fast alternative processing for medical polymer materials. The correlation between microbiological and physicochemical investigations demonstrated that bacteria presenting hydrophilic surface (here

exemplified by KPC-producing enterobacteriaceae and MRSA) had biofilm formation significantly inhibited on treated PS surfaces due to the prevention of bacterial adhesion. Experiments evaluating the shelf life of plasma-treated PS indicated that the surface nitrogen concentration in excess of 8.8 at.%, which was equivalent to polar component of the surface energy above 15 mJ/m^2, is critical for limiting bacterial adhesion. *Staphylococcus epidermidis* hydrophobic strains were not affected, allowing us to suggest that these effects may be explained in terms of electrostatic repulsion. The heterogeneity and complexity of bacterial surfaces, including the variability across different species and strains, contribute to the difficulty in generalizing findings considering the impairment of bacterial attachment on substrata. Thereby, the potential application of a fast technique, as PSM, extends the possibility of controlling the adhesion of various bacterial species and genera, including clinically important pathogens, since it does not target specific microbial structure or substrate, unlike traditional antimicrobial agents. Moreover, compared to the antimicrobial-based coating approach that kill microorganisms to mitigate bacterial colonization, the PSM treatments presented in this work would be associated with minimal development of bacterial resistance, interfering just with bacterial surface - material surface interactions. Regarding the dramatic situation in the hospital environment, where the usual management to combat pathogenic biofilms is the removal of thousands infected artificial devices, the results presented herein should be considered as a clue and a contribution to explore PSM methods for future material development.

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Keywords: bacterial adhesion; multidrug resistant bacteria; plasma treatment; polystyrene; surface modification

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V – DISCUSSÃO GERAL

O presente trabalho, conduzido de forma multidisciplinar, utilizou microrganismos e superfícies modelo, com o objetivo principal de demonstrar provas de conceito mais do que aplicações propriamente ditas. Neste sentido, foram abordadas duas estratégias para o combate da formação de biofilmes de bactérias: (i) a busca por fitocompostos com atividade antiformação de biofilmes, guiado por relatos etnofarmacológicos, e o posterior recobrimento de superfície polimérica com estes compostos e, (ii) a modificação de propriedades de superfície polimérica, através da técnica de plasma iônico, para a obtenção de superfícies antiadesivas. O principal modelo de superfície utilizado foi o poliestireno. O poliestireno é um material de hidrocarboneto aromático e, como tal exibe caráter hidrofóbico de hidrocarbonetos, juntamente com a estabilidade química dos compostos aromáticos. Este polímero é largamente empregado em culturas de células por não apresentar toxicidade, ser transparente e ter baixo custo de produção, tal como indicado pelas inúmeras marcas disponíveis na área de pesquisa para este propósito. Embora o uso deste polímero como biomaterial na prática clínica seja raro, o poliestireno é utilizado em aplicações médicas em sistemas artificiais de função hepática e em dispositivos de liberação controlada (PAVITHRA e DOBLE, 2008), e o seu uso é descrito em curativos com liberação controlada de agentes terapêuticos e em revestimentos para dispositivos médicos implantáveis (VACHON e WNEK, 2001; SCHWARZ, 2011).

Após a seleção, coleta de plantas e preparo dos 45 extratos aquosos, a fim de reproduzir o uso tradicional das plantas da Caatinga, foram realizados os rastreamentos para a busca de atividade antibiofilme e atividade antimicrobiana utilizando duas bactérias-modelo formadoras de biofilme: *S. epidermidis* ATCC 35984 e *P. aeruginosa* ATCC 27853. Considerando *S. epidermidis*, 15,5% dos extratos apresentaram uma expressiva atividade antiformação de biofilme (variando de 75 a 100%) e apenas 2,2% inibiram o crescimento bacteriano (TRENTIN *et al*., 2011). Entretanto, quando testados frente à *P. aeruginosa* o número de extratos com altas atividades foi bem menor (4,4 e 6,7% para antibiofilme e antimicrobiana, respectivamente). Estes trabalhos iniciais demonstraram o alto potencial antibiofilme de extratos aquosos da Caatinga contra *S. epidermidis* e indicaram três plantas com

atividade antimicrobiana para *P. aeruginosa*, sendo importantes para direcionar nossa posterior investigação.

Subsequentemente, o estudo foi focado na purificação dos compostos bioativos de quatro plantas: *A. colubrina*, *C. leptophloeos* e *M. urundeuva* (ativas contra *P. aeruginosa*) e *P. moniliformis* (ativa contra *S. epidermidis*). Considerando a metodologia de extração (maceração aquosa), a origem do material vegetal (cascas e folhas), os dados sobre o alto teor de taninos das espécies *A. colubrina* e *M. urundeuva* (SIQUEIRA *et al*., 2012), e o fato de que as plantas da Caatinga estão submetidas a elevada radiação solar, a obtenção de taninos pelo processo de fracionamento bioguiado era, de certa forma, esperada.

Taninos representam uma classe complexa de produtos naturais, sendo que muitos trabalhos que direcionam a busca de compostos bioativos realizam processos extrativos para a remoção dos mesmos. Esta complexidade pode ser explicada devido à grande heterogeneidade dos taninos, os quais diferem quimicamente e no grau de oligomerização, e devido à ausência de padrões apropriados para aplicação em técnicas analíticas. Desta forma, a quantificação de taninos frequentemente é sub ou superestimada dependendo do padrão escolhido (SCHOFIELD *et al*., 2001). Considerando a cromatografia líquida de alta eficiência, a limitada resolução cromatográfica dos taninos em colunas de fase reversa é atribuída à presença de isômeros com polaridades similares e diferentes graus de polimerização, resultando em tempos de retenção sobrepostos (SCHOFIELD *et al*., 2001; YANAGIDA *et al.*, 2003), além da elevada massa molecular desses metabólitos, o que prejudica a resolução do cromatograma. O uso de fases estacionárias normais, entretanto, parece ser útil para separar taninos hidrolisáveis de acordo com a massa molecular (OKUDA *et al.*, 1989) assim como oligômeros de taninos condensados e polímeros (SCHOFIELD *et al.*, 2001). Considerando as técnicas cromatográficas preparativas, os taninos adsorvem-se fortemente em sílica gel, justificando o baixo rendimento de frações ativas na coluna de RP-C18 e a presença de material retido na coluna após a limpeza da mesma (dados não mostrados). Desta forma, a separação dos compostos ativos neste trabalho foi conduzida em Sephadex LH-20, sendo induzida pela diferença de adsortividade de

cada tanino no gel e não pelo princípio de gel filtração através da separação por tamanho molecular (OKUDA *et al*., 1989).

As análises por MALDI MS MS, uma das ferramentas mais apropriadas para a caracterização de macromoléculas (MONAGAS *et al*., 2010), confirmaram a presença de taninos como compostos ativos. Estruturas complexas de proantocianidinas (composta principalmente por profisetinidina para *A. colubrina* e por prorobinetinidina para *C. leptophloeos*) foram descritas pela primeira vez para o gênero *Anadenanthera* e *Commiphora*, e de taninos hidrolisáveis (constituído por unidades de ácido gálico em *M. urundeuva*) foram identificadas. Estes taninos inibiram a formação de biofilme de *P. aeruginosa* através da ação bacteriostática, causando danos de membrana e excesso na proliferação de vacúolos bacterianos, embora a membrana de eritrócitos tenha sido preservada. Com relação à *P. moniliformis*, proantocianidinas ricas em prodelfinidina foram os compostos responsáveis pela completa inibição da formação de biofilme de *S. epidermidis*, sem afetar a viabilidade do microrganismo. Esta investigação é o primeiro estudo fitoquímico desta abundante planta da Caatinga. Todas as frações obtidas da coluna de Sephadex que continham proantocianidinas foram capazes de prevenir a formação de biofilmes, embora a inibição tenha sido completa nas frações cujas estruturas apresentavam grau de polimerização maior que 3 (trímero), destacando a importância do tamanho molecular para a atividade biológica. Estes resultados corroboram com o estudo de Wittschier e colaboradores (2007), o qual destaca o potencial de grandes moléculas, como proantocianidinas e carboidratos provenientes de plantas, como antagonistas de interações adesivas. Interessantemente, estas proantocianidinas ricas em prodelfinidina derivadas da *P. moniliformis*, apresentam estrutura linear com ligações interflavanil tipo B, diferentemente das proantocianidinas do cranberry, ativas contra a adesão de *E. coli*, cuja ligação tipo A parece ser um pré-requisito para a atividade (HOWEL *et al*., 2005). Neste aspecto, nossos resultados vão ao encontro dos achados de Janecki e Kolodziej (2010), onde a redução da adesão de *Streptococcus* foi observada exclusivamente para prodelfinidinas, sugerindo que a estrutura do pirogalol (anel B) constitui um grupamento estrutural importante para a atividade.

Experimentos visando um maior detalhamento da atividade biológica das proantocianidinas derivadas de *P. moniliformis* foram realizados com a fração F7, a qual apresentou maior rendimento frente às demais. Os resultados indicaram que tanto a superfície bacteriana (*S. epidermidis*) quanto a superfície dos materiais testados (vidro e poliestireno) são espontaneamente recobertos pelas proantocianidinas, tornando-se superfícies hidrofílicas. Diversos trabalhos vêm demonstrando que a hidrofobicidade é um dos principais parâmetros envolvidos na adesão bacteriana a materiais (VACHEETHASANEE *et al*., 1998; PASCUAL, 2002; ROOSJEN *et al*., 2006; JACOBS *et al*., 2007; OTTO, 2009) e, que a atividade antiadesiva de compostos, como a heparina e o ácido hialurônico, bem como de superfícies modificadas está sendo atribuída à hidrofilicidade dos mesmos (CASSINELLI *et al*., 2000; FU *et al*., 2006; GAO, *et al*., 2011). Quando as superfícies foram recobertas com estas proantocianidinas via *spin coating*, a habilidade de prevenir a adesão de *S. epidermidis* se manteve e a superfície do material apresentou-se fortemente hidrofílica.

Um dos grandes desafios atuais na área de *ciências de materiais* é a criação de superfícies antiaderentes para microrganismos e que, simultaneamente, permitam a adesão e a colonização de células mamíferas. Uma vez que as células hospedeiras superem as bactérias na colonização de uma superfície, o risco de infecção do biomaterial deve diminuir significativamente devido à integração do tecido do hospedeiro (BUSSCHER *et al*., 2012). As superfícies recobertas com taninos se mostram compatíveis com células epiteliais de mamífero, indicando o grande potencial destes produtos naturais como agentes funcionais de recobrimento de superfícies. Entretanto, a habilidade destas superfícies revestidas com as proantocianidinas em mediar diferentemente a adesão de *S. epidermidis* e de células de mamífero concomitantemente incubadas permanece por ser avaliada. Embora o rendimento destas frações ativas não seja alto (aproximadamente 0,25% do pó da planta para a fração F7), recentes avanços na síntese de proantocianidinas mostram-se como outras opções para a obtenção destes compostos e ainda, possibilitam um futuro estudo de relação estrutura-atividade considerando o grau de polimerização estrutural (FERREIRA e COLEMAN, 2011; QUIDEAU *et al*., 2011).

Os resultados aqui apresentados identificando os taninos como compostos antimicrobianos ou antibiofilmes vêm ao encontro dos trabalhos realizados com a comunidade rural da Caatinga. Segundo Albuquerque e colaboradores (2012), existem evidências da correlação entre a produção de compostos fenólicos e a atribuição de valor terapêutico às plantas da Caatinga pela comunidade local. Neste sentido, plantas indicadas para o tratamento de inflamações e que são popularmente usadas como antimicrobianos possuem altos teores de taninos em comparação às plantas indicadas para outros usos terapêuticos (ARAÚJO *et al*., 2008; SIQUEIRA *et al*., 2012). Ainda, a população da Caatinga frequentemente utiliza as cascas de árvores para diferentes "alvos terapêuticos" mesmo quando outras estruturas (como folhas) estão disponíveis para a mesma proposta (ALBUQUERQUE *et al*., 2012).

Com relação à segunda abordagem, o trabalho realizado em colaboração com o Instituto de Física da UFRGS mostrou que a descarga de plasma de N_2/H_2 produz, de maneira rápida e bastante efetiva, superfícies de poliestireno capazes de impedir a adesão de bactérias altamente resistentes aos antimicrobianos. Através da caracterização por análise de XPS, verificou-se que quando a concentração de nitrogênio da superfície alcança valores superiores a 8,8% e o componente polar da energia de superfície se torna superior a 15 mJ/m^2, bactérias que apresentam superfície hidrofílica (como, enterobactérias produtoras de carbapenemase e MRSA) apresentaram reduzida adesão nestas superfícies, enquanto que cepas hidrofóbicas (exemplificadas pelo *S. epidermidis*) mantiveram a capacidade de aderir e formar biofilme. A atividade antiadesiva permaneceu após a estocagem das superfícies por 15 dias, mas foi diminuída após 30 dias para alguns dos microrganismos testados, indicando a necessidade de manter estes materiais sob vácuo. Interessantemente, a hidrofobicidade da superfície de *S. epidermidis* apresenta grandes variações, apresentando valores de 3 a 89% no teste de afinidade microbiana ao hidrocarboneto (MATH) (HOGT *et al*., 1985). Estes achados suportam a ideia de que a adesão de muitos isolados de *S. epidermidis*, um dos principais agentes etiológicos de infecções envolvendo biomateriais, seja suprimida nestas superfícies tratadas com plasma iônico. É importante destacar que os resultados encontrados com os tratamentos a plasma representam uma estratégia alternativa "limpa" para o processamento de materiais de

uso médico e, ampliam a possibilidade de controlar a adesão de diversos gêneros e espécies bacterianas, uma vez que não faz uso de compostos ou substratos específicos para determinados microrganismos.

Considerando os resultados obtidos com as superfícies recobertas com proantocianidinas, capazes de inibir a adesão de *S. epidermidis*, bem como os resultados obtidos com as superfícies tratadas a plasma, antiaderentes para *K. pneumoniae*, *E. cloacae*, *S. marcescens* e *S. aureus*, ambos achados podem ser explicados em termos da repulsão eletrostática - interação físico-química não específica importante no processo inicial da adesão bacteriana - levando à criação de superfícies antiadesivas. O desempenho de ambas as superfícies, recobertas com proantocianidinas e tratadas por plasma, após a formação de filme condicionamente de matéria orgânica, permanece por ser investigado. Entretanto, existe uma boa expectativa, uma vez que o tratamento a plasma bem como o recobrimento de superfícies com polímeros podem ser utilizados para gerar substratos com alta energia de superficie através de grupos hidrofílicos, os quais são repelentes para proteínas (KUMAR *et al*., 2010), exemplificado pela dimunição da adsorção protéica em superfícies com filme de polietilenoglicol (KUMAR *et al*., 2010) e em membranas polietersulfônicas tratadas com plasmas baseados em nitrogênio (KULL *et al*., 2005).

O controle da formação de biofilmes é uma tarefa muito complexa, como demonstrada pela incapacidade da produção de biomateriais implantáveis clinicamente eficazes em resistir à infecção (BUSSCHER *et al*., 2012). Esta complexidade pode ser atribuída (i) à heterogeneidade das superfícies bacterianas, incluindo a variabilidade entre diferentes espécies e cepas, o que impede a generalização de achados considerando a diminuição da adesão bacteriana sobre os substratos e, (ii) a dificuldade em reunir várias áreas do conhecimento científico para abordar o problema de forma suficientemente interativa.

A fim de abranger estratégias para o controle da formação de biofilmes a presente tese contou com o envolvimento de diferentes áreas do conhecimento (Figura 1), uma vez que o estudo de biofilmes, recentemente cunhado pelo termo biofilmologia, é cada vez mais reconhecido como uma ciência multidisciplinar onde a

visão e cooperação mútuas de diferentes disciplinas se tornam imprescindíveis (KARUNAKARAN *et al*., 2011).

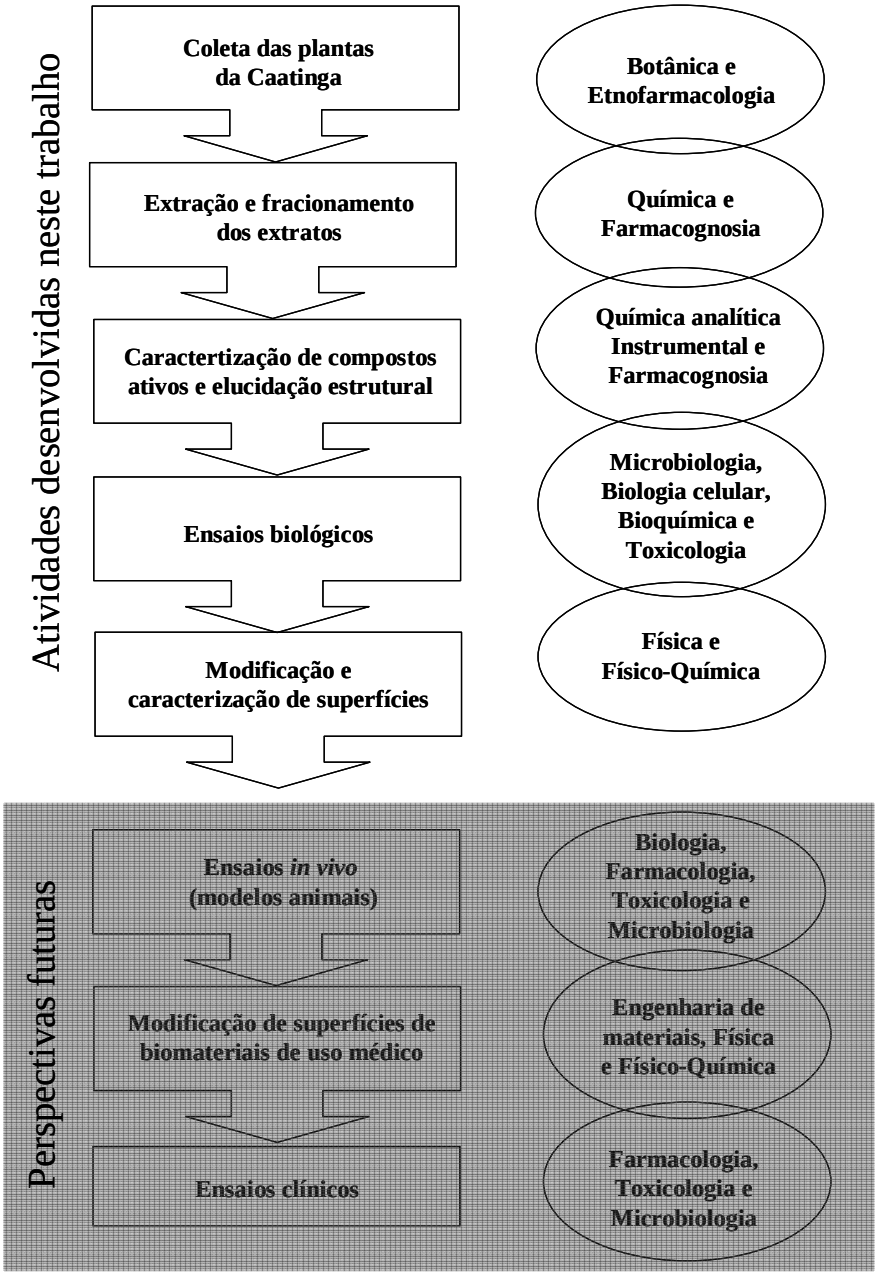

Figura 12: Interfaces das diferentes áreas envolvidas nesta investigação para o combate da formação de biofilmes.

No que diz respeito à dramática situação no ambiente hospitalar, em que o manejo habitual para o combate de biofilmes patogênicos é a remoção de milhares de dispositivos infectados, os resultados aqui apresentados devem ser considerados como guias para o direcionamento de futuros estudos e para o desenvolvimento de pesquisa na área de biofilmes.

VI – CONCLUSÕES

Em resumo, os resultados obtidos nesta tese:

- Fornecem uma base científica que pode justificar alguns dos usos etnofarmacológico das plantas da Caatinga e destacam o papel de taninos como importantes compostos com ação biológica;

- Elucidam pela primeira vez a estrutura de taninos dos gêneros *Anadenanthera* e *Commiphora*;

- Relatam pela primeira vez a atividade antibiofilme e a investigação fitoquímica de folhas de *Pityrocarpa moniliformis*;

- Demonstram altas taxas de inibição da formação de biofilme de *P. aeruginosa* devido à inibição do crescimento bacteriano por taninos condensados e hidrolisáveis e altas taxas de inibição da formação de biofilme de *S. epidermidis* devido à inibição da adesão bacteriana por taninos condensados ricos em prodelfinidina;

- Apresentam a aplicabilidade de revestimentos à base de proantocianidinas como uma abordagem para o desenvolvimento de superfícies antiaderentes para bactérias e biocompatíveis com células de mamíferos;

- Exibem a criação de superfícies antiaderentes e não biocidas para bactérias com superfícies hidrofílicas (*K. pneumoniae, E. cloacae, S. marcescens* e *S. aureus*), através de tratamentos do material polimérico com descarga de plasma de N_2/H_2;

- Combinam a caracterização físico-química de superfície do polímero e as propriedades da superfície bacteriana para propor que a hidrofilicidade e a repulsão eletrostática são os parâmetros responsáveis pelos efeitos antiaderentes alcançados;

- Evidenciam a necessidade de trabalhos multidisciplinares para que medidas de controle de biofilmes sejam obtidas com sucesso.

VII - REFERÊNCIAS

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