**UNIVERSIDADE FEDERAL DE SANTA MARIA**
**CENTRO DE CIÊNCIAS DA SAÚDE**
**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS**

**Camilla Filippi Dos Santos Alves**

# IDENTIFICAÇÃO DE SUBSTÂNCIAS MAJORITÁRIAS PRESENTES NA *Equisetum hyemale* E AVALIAÇÃO DAS ATIVIDADES ANTIMICROBIANA, ANTIBIOFILME, CITO E GENOTÓXICAS.

**Santa Maria, RS, Brasil**
**2020**

**Camilla Filippi Dos Santos Alves**

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Tese apresentada ao Curso de Doutorado do Programa de Pós Graduação em Ciências Farmacêuticas. Área de concentração Análises Clínicas e Toxicológicas: Desenvolvimento e Aplicação de Marcadores no Diagnóstico Laboratorial, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Doutora em Ciências Farmacêuticas.**

Orientadora: Profª. Drª. Marli Matiko Anraku de Campos
Co-orientador: Prof, Dr Roberto Christ Vianna Santos

Santa Maria, RS, Brasil

This study was financied in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001

Alves , Camilla Filippi dos Santos Alves
IDENTIFICAÇÃO DE SUBSTÂNCIAS MAJORITÁRIAS PRESENTES NA Equisetum hyemale E AVALIAÇÃO DAS ATIVIDADES ANTIMICROBIANA, ANTIBIOFILME, CITO E GENOTÓXICAS. / Camilla Filippi dos Santos Alves Alves .- 2020.
110 p.; 30 cm

Orientadora: Marli Matiko Anraku de Campos Campos
Coorientador: Roberto Christ Vianna Santos Santos
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós Graduação em Ciências Farmacêuticas, RS, 2020

1. Atividade antimicrobiana 2. Biofilmes 3. Plantas 4. compostos fenólicos I. Campos , Marli Matiko Anraku de Campos II. Santos , Roberto Christ Vianna Santos III. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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aprova a Tese de doutorado:

**IDENTIFICAÇÃO DE SUBSTÂNCIAS MAJORITÁRIAS PRESENTES NA *Equisetum hyemale* E AVALIAÇÃO DAS ATIVIDADES ANTIMICROBIANA, ANTIBIOFILME, CITO E GENOTÓXICAS.**

elaborada por
**Camilla Filippi dos Santos Alves**

como requisito parcial para obtenção do grau de
**Doutora em Ciências Farmacêuticas**

**COMISSÃO EXAMINADORA:**

Dr$^{a.}$ Liliane de Freitas Bauermann

Dr$^{a}$ . Michele Rorato Sagrillo

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Santa Maria, 30 de março de 2020

## DEDICATÓRIA

*Dedico este trabalho a minha amada família, minha mãe, meu pai, meus irmãos e meus avós. Todos me proporcionaram a base fortalecedora durante esta etapa. Amo vocês!*

## AGRADECIMENTOS


*Inicialmente, gostaria de agradecer a Deus e aos espíritos protetores que sempre guiam, protegem e iluminam o meu caminho.*

*De uma forma geral, agradeço a todos que de alguma forma me apoiaram e incentivaram nesta etapa. De uma maneira especial, agradeço:*

*- a Universidade Federal de Santa Maria, por me proporcionar desenvolver e concretizar este estudo;*

*- a todo o corpo docente do Programa de Pós graduação em Ciências Farmacêuticas, pelos ensinamentos transmitidos;*

*- a CAPES (Coordenação de Aperfeiçoamento de Pessoal de nível Superior), pela bolsa de estudos e benefícios financeiros concedidos;*

*- aos professores que compõe a banca, agradeço a disponibilidade em avaliar e contribuir com este trabalho. Vocês são inspiração para mim;*

*- a minha querida orientado Marli Matiko Anraku de Campos, por sempre estar disponível para estender a mão e me acolher, pela confiança, ensinamentos e dedicação para a concretização deste estudo. Muito obrigada!*

*- ao co-orientador Dr. Roberto Christ Vianna, que me acompanha a 9 anos, muito obrigada pelo apoio, incentivo, ensinamentos e incansável colaboração na construção da minha profissão. Gratidão!*

*- aos meus pais, Marco Antonio e Adriane, que são os maiores responsáveis pela conclusão desta etapa. Gratidão pela educação, amor, carinho e incentivo. Amo vocês!*

*- aos meus irmãos Pedro Henrique e Marcos Vinicius pelo apoio, amor e amizade. Muito obrigada, amo vocês.*

*- aos meus avós Pedrolina, Celita e Morai, gratidão pelo esteio e amor. Amo vocês!*

- *a toda a minha família, tios/tias e primos/primas, pelo apoio e carinho, muito obrigada. Amo voçes!*

*- ao meu namorado Marcelo Pedroso, pela parceria, amor e amizade. Te amo!*

*- aos meus queridos colegas do Lapmicro (laboratório de pesquisa em microbiologia oral), pela cooperação e amizade. Vocês foram responsáveis por grande parte desta pesquisa, muito obrigada, contem sempre comigo!*

*- à Pauline Bonez, que além de amiga foi minha "orientadora". Você é minha inspiração. Gratidão!*

*- ao Prof Paulo Edelvar Peres, pela empatia, simpatia e todo o conhecimento transmitido. Você é uma pessoa iluminada! Muito obrigada!*

*- aos colegas do labmyco (Laboratório de micobacteriologia) pela receptividade, amizade e auxilio, muito obrigada;*

*For fim, agradeço imensamente a todos que estiveram do meu lado durante esta trajetória e que me incentivaram a seguir e sempre ser uma pessoa melhor.*

*GRATIDÃO!*

## RESUMO

**IDENTIFICAÇÃO DE SUBSTÂNCIAS MAJORITÁRIAS PRESENTES EM *Equisetum hyemale* E AVALIAÇÃO DAS ATIVIDADES AMTIMICROBIANA, ANTIBIOFILME, CITO E GENOTÓXICAS.**

AUTOR: CAMILLA FILIPPI DOS SANTOS ALVES
ORIENTADOR: MARLI MATIKO ANRAKU DE CAMPOS
CO-ORIENTADOR: ROBERTO CHRIST VIANNA SANTOS
Santa Maria, 30 de março de 2020.

Os biofilmes são aglomerados de células microbianas, aderidos a uma superfície e protegidos por camadas de substâncias poliméricas extracelulares (SPE). Uma grande parte das infecções relacionadas ao uso de implantes e dispositivos médicos está associada à formação desses biofilmes. Aceitar a complexa arquitetura dos biofilmes, o controle e o tratamento são complexos e dificultosos. A formação de biofilme decorre as etapas sucessivas, obedece às condições determinantes (motilidade microbiana, auto e co-agregação e sinalização *quorum sensing* (QS)). Por esse motivo, torna-se importante buscar alternativas que possam interferir nessas etapas e, de algum modo, inibir a formação de biofilmes. Nesse caso, este estudo utilizou a *Equisetum hyemale*, popularmente conhecido como Cavalinha, trata-se de uma planta típica de clima temperado que é amplamente utilizada na medicina popular como diurético e digestivo. Este estudo teve como objetivos, identificar as principais substâncias presentes na *E.hyemale*, avaliar atividades antimicrobianas, antibiofilme, anti-QS, citações e genotóxicas. Uma atividade antibacteriana foi avaliada pelo método de microdiluição em caldo diante da P. aeruginosa 01. Os ensaios de citologia e genotoxicidade foram realizados pelo teste de MTT e o teste de Cometa, respectivamente. A inibição do biofilme foi avaliada em placas de poliestireno através da técnica de cristal violeta e também da Microscopia de Força Atômica (MFA). Fatores essenciais à fisiologia do biofilme, como, motilidade (*Swimming, swarming e twitching*), foram analisados na presença e ausência de extratos e substâncias isoladas. A inibição do *quorum sensing* foi observada usando o padrão de *Chromobacterium violaceum*. Os extratos e compostos fenólicos utilizados foram capazes de inibir a formação de biofilme e interferir em fatores cruciais importantes. Além disso, foi inibida a produção de violaceína (produto QS em *Chromobacterium violaceum*, sugerindo que extrato bruto, frações e compostos majoritários de *E. hyemale* exibem atividade antibiofilme importante e anti-QS. *E. hyemale* surge, portanto, como uma maneira promissora para reduzir a adesão microbiana em superfícies, haja vista que não observamos efeitos tóxicos citados e genotóxicos em células sanguíneas mononucleares.


**Palavras-chave:** Atividade antimicrobiana; Biofilme; Plantas; compostos fenólicos.

# ABSTRACT

## IDENTIFICATION OF MAJORITARY SUBSTANCES PRESENT IN *Equisetum hyemale* AND EVALUATION OF OF AMTIMICROBIAL, ANTIBIOFILM, CITO AND GENOTOXIC ACTIVITIES.



AUTHOR: CAMILLA FILIPPI DOS SANTOS ALVES
ADVISER: MARLI MATIKO ANRAKU DE CAMPOS
CO-SUPERVISOR: ROBERTO CHRIST VIANNA SANTOS
Santa Maria, March, 30, 2020.



Biofilms are clusters of microbial cells, adhered to a surface and protected by layers of extracellular polymeric substances (SPE). A large proportion of infections related to the use of implants and medical devices are associated with the formation of these biofilms. Accepting the complex biofilm architecture, control and treatment is complex and difficult. The formation of biofilm follows successive stages, obeying determining conditions (microbial motility, self and co-aggregation and *quorum sensing* (QS) signaling). For this reason, it is important to look for alternatives that can interfere in these steps and, in some way, inhibit the formation of biofilms. In this case, this study used *Equisetum hyemale*, popularly known as Horsetail, a typical plant of temperate climate that is widely used in folk medicine as a diuretic and digestive. This study aimed to identify the main substances present in *E.hyemale*, to evaluate antimicrobial activities, antibiofilm, anti-QS, citations and genotoxic. An antibacterial activity was evaluated by the microdilution method against a broth of *P. aeruginosa* 01. The cytology and genotoxicity tests were performed by the MTT test and the Comet test, respectively. Biofilm inhibition was evaluated in polystyrene plates using the violet crystal technique and also with Atomic Force Microscopy (AFM). Factors essential to the biofilm's physiology, such as motility (swimming, swarming and twitching), were analyzed in the presence and absence of extracts and isolated substances. Inhibition of quorum sensing was observed using the Chromobacterium violaceum pattern. The extracts and phenolic compounds used were able to inhibit biofilm formation and interfere with important crucial factors. In addition, the production of violacein (QS product in Chromobacterium violaceum) was inhibited, suggesting that crude extract, fractions and major compounds of *E. hyemale* exhibit important antibiofilm and anti-QS activity. *E. Hyemale* therefore appears as a promising way to reduce microbial adhesion on surfaces, since we have not observed the mentioned toxic and genotoxic effects on mononuclear blood cells


Key words: Antimicrobial activity; Biofilm; Plants; phenolic compounds.

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# APRESENTAÇÃO

O item **INTRODUÇÃO** apresenta uma detalhada revisão bibliográfica acerca dos temas abordados nesta tese. O presente trabalho originou o **ARTIGO CIENTÍFICO 1 e MANUSCRITO 1**, os quais englobam os itens: Resultados, Materiais e Métodos, Discussão e Referencias. Ambos os trabalhos estão formatados em conformidade com os periódicos aos que foram publicados e/ou submetidos.

Nos itens: **DISCUSSÃO**, **CONCLUSÕES** e **PERSPECTIVAS**, estão expostas as interpretações, comentários gerais e sugestões de abordagens futuras a respeito dos assuntos tratados nesta tese. O item **REFERENCIAS** reporta-se somente às citações que aparecem nos itens Introdução e Discussão.

# 1 INTRODUÇÃO

## *1.1 Equisetum hyemale*

A vasta biodiversidade brasileira ainda não é totalmente conhecida devido a sua grande complexidade. Estima-se a existência de mais de dois milhões de espécies distintas de plantas, animais e microrganismos. O Brasil é dono da maior diversidade genética vegetal do mundo, contando com mais de 55 000 espécies catalogadas, sendo que a metade se encontram nas florestas tropicais (GECHEV et al., 2014; MARTIN et al., 2013). Apenas 8% da flora brasileira foi estudada de modo a encontrar novos compostos bioativos, e mais de 1100 espécies foram avaliadas em suas propriedades medicinais. Destas, 590 plantas foram registradas no ministério da saúde para a comercialização (BARCELOUX, 2008; HAVSTEEN, 2002).

*Equisetum hyemale* pertence ao filo *Sphenophyta* e família *Equisetaceae,* comumente encontrada em climas tropicais e temperados. É uma planta de pequeno porte que raramente atinge um metro de altura. Sua morfologia é de folhas muito reduzidas e caule aéreo fistuloso que possui nós e entrenós característicos de 0,7 a 4 mm de diâmetro e 10 cm de comprimento. Os brotos são aéreos e perpendiculares, com rizomas subterrâneos, são quase uniformes quanto à altura, ascendentes e com nervuras. Assemelha-se com um rabo de cavalo, e por este motivo, todas as espécies do gênero são conhecidas popularmente como "Cavalinha" (figura 1). (RADULOVIĆ; STOJANOVIĆ; PALIĆ, 2006).

Figura 1- Imagem da *Equisetum hyemale* colhida

Fonte: (Arquivo pessoal do autor)

Em geral, na medicina popular são utilizadas as hastes da *Equisetum* para a produção de chás, que "funcionam" como diurético, digestivo, anti-anêmico, anti-inflamatório, antimicrobiano, sendo empregada também no tratamento de gonorreia, diarreia, infecções dos rins, bexiga e doenças oculares. No Brasil, a espécie *Equisetum arvense* é utilizada em programas do Sistema Único de Saúde (SUS) para o tratamento da hipertensão arterial, apesar de não fazer parte da Farmacopeia brasileira. Altos teores de minerais, principalmente silício e metabólitos secundários estão relacionados com utilização dessa planta no tratamento de algumas patologias. A presença de compostos fenólicos, saponinas, flavonoides, taninos e alcaloides na composição da planta podem agir em sinergismo e potencializar as atividades biológicas dessa espécie (GARCIA et al., 2012; SAPEI et al., 2007; YAMANAKA et al., 2012).

O extrato bruto da *E. arvense* apresenta atividade antimicrobiana com concentração inibitória mínima (CIM) de 50 mg/mL frente a *Staphylococcus. aureus, Staphylococcus. epidermidis, Candida. albicans, Candida. tropicalis, e Candida. glabrata* e CIM (concentração inibitória mínima) de 25 mg/mL frente à *Streptococcus mutans,* essa atividade foi atribuída à presença de compostos fenólicos. Também foram testados os óleos essenciais que demostraram uma grande efetividade frente à Gram negativos como *Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enteritidis* e *Escherichia coli* (DE OLIVEIRA et al., 2013; RADULOVIĆ; STOJANOVIĆ; PALIĆ, 2006).

Embora existam poucos relatos acerca da atividade antimicrobiana do *E. hyemale*, um trabalho identificou que os extratos etanólicos e metanólicos da *E. hyemale* são constituídos principalmente por compostos fenólicos. Esses compostos são relacionados com as atividades de eliminação de radicais livres, antimicrobiana e anti-fúngica com CIM de 0,62 mg/mL para *Trichophyton rubrum* e *Microsporum canis* (DE QUEIROZ et al., 2015; GARCIA et al., 2013).

Neste contexto, já foram realizados alguns estudos que identificaram a presença de compostos fenólicos em algumas espécies do gênero *Equisetum*. Os compostos fenólicos são ativos biologicamente contra bactérias, fungos, vírus, além de apresentarem atividade anti-inflamatória e antioxidante (GIERLER; SAPEI; PARIS, 2008; KUKRIC et al., 2013).

Compostos como canferol e quercetina foram identificados nos extratos de *Equisetum giganteum*. As espécies *Equisetum giganteum* e *Equisetum bogotense* possuem valores dez vezes mais elevados de canferol comparados com a quercetina. Por outro lado, a espécie *E. arvense* demonstrou valores mais elevados nas concentrações de quercetina do que canferol e maiores dosagens de flavonóides totais (FRANCESCATO et al., 2013).

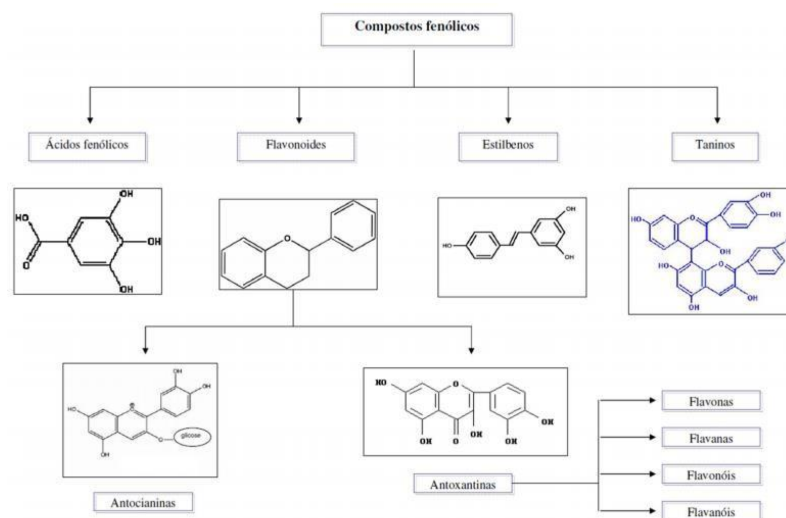
## 1.2 METABÓLITOS SECUNDÁRIOS

Os metabólitos secundários possuem as funções de adaptar e proteger o vegetal, bem como a função reprodutiva (VANHAELEN et al., 1994). São substâncias químicas distintas dos intermediários e dos produtos do metabolismo primário. Eles variam de acordo com a espécie vegetal, e alguns são restritos a determinada família, gênero ou espécie, possibilitando o emprego como marcador taxonômico (COCK, 2015; GECHEV et al., 2014).

As relações entre as atividades biológicas dos produtos naturais e seus componentes são responsáveis pelo avanço das pesquisas na descoberta de novas alternativas terapêuticas. Como exemplo, as plantas que contém em sua composição os polifenóis, que são substâncias caracterizadas por possuírem uma ou mais hidroxilas ligadas a um anel aromático. Fazem parte desse grupo: Taninos, ligninas, derivados do ácido caféico e flavonóides (PIANA et al., 2015; SULTANA; ANWAR, 2008). As pesquisas envolvendo plantas medicinais são de grande importância e nos últimos anos, estudos químicos e farmacológicos, visam obter novos compostos que possam ser utilizados como alternativa de tratamento para diversas patologias (BOLIGON et al., 2013; CORRÊA; AGERTT; BOLIGON, 2012).

Os compostos fenólicos estão presentes em uma vasta gama de frutas e vegetais. Sua estrutura química caracteriza-se por possuir uma hidroxila ligada diretamente ao um grupo hidrocarboneto aromático. São classificados como fenóis simples e polifenóis com base nos números de unidades desta estrutura, na molécula. De acordo com seus arranjos, esses compostos são classificados em: Ácidos fenólicos, flavonoides e não flavonoides como demosntrado no esquema da figura 2 (BEN YAKOUB et al., 2018; FACCIN et al., 2016).

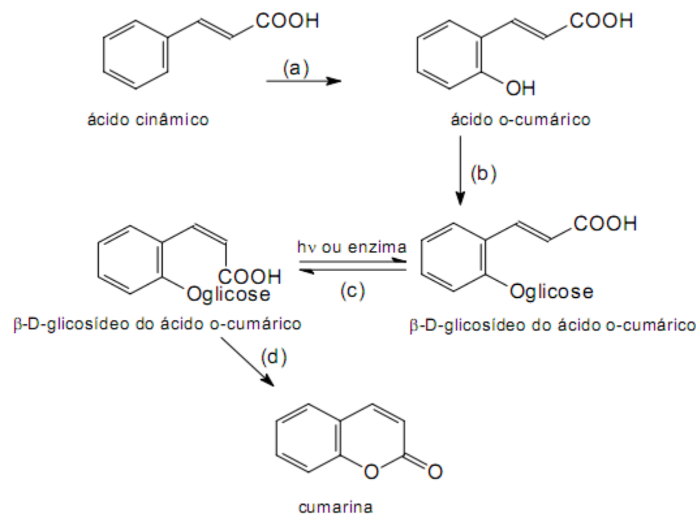
Figura 2- Classe de compostos fenólicos




Fonte: JANIQUES et al.,2013

Os flavonoides representam uma extensa família de compostos fenólicos de baixo peso molecular, de acordo com suas estruturas abrangem diversas classes químicas, como flavonas, flavanonas, isoflavonas, antocianinas, flavonóis (ou catequinas), entre outros. Os ácidos fenolicos são derivados de hidroxila de ácidos carboxílicos aromáticos que possuem um único anel e pode ser dividido em dois tipos principais, os ácidos benzoicos e ácidos cinâmicos. Os compostos fenólicos podem se ligar entre si ou com outros ácidos, como por exemplo, a ligação com ácido caféico, o qual, associado a um álcool-ácido cíclico, origina o ácido clorogênico e ácido ferulico (KUMAR; PRUTHI, 2014; SONAR et al., 2017).Os ácidos cinâmicos que possuem nove átomos de carbono, sendo sete os mais comumente encontrados no reino vegetal, a partir da ciclização da cadeia lateral do ácido o-cumárico, que dão origem as cumarinas mo ilustra a figura 3 (VENUGOPALA; RASHMI; ODHAV, 2013).

Figura 3- Rota Biosintética da cumarina




Fonte: Disponível em: http// adamogama.blogspot.com/2012/01/cumarinas.html

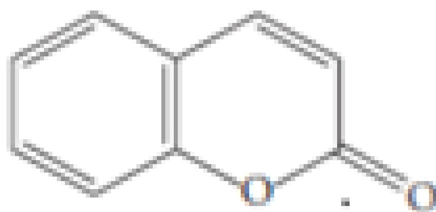
### 1.2.1 Atividade antimicrobiana dos compostos fenólicos

O uso inadequado de antimicrobianos resulta no aumento da pressão seletiva sobre os microrganismos para que adquiram resistência. A resistência microbiana gera altos custos para a saúde pública além de ser a responsável por milhares de morte por infecções persistentes. Diante disso, torna-se necessária a busca por novas substâncias com efeitos antimicrobiano (BARCELOUX, 2008; FRIEDRICH et al., 2016) Deste modo, se apresentam os compostos fenólicos que são ricas fontes de pesquisa que envolvem efeitos biológicos, dentre elas a

capacidade de inibir o crescimento microbiano por diferentes mecanismos de ação (FACCIN et al., 2016; GECHEV et al., 2014).

Neste contexto, apresentam-se as Cumarinas que são compostos orgânicos e consistem em um anel aromático 1,2 benzopironas e um benzeno fundido ao anel de α-pirona como demosntra a Figura 4. Apesar de ser originalmente encontradas em *tonka bean (Dipteryx odorata Wild),* estão distribuídas em quase 30 famílias e 150 espécies de plantas e são caracterizadas por apresentarem baixa massa molecular, serem facilmente sintetizadas e possuírem alta biodisponibilidade e baixa toxicidade (VENUGOPALA; RASHMI; ODHAV, 2013). Suas atividades farmacológicas mais estudadas são: anti-HIV (Vírus da Imunodeficiência Humana), antitumoral, anti-hipertensivo, anti-inflamatório, analgésico e antimicrobiano (KERI et al., 2015; SANDHU et al., 2014).

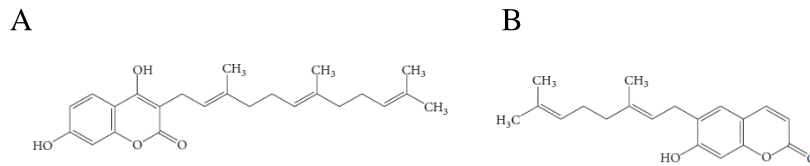
Figura 4- Estrutura química da cumarina simples.




Fonte Adaptado de (VENUGOPALA; RASHMI; ODHAV, 2013).

A estrutura simples da Cumarina, possui baixa atividade, porém, compostos com substituições de hidrocarbonetos de cadeia longa, como ammoresinol e ostruthin ( ilustrados na figura 5 A e B), apresentam atividade contra um amplo espectro de bactérias Gram positivas, pois uma estrutura que apresenta cadeias longas e planas, tendem a ser mais lipofílicas e por isso penetram mais facilmente na membrana celular bacteriana (VENUGOPALA; RASHMI; ODHAV, 2013). Cumarinas isoladas das raízes de *Ferulago campestris* foram reportados contra *Staphylococcus aureus*, *Salmonella enterica, Enterobacter cloacae* e *E. aerogenes* (BASILE et al., 2009). Atividade frente aos micro-organismos *Bacillus subtilis* e *Klebsiella pneumoniae* também foram evidenciados em cumarinas isolados a partir do extrato de raízes de *Prangos hulusii* (TAN et al., 2017).

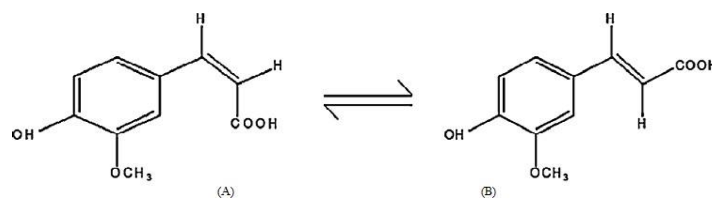
Figura 5- Estrutura química da Ammoresinol (A) e Ostruthin (B)




Fonte: Adaptado de (VENUGOPALA; RASHMI; ODHAV, 2013)

Outro composto fenólico bastante pesquisado acerca de sua atividade antimicrobiana é o Ácido ferúlico (ácido 4-hidroxi-3-metoxicinâmico) apresenta uma cadeia lateral insaturada e também existência de isômeros cis e trans como representa a figura 6, foi isolado pela primeira vez da *Ferula foetida*. Está presente em ervas da medicina tradicional chinesa e exibe muitas funções fisiológicas (BENTO-SILVA; VAZ PATTO; DO ROSÁRIO BRONZE, 2018; ERGÜN et al., 2011). Foi capaz de inibir o crescimento de diferentes cepas de *C. sakasakii* (bacilo Gram negativo que acomete principalmente neonatos) na concentração de 2,5 mg/ml (SHI et al., 2016).

Figura 6- Representação esquemática de duas formas isoméricas diferentes de ácido ferúlico encontradas na natureza (A) conformação cis e (B) conformação trans do ácido ferúlico

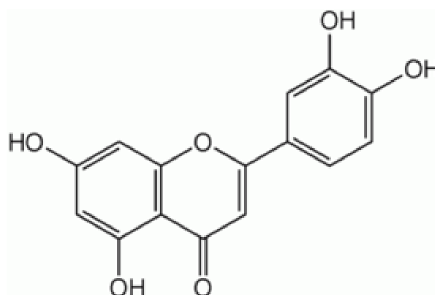



Fonte : adaptado de KUMAR; PRUTHI, 2014.

A luteolina pertence ao grupo dos flavonoides e também está presente em diversas plantas medicinais. Sua estrutura química é constituída por um grupo hidroxil (-OH) ligado nas posições 5, 7, 3 e 4 da espinha dorsal da flavona como ilustra a figura 7. Apresenta atividade antimicrobiana contra cepas ATCC (American Type Culture Collection- Uma organização sem fins lucrativos que coleta, armazena e distribuí microrganismos de referência padrão) de bactéria Gram-positiva *S. aureus* e Gram-negativas bactéria *E. coli* (BUSTOS et al., 2018). Alguns autores sugerem que o mecanismo de ação dos compostos fenólicos baseia-se principalmente na capacidade de alterar a permeabilidade da membrana celular das bactérias, e

dessa forma interromper o funcionamento metabólico da célula. Como por exemplo, transporte de elétrons, absorção de nutrientes, síntese de proteínas e ácidos nucleicos e atividade enzimática (DE QUEIROZ et al., 2015; ERGÜN et al., 2011; SHI et al., 2016).

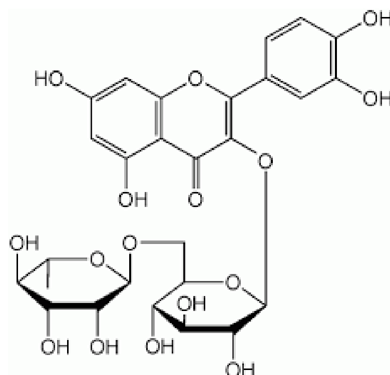
Figura 7- Estrutura química da Luteolina




Fonte : AZIZ; KIM; CHO, 2018

Na classe dos flavonoides também faz parte a rutina (3,30,40,5,7-penta-hidroxiflavona-3-ramnoglucósido) como mostra a figura 8. A rutina é uma substância encontrada em flores, chás e maças, seu nome é originado da planta *Ruta graveolens*. É extensivamente estudado quanto à atividade antimicrobiana. Foi capaz de inibir significativamente o crescimento de *Escherichia coli* (ARARUNA et al., 2012), *P. aeruginosa* e *Bacillus subtilis* (GANESHPURKAR et al., 2013). O principal mecanismo de ação da rutina é a inibição da *DNA isomerase IV* demonstrou, impedindo a replicação do material genético microbiano (Bernard et al., 1997). Outros estudos relatam que a rutina age sinergicamente melhorando a atividade de outros flavonoides contra *Bacillus cereus* e *Salmonella enteritidis* (ARIMA; ASHIDA; DANNO, 2002).

Figura 8 - Estrutura química da Rutina




Fonte : GANESHPURKAR; SALUJA, 2017)

Além disso, a resistência microbiana está intimamente relacionada com a capacidade de formação de biofilmes. Os biofilmes são massas microbianas aderidas a uma superfície biótica ou abiótica e são cobertas por uma camada espessa de exopolissacarídeos, o que dificulta a remoção dessas bactérias (SELEZSKA et al., 2012; STEWART; WILLIAM COSTERTON, 2001). Algumas pesquisas têm como foco investigar a atividade anti-biofilme dos metabólitos secundários, afim de impedir e/ou remover essas estruturas (KOT et al., 2015; SAMPATHKUMAR et al., 2019; SLOBODNÍKOVÁ et al., 2016).

## 1.3 BIOFILME

### 1.3.1 Estrutura

Os biofilmes foram observados pela primeira vez em meados do século XVII, onde Van Leeuwenhoek examinou as placas formadas em seus próprios dentes. Porém, a teoria geral sobre essas estruturas, somente foi fundamentada no ano de 1978 (COSTERTON; VEEH, 2003; DONLAN; COSTERTON, 2002). Esta teoria afirmava que os microrganismos podem apresentar-se na forma planctônica, ou seja, em suspensão circulando isoladamente, ou séssil onde quando estão aderidos á superfícies em condições nutritivas favoráveis. Os microrganismos sesseis apresentavam um comportamento diferente ao daqueles de vida planctônica (STOODLEY et al., 2002a).

Atualmente os biofilmes são definidos como o resultado de um ecossistema microbiológico aderido a uma superfície, formado por populações desenvolvidas a partir de uma única ou de múltiplas espécies. Sendo assim, essas estruturas podem ser constituídas por bactérias, fungos e/ou protozoários. Em consequência da associação entre os microrganismos, ocorre a formação de uma matriz de polímeros orgânicos (RYBTKE et al, 2015). Através de análises microscópicas em ecossistemas aquáticos naturais, observou-se que mais de 99,9% das bactérias crescem em biofilmes e em tese, praticamente não exista superfície - seja ela natural ou sintética que não possa vir a ser colonizada por microrganismos (HALL-STOODLEY; COSTERTON; STOODLEY, 2004).

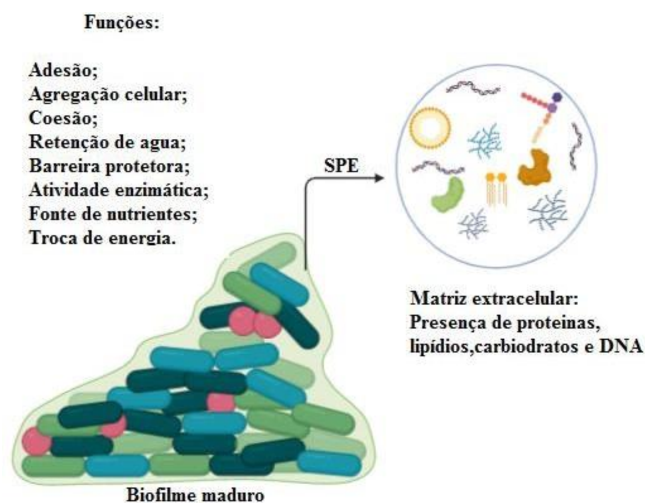
Embora os biofilmes sejam definidos por massa de aglomerados microbianos, também fazem parte dessa estrutura, os resíduos do ambiente colonizado, material genético, proteínas, lipídios, enzimas, íons e água. Tudo isso coberto por camadas de Substâncias Poliméricas Extra celulares (SPE) formando uma estrutura porosa e hidratada (STOODLEY et al., 2002b). Os poros permitem que o interior do biofilme se mantenha hidratado e nutrido. Além disso, as condições

hidrodinâmicas do ambiente físico determinam a arquitetura dos biofilmes. Pois, a flexibilidade das estruturas permite que os aglomerados microbianos resistam a diferentes fluxos, como por exemplo, o fluxo sanguíneo dentro de um cateter venoso ou de água dentro de uma tubulação industrial (NITHYANAND et al., 2015).

Os microrganismos produzem a SPE, que no que lhe concerne, determina as propriedades físicas, químicas e biológicas do biofilme. As SPEs representam 90% da massa dos biofilmes e são responsáveis pela morfologia, estrutura, coesão, integridade funcional (CISZEK-LENDA et al., 2011). A matriz extracelular confere a essas estruturas, uma grande vantagem a este modo de vida, pois, oferece um ambiente protetor às células microbianas a entrada de fármacos e agentes germicidas. Além disso, também protege os microrganismos contra a dissecação, oxidação, radiação ultravioleta e defesa imunitária (SHARMA et al., 2014).

A camada de SPE, funciona como uma barreira e regula o transporte de moléculas para o interior dos biofilmes, com isso, impede e/ou retarda a penetração de fármacos antimicrobianos a estrutura. Suci e colaboradores (1994) demonstraram uma penetração tardia da ciprofloxacina nos biofilmes de *P. aeruginosa*; o que normalmente exigia 40s para uma superfície estéril levou 21min para penetrar uma superfície contendo biofilme. Hoyle e colaboradores (1993) descobriram que as células bacterianas dispersas eram 15 vezes mais suscetíveis à tobramicina do que as células dos biofilmes intactos. Outros estudos examinaram a penetração de agentes antimicrobianos e interação com o material extracelular da substância polimérica de biofilmes, uma suspensão de 2% de alginato isolado de *P. aeruginosa* inibiu a difusão gentamicina e tobramicina (HATCH; SCHILLER, 1998). A figura 9 representa de forma didática a composição da camada de SPE e suas funções.

Figura 9- Representação didática da camada de Substância Polimérica Extracelular (SPE).



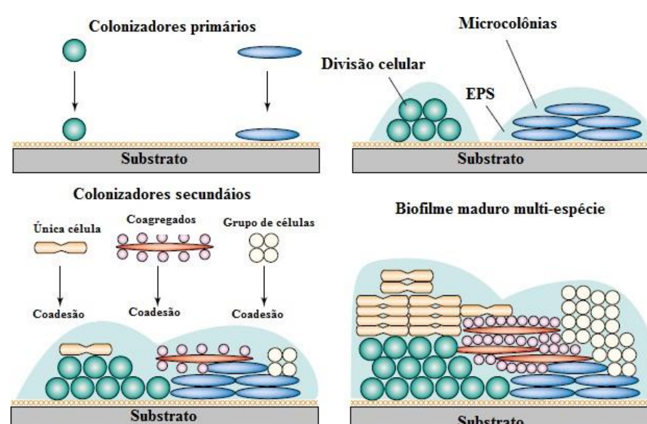

Fonte: Autoria própria

### 1.3.2 Etapas da formação do biofilme

A formação do biofilme é orquestrada por uma série de processos químicos, físicos e biológicos. Cada um desses mecanismos, dependerá dos organismos envolvidos, natureza da superfície a ser colonizada e características do meio ambiente (BONEZ et al., 2013; CHATTERJEE et al., 2014a). A primeira etapa, é chamada de adesão primária, onde ocorre a aproximação de células planctónicas aleatórias através de movimentos Browniano e força gravitacional(STOODLEY et al., 2002a). Ou de modo induzido, da motilidade superficial, onde a contração da píli superam as forças de repulsão e facilitam a adesão das células na superfície. Além disso, pode ocorrer a divisão binária das células conectadas e o recrutamento de microrganismos livres para o biofilme em desenvolvimento (DONLAN; COSTERTON, 2002; SHARMA et al., 2014).

Este processo inicial ainda é reversível, porém, à medida que aumenta o acúmulo de nutrientes, as células pioneiras se reproduzem originando micro colônias e a adesão torna-se irreversível. As micro colônias sintetizam sustâncias extracelulares que atuam como substrato para a adesão de outros microrganismos, que são chamados colonizadores secundários (PASICH et al., 2013; PEREIRA et al., 2015). Depois de aderidas, as células bacterianas produzem a superfície polimérica extracelular (SPE). Alguns estudos também mostraram que a ruptura do SPE resulta em menor complexidade estrutural e maior suscetibilidade a agentes antimicrobianos, enquanto a superprodução induzida teve o efeito oposto (CISZEK-LENDA et al., 2011; NITHYANAND et al., 2015). A figura 10 ilustra todas as etapas de formação do biofilme.

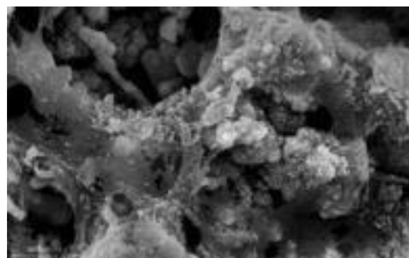
Figura 10 - Etapas da formação do biofilme




Fonte: Adaptado de (RICKARD et al., 2002)

O biofilme torna-se maduro, quando há um aumento da densidade populacional e deposição de componentes extracelulares gerados pelos microrganismos. E caracteriza-se por ser uma estrutura altamente hidratada e viscoelástica, constituindo-se de um aspecto gelatinoso, escorregadio e que possuem canais para a passagem água e nutrientes (DE SOUZA et al., 2017; STOODLEY et al., 2002a). A figura 11 mostra a imagem de um biofilme maduro, captada através de Microscopia Eletrônica de Varredura (MEV).

Figura 11- Imagem do biofilme maduro (MEV)



Fonte: Adaptado de (CRAVO JUNIOR et al, 2010).

Com base em dimensões físicas, um biofilme pode levar mais de 10 dias para atingir a maturidade estrutural. No interior desses aglomerados de células, há uma diminuição na atividade celular à medida que o biofilme amadurece. No entanto, essas células inativas podem ser reativadas pela adição de uma fonte de carbono mais facilmente utilizável. Isso indica que a atividade celular no interior do biofilme pode ser controlada pela disponibilidade de nutriente, e com isso também demonstraram que os canais ao redor da estrutura aumentam o suprimento de oxigênio (e outros nutrientes) para as bactérias. Por fim, quando o biofilme atinge uma etapa de amadurecimento, a massa microbiana é liberada e os microrganismos desprendidos poderão colonizar novos ambientes causando contaminação (Figura 11) (DONLAN; COSTERTON, 2002; RICKARD et al., 2003).

### 1.3.3 Fatores que influenciam da formação dos biofilmes

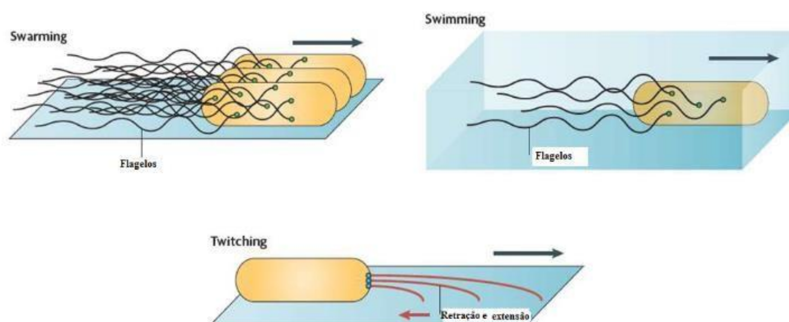
Tendo em vista que a formação dos biofilmes ocorre em etapas, diversos fatores influenciam neste processo. Como por exemplo, a espécie do microrganismo, ou seja, se possui motilidade (presença de flagelos e pili), se apresenta características hidrofóbicas ou consegue produzir SPE. Além disso, tipo de superfície a ser colonizada também é um fator, pois, variáveis como porosidade, pH, temperatura, oxigênio, carga e nutrientes determinam a adesão microbiana (MAUNDERS; WELCH, 2017).

1.3.3.1 Motilidade

As bactérias conseguem colonizar uma ampla diversidade de superfícies que ofereçam as condições nutritivas favoráveis. Os microrganismos podem, permanecerem aderidos ou mudarem-se para colonizar áreas maiores, invadir tecido do hospedeiro, ou elaborar corpos de frutificação para produzir esporos. Diante disso, as bactérias desenvolvem os mecanismos de motilidade que fornece uma vantagem sob uma grande variedade de ambientes, ou seja, esses microrganismos conseguem se locomover de um ambiente desfavorável até um mais adequado para sua sobrevivência. Desta maneira, as bactérias desenvolvem sistemas diferentes de motilidade para cada tipo de superfície. (MAURIELLO et al., 2010; OURA et al., 2015).

São citados na literatura três sistemas de motilidade: *swarming* (pulular), *swimming* (natação) *ou twitching* (extensão e retração) como demonstrado na figura 12. A motilidade do tipo *swarming* ocorre geralmente em bactérias que possuem flagelos peritríquios, é um movimento contínuo e em grupos (KEARNS; LOSICK, 2003). Este tipo de movimento aumenta a efetividade dos microrganismos de locomoverem-se em líquidos viscosos. Diferente da *swarming*, no tipo *swimming* as células se movem individualmente e de forma aleatória e só ocorrem quando a superfície é suficientemente espessa. O tipo de translocação *twitching* ocorre em uma variabilidade de bactérias que podem ser flageladas ou não. As células se movem predominantemente isoladas, embora possa ocorrer agregados móveis. O movimento é intermitente e espasmódico e não acompanha regularmente o eixo ao longo da célula.(COPELAND; WEIBEL, 2009; DUAN et al., 2013; REMIS; COSTERTON; AUER, 2010).

Figura 12: Ilustração dos tipos de motilidade *swarming, swimming* e *twitching*.




Fonte: (Adaptado de KEARNS, 2010).

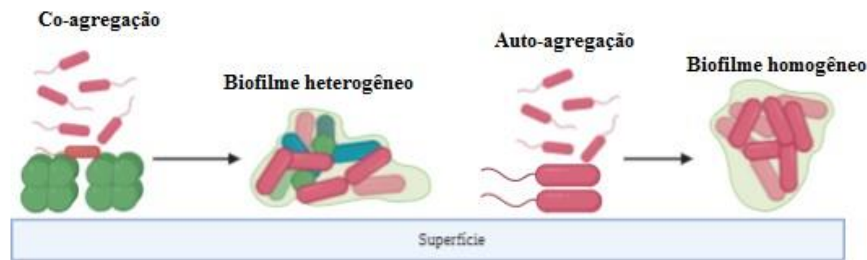
A motilidade bacteriana é um componente chave para a formação do biofilme. Na fase inicial de adesão do microrganismo à superfície, a presença de pilis e flagelos são essenciais, deste modo, relaciona-se o movimento do tipo *swarming* com a primeira etapa da formação dos aglomerados. Nas fases posteriores, especula-se que há a influência da motilidade *twitching,* pois, ela facilita que ocorra a propagação e crescimento microbiano no interior do biofilme maduro (KEARNS, 2010; MERZ; SO; SHEETZ, 2000; RASMUSSEN et al., 2005).

Diante disso, alguns estudos avaliam a capacidade dos compostos fenólicos em inibir a motilidade microbiana. Meios de culturas para o tipo de motilidade *swarming* foram suplementadas com ácidos fenólicos e inoculados em pontos, utilizando cepas de *P. aeruginosa* e então através da medida dos halos de crescimento, foi determinado que os compostos fenólicos inibiram significativamente a motilidade (UGURLU et al., 2016). Outro estudo demonstrou que o ácido gálico causou inibição total das motilidades do tipo *swimming* (*L. monocytogenes*) e do tipo *swarming* (*L. monocytogenes* e *E. coli*). O ácido ferúlico também é citado por inibir a motilidade *swarming* em *L. monocytogenes* e *swimming* em *L. monocytogenes e E. coli* (BORGES; SAAVEDRA; SIMÕES, 2012). Cepas de *E. coli* uropatogênicas, quando testadas frente à luteolina apresentou uma diminuição na expressão do gene que codificam fímbrias do tipo adesina (*fimH tipo 1*) e também menor hidrofobicidade da superfície bacteriana e motilidade *swimming* (SHEN et al., 2014).

1.3.3.2 Auto-agregação e co-agregação

Tendo em vista que os biofilmes podem ser homogêneo ou heterogêneo, os processos de auto-agregação e co-agregação também são fatores importantes para a formação dessas estruturas, pois, são altamente específicos (HSU et al., 1990; KOMIYAMA; GIBBONS, 1984). Em virtude de que mudanças ambientais ocorrem no interior do biofilme jovem, colonizadores secundários iniciam o processo de auto/coagregação e então os aglomerados passam a ser estruturas organizadas e complexas (homogênea ou heterogênea) (figura 13) (MONTEIRO et al., 2013; RICKARD et al., 2002, 2003; RØDER; SØRENSEN; BURMØLLE, 2016).

Figura 13- Processos de Co-agregação e Auto-agregação.




Fonte: Autoria própria

Além disso, a arquitetura compacta dos biofilmes permite que as células fiquem bem próximas umas das outras, o que favorece a troca de informações genéticas. Isso tudo ocorre através de sinalizações químicas que é denominado *Quorum sensing* (QS).

*1.3.3.3 Quorum sensing*

O *Quorum sensing* (QS) é a comunicação célula-célula que as bactérias utilizam para regular, comportamentos coletivos e com isso controlam a produção de fatores de virulência, como exemplo, a capacidade de algumas espécies bacterianas formarem biofilmes (FUQUA; GREENBERG, 1998; WANG et al., 2019).

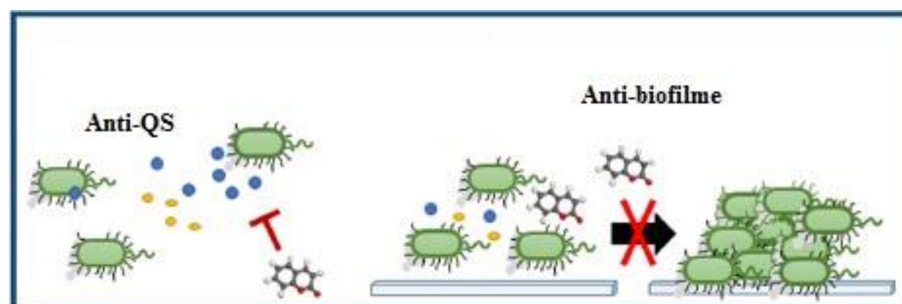
Todo esse processo depende da produção, detecção e resposta de um grupo de moléculas sinalizadoras extracelulares chamadas auto indutores (AHLs), essas moléculas são constantemente produzidas e recebidas ao nível basal na célula bacteriana, quando a densidade populacional das bactérias aumentam, consequentemente a concentração de AHLs também aumenta e então elas difundem-se e acumulam-se no ambiente circundante quando atinge um limiar (nível de QS), e então, difundem-se novamente para o interior da célula microbiana e induzem a expressão de um conjunto de genes alvo, que alteram o comportamento do microrganismo (PACZKOWSKI et al., 2017a; RASMUSSEN et al., 2005) .

Diante disso, atualmente alguns estudos buscam compostos que sejam capazes de interromper o *quorum sensing*, considerado que essa é uma estratégia para combater a patogenicidade dos microrganismos. Tendo em vista que diversas espécies de plantas que produzem compostos fenólicos como metabólitos secundários, são relatadas demonstrando potente ação antimicrobiana e antibiofilme, esses metabolitos tornaram-se alvo de pesquisas como promissores inibidores de QS (BORGES et al., 2014a; REEN et al., 2018; ZENG et al., 2008).

Recentemente, pesquisas demonstraram que o grupo das cumarinas possuem atividade antibiofilme e capacidade de inibir a sinalização QS (KOT et al., 2015; REEN et al., 2018; SLOBODNÍKOVÁ et al., 2016; ZHANG et al., 2017). Um estudo realizado por ZENG e colaboradores (2008) através de um modelo virtual, demonstrou que a esculetina (6,7-di-hidroxicumarina) e esculina foram estruturalmente compatíveis com o local de ligação do sinal TraR (ativador transcricional de *Agrobacterium tumefaciens* - QS). Embora este estudo não tenha explorado a função inibitória do QS experimentalmente, os autores demonstraram que esses derivados foram capazes de inibir os biofilmes de *P. aeruginosa*.

Corroborando com esses resultados, Reen e colaboradores (2015) evidenciaram que na presença de concentrações crescentes de cumarina, houve significativa redução de pigmentos produzidos pelas sinalizações QS nas cepas de *Serratia marcescens* SP15, *Chromobacterium violaceum* DSM 30191 *A. tumefaciens* NTL4. Além disso, realizaram ensaios de competição de sinal por biosensor, onde sugiu a interferência específica da cumarina com a AHL. Tendo em vista a importância clínica, os pesquisadores observaram que a cumarina diminuiu a expressão de genes relacionada a QS em *P. aeruginosa PA14* e também inibiu a formação de biofilme e motilidade deste patógeno (figura 14).

Figura 14- Ilustração da atividade anti-QS e anti-biofilme do grupo cumarinas.




Fonte: (Adaptado de REEN et al., 2018).

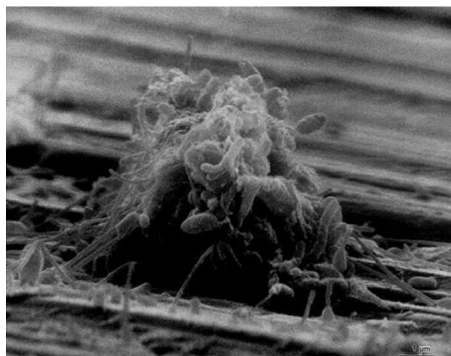
O composto fenólico rutina interfere no QS através da redução da secreção de auto indutores, reduzindo a produção de genes de virulência de *E.coli* (APEC) e também inibindo a formação de biofilme desse microrganismo (PENG et al., 2018). O carvacrol, composto fenólico presente no óleo essencial de orégano, em concentrações sub-MIC reduziram a expressão de *cviI* (um gene de codificação da N-acil-L-homosserina lactona sintase), produção de violaceína, atividade de quitinase (ambos regulado pelo QS) e também inibiram a formação de biofilme. Os autores sugeriram que a atividade antibiofilme do carvacrol pode estar

relacionada com o rompimento da sinalização QS (BURT et al., 2014). A presença de duas porções hidroxilas no esqueleto do anel aromático das flavonas é fundamental para a inibição dos receptores de ligação do auto indutor, pois, ligam-se especificamente a detecção do QS via antagonismo. Além disso, a administração dos flavonóides à *P. aeruginosa* altera a transcrição dos promotores-alvo controlados pelo sensor de QS e suprimem a produção do fator de virulência, confirmando seu potencial como anti-QS (PACZKOWSKI et al., 2017a).

### 1.3.4 Impactos clínicos e ambientais da formação de biofilmes

Os biofilmes estão presentes na maioria dos ecossistemas naturais e são responsáveis por grande parte dos processos fisiológicos que ocorrem nesses ambientes. Por outro lado, a formação das massas microbianas ocorre com bastante frequência em sistemas de tubulações aquáticas industriais, e isso resulta em grandes prejuízos, pois, os biofilmes provocam corrosões e obstruções do fluxo de água. Diante disso, as indústrias foram os pioneiros no desenvolvimento de métodos e estratégias para o controle da formação das massas microbianas e suas depredações (DONLAN; COSTERTON, 2002; RØDER; SØRENSEN; BURMØLLE, 2016). A figura 15 mostra a microscopia eletrônica de varredura do biofilme formado em uma superfície metálica no interior de um sistema aquático industrial.

Figura 15- Imagem de microscopia eletrônica de varredura do biofilme formado em uma superfície metálica no interior de um sistema aquático industrial.



Fonte: (DONLAN; COSTERTON, 2002)

Clinicamente, os biofilmes desenvolvem-se preferencialmente em implantes de dispositivos médicos (cateteres e próteses), superfícies inertes, tecidos mortos, ou vivos como

é o caso de endocardites. A utilização de implantes biomédicos é indispensável em grande parte dos casos clínicos, uma vez que esses dispositivos são fundamentais no reparo e manutenção das funções fisiológicas do paciente. Diante disso, a indústria de biomateriais investe aproximadamente 28 bilhões de dólares ao ano nos avanços tecnológicos desses materiais, e mesmo assim os microrganismos conseguem colonizar esses instrumentos (HOLZAPFEL et al., 2013).

Essas colonizações ocorrem, devido, principalmente, à existência de déficit imunológico que ocorre na interfase implante-hospedeiro. Neste contexto, relaciona-se o desenvolvimento de pneumonias em pacientes que utilizam ventilação mecânica, com a formação de biofilmes no interior desses aparelhos. Bem como a ocorrência de infecções persistentes nos casos que necessitam inserção de cateter urinário permanente, onde metade dos eventos decorrem nos primeiros 10 - 12 dias após a colocação dos dispositivos. Também são associadas a formação de biofilmes, infecções de pele e sepse, quando o paciente faz uso de cateteres venosos (STICKLER, 2008).

Geralmente, as infecções por biofilme são mais lentas para produzir sintomas evidentes do que as células planctônicas. Mesmo que as células sésseis façam a liberação de antígenos e estimulem a produção de anticorpos, esses, não serão eficazes em matar o microrganismo dentro de biofilmes, mesmo em indivíduos com excelentes reações imunológicas celulares e humorais. Sendo assim, infecções relacionadas com biofilme, raramente serão resolvidas por mecanismos de defesa do hospedeiro (MARCINKIEWICZ; STRUS; PASICH, 2013; PASICH et al., 2013).

O tratamento de infecções associadas aos biofilmes formados em dispositivos médicos resulta na necessidade de o paciente permanecer por um período maior sob internação hospitalar, bem como, impacta diretamente no aumento de custos das intervenções. Uma diversidade de mecanismos que atuam simultaneamente, explica a resistência das células sésseis aos recursos disponíveis atualmente. Esses mecanismos incluem a baixa penetração dos antimicrobianos e antissépticos, pois, não conseguem ultrapassar a camada de exopolissacarídeos (SPE) (SHARMA et al., 2014). O crescimento lento das células dentro do biofilme, ou seja, a baixa atividade metabólica dos micro-organismos no interior da SPE (provocada por falta de nutriente ou acumulo de resíduos) pode antagonizar os efeitos dos fármacos. A transferência de genes de resistência e falha na resposta imune humana (SINGH et al., 2017).

Algumas bactérias ganham destaque acerca da formação de biofilme, e isso se deve às características fenotípicas, como por exemplo a presença de flagelos. Dentre os mais reportados

como formadores de biofilme, pode-se incluir *P. aeruginosa* frequentemente encontradas em cateteres cardiovasculares, pacientes com fibrose cística. Posiciona-se entre as principais bactérias causadoras de infecções hospitalares, afetando mais de dois milhões de pacientes todos os anos, sendo responsável por mais 90.000 mortes (BIELECKI et al., 2008).

*1.3.4.1 Pseudomonas aeruginosa*

*P. aeruginosa* é uma bactéria Gram negativa, não fermentadora da glicose, que pode ser encontrada em diferentes nichos no ambiente, como por exemplo: Água, solo, plantas e animais. É conhecida por ser um patógeno oportunista, que acometendo principalmente pacientes imunocomprometidos. Comumente identificada como agente causador de infecções do trato urinário e do sistema respiratório, assim como dermatites, bacteremias, infecções dos tecidos moles e sistêmicas (SELEZSKA et al., 2012).

Acerca de adesão às superfícies, a família das *Pseudomonadaceae* é a mais pesquisada. Isso se deve ao fato de que a *P. aeruginosa* tem habilidade para formar biofilmes em uma ampla variedade de materiais, principalmente em implante de dispositivos médicos (cateteres, tubos e sondas), assim como em tecidos vivos (pulmões, feridas e queimaduras), matérias de alumínio (encontradas em aparelhos odontológicos ) e lentes de contato, ocasionando dano ocular (BIELECKI et al., 2008; BORGES et al., 2014a; WALKER; MOORE, 2014). Além disso, a formação de biofilme de *P. aeruginosa* pode causar sérias complicações em pacientes com fibrose cística. Uma vez que a antibioticoterapia se torna altamente ineficaz frente à fixação dessas películas no tecido pulmonar (DAVIES, 2002; HØIBY; CIOFU; BJARNSHOLT, 2010).

O desenvolvimento de biofilmes está estreitamente relacionado com a dificuldade terapêutica encontrada nestes casos, tento em vista que os biofilmes formados por de *P. aeruginosa* não são tolerantes a uma série de antimicrobianos e biocidas sob uma variedade de condições de crescimento, o problema se agrava pelo fato de que isolados clínicos multirresistentes, possuem maior habilidade de desenvolver biofilmes (SHARMA et al., 2014).

No processo de infeção por *P. aeruginosa* está relacionada a produção de diversos fatores de virulência extracelulares, como proteases, lectinas, sideróforos, piocianina e ramnolipídios A regulamentação da produção desses fatores de virulência extracelular é controlado principalmente pelo QS (NADAL JIMENEZ et al., 2012). Especula-se que existem no mínimo quatro sistemas regulatórios clássico do QS em *P. aeruginosa,* que envolvem grande número de genes (até 300, cerca de 6% do genoma) relacionados com fatores de virulência e formação de biofilme (VENTURI, 2006; WHITELEY; DIGGLE; GREENBERG, 2017).

Um recente estudo realizado por Wei e colaboradores (2019), avaliou os efeitos de três diferentes ervas utilizadas na medicina tradicional chinesa (folhas de *A. argyi*, a casca de raiz de *C. dictamni* e a raiz de *S. melongena*) frente à *P. aeruginosa.* O grupo obteve resultados que demonstraram, que quando os extratos das plantas foram colocados junto às células microbianas, não causaram a morte total da bactéria, porém, houve a inibição do sistema de sinalização (QS) de *Pseudomonas*.

Diante disso muitas pesquisas buscam nos produtos de origem natural uma alternativa de tratamento e/ou prevenção para os danos causados pela formação de biofilmes. Porém é de extrema relevância que além dos efeitos antibiofilme e antimicrobiano, também seja feita uma cuidadosa avaliação acerca da toxicidade das plantas e/ou substancias utilizadas na pesquisa(NJUGUNA; MASIMIREMBWA; CHIBALE, 2012).

## 1.4 SEGURANÇA DE USO

Uma ampla variedade de produtos naturais são utilizados popularmente para tratamento de diversas patologias (FENNER et al., 2006; MARTIN et al., 2013). Diante disso, há centenas de anos, esses produtos proporcionam uma das principais fonte de drogas. Contudo, nas últimas décadas, o desenvolvimento de fármacos a partir de fontes naturais sofreu um declínio perceptível, apesar de existirem inúmeras pesquisas com finalidade de identificar metabólitos secundários e esclarecer mecanismos de ação e atividades biológicas. Em partes, isso se deve preocupação com a biodisponibilidade e possível toxicidade dos produtos naturais (GECHEV et al., 2014; NJUGUNA; MASIMIREMBWA; CHIBALE, 2012).

A maioria dos compostos fenólicos identificados em plantas e alimentos, aparecem na forma glicosilada. Ao nível intestinal, eles sofrem diferentes biotransformações dependendo da natureza dos resíduos de açúcar. No intestino, esses compostos são hidrolisados e também sofrem a ação da microbiota intestinal, com isso as porções de açúcar são clivadas e as agliconas são absorvidas. Os compostos absorvidos podem ainda sofrer transformações hepáticas e após atingirem o órgão alvo caem na corrente sanguínea e são excretados via urina (CORCORAN; MCKAY; BLUMBERG, 2012; FRAGA et al., 2010).

Independente da natureza, qualquer composto estranho que ingerimos, são alvos de biotransformação metabólica pelo nosso organismo, isto ocorre com a finalidade de converter esses compostos em produtos químicos que possam ser mais facilmente eliminados pelo corpo (PATRICK, 2009; THOMAS, 2003). Após serem metabolizados, as substâncias podem tornar-se - inativos (não possuem atividades farmacológicas), ativos (possuem propriedades farmacológicas de menor, igual ou maior magnitude do que seus compostos de origem) ou

reativos (Reagem com o organismo, resultando em efeitos tóxicos indesejáveis) (HAND; HONEK, 2005; TESTA; KRÄMER, 2007).

Os efeitos tóxicos podem ser causados pela formação de espécies reativas de oxigênio (EROS). A produção das EROS é um efeito típico do metabolismo humano e está envolvida em vários processos fisiológicos, como por exemplo, na fagocitose (mecanismo de defesa celular frente a patógenos) (BACANL; AYD; BAS, 2017). Porém, quando ocorre uma produção abundante de EROS o organismo dispõe de um eficiente mecanismo antioxidante que controla e estabelece o equilíbrio, evitando que ocorra o estresse oxidativo (desequilíbrio entre pro oxidantes (EROS)e antioxidantes) e consequentemente dano (VASCONCELOS et al, 2007).

Um indivíduo, dependendo da dieta, pode ingerir de 50 a 800 mg / dia de fitoquímicos que estão presentes em legumes, frutas e bebidas específicas. Eles têm sido considerados como possíveis antioxidantes. Conforme os achados na literatura, os autores relatam que os compostos, possuem uma atividade protetora de dano contudo, em certas ocasiões, na presença de metais (cobre, ferro, etc), esses compostos podem agir como pró-oxidantes, produzindo espécies reativas (peróxido de hidrogênio, radicais hidroxila)(PROCHÁZKOVÁ; BOUŠOVÁ; WILHELMOVÁ, 2011).

Esses compostos quando consumidos em altas doses torna-se potencialmente tóxicos, especialmente em grupos de indivíduos vulneráveis, como por exemplo, os idosos. Isso se deve, principalmente pelo fato de os compostos fenólicos interagirem com medicamentos. Além disso, o consumo exagerado pode desencadear problemas na tireoide. Alimentos ricos em quercetina, consumido em exagero possuem efeitos tóxicos, que pode ser relacionado à mutagenicidade e toxicidade mitocondrial. Este mecanismo é confirmado por resultados experimentais *in vitro* utilizando células humanas (fibroblastos embrionários, células umbilicais endoteliais e glóbulos vermelhos), onde a quercetina (300mM) demonstrou ser moderadamente citotóxica, contribuindo para um aumento da geração de Espécies Reativas de Oxigênio (EROS) (BOOTS et al., 2007; GALATI et al., 2002; WILMS et al., 2005).

Apesar das diversas pesquisas realizadas principalmente com plantas, uma grande maioria da população ainda utiliza esses vegetais sem conhecimento prévio acerca da composição desses produtos (CORRÊA; AGERTT; BOLIGON, 2012; GECHEV et al., 2014). Muitos desses compostos que possuem atividades benéficas para a saúde, em altas doses podem apresentar efeitos tóxicos e prejudicais. Diante disso, é de extrema importância de que além dos estudos de atividades biológicas de plantas também seja realizada uma avaliação dos efeitos

cito e genotoxicas desses vegetais, de modo a oferecer uma maior segurança de uso à população. (BARCELOUX, 2008; MARGINĂ et al., 2015).

# 2. OBJETIVOS

## 2.1 OBJETIVO GERAL

Identificar as substâncias majoritárias presentes no extrato bruto e frações de *Equisetum hyemale*, bem como avaliar as atividades antimicrobiana, antibiofilme, citotóxica e genotóxica dos compostos majoritários em cada fração

## 2.2 OBJETIVOS ESPECÍFICOS

2.2.1 Identificar os compostos majoritários presentes no extrato bruto e frações de *Equisetum Hyemale*;

2.2.2 Determinar a concentração inibitória mínima dos extratos e compostos majoritários frente a diferentes microrganismos

2.2.3 Avaliar a capacidade de inibição de biofilme formado por PA01 frente aos extratos e compostos majoritários;

2.2.4 Avaliar a influência dos extratos e compostos na co-agregação da *PAO1e E. coli*;

2.2.5 Determinar a inibição da motilidade da *PAO1* frente aos extratos e compostos majoritários;

2.2.6 Analisar a capacidade dos extratos e compostos majoritários em inibir o sistema de *Quorum Sensing*,

2.2.7 Avaliar a capacidade cito e genotoxica dos extratos e compostos majoritário;

# 3 PRODUÇÕES CIENTÍFICAS

As produções científicas incluem um artigo e um manuscrito. O artigo foi publicado na *Pharmacognosy Journal* e está formatado de acordo com as normas exigidas pelo mesmo, acessadas no site: https://www.phcogj.com/information-authors.

O manuscrito foi submetido ao periódico *Biofouling* e está formatado de acordo com as normas exigidas pelo mesmo, acessadas no site: https://www.tandfonline.com/action/authorSubmission?show=instructions&journalCode=gbif20

## 3.1 ARTIGO 1

Título: **Antimicrobial, cyto and genotoxic activities of *Equisetum hyemale***

Autores: Camilla Filippi dos Santos Alves, Pauline Cordenonsi Bonez, Marcia de Souza Ebling, Camila Casagrande, Litiane Freitas, Carolina Dolwitsch, Fernanda Pires, Michele Rorato Sagrillo, Gerson Fernandes de Brum, Marli Matiko Anraku de Campos, Roberto Christ Vianna Santos

Artigo publicado no periódico ***Pharmacognosy Journal*** (Pharmacognosy Journal, n. 6, V. 11, p. 1-9, 2019) Doi: 10.5530/pj.2019.11. Disponível em: http://www.phcogj.com/v11/i6s.

**Antimicrobial, cyto and genotoxic activities of *Equisetum hyemale***

**Camilla Filippi dos Santos Alves[1], Pauline Cordenonsi Bonez[2], Marcia de Souza Ebling[3], Camila Casagrande[2], Litiane Freitas[2], Carolina Dolwitsch[5], Fernanda Pires[5], Michele Rorato Sagrillo[4], Gerson Fernandes de Brum[4], Marli Matiko Anraku de Campos[1], Roberto Christ Vianna Santos[2]**

**Affiliations**

[1] Department of Clinical and Toxicological Analysis, Mycobacteriology Laboratory, Universidade Federal de Santa Maria (UFSM), Santa Maria, Brazil

[2] Oral Microbiology Research Laboratory, Microbiology and Parasitology Department, Universidade Federal de Santa Maria (UFSM), Santa Maria, Brazil

[3] Graduate Program in Nanoscience, Franciscan University (UFN), Santa Maria, RS, Brazil

[4] Cell Culture Laboratory, Universidade Franciscana (UFN), Santa Maria, Brazil

[5] Graduate Program in Pharmaceutical Sciences, Universidade Federal de Santa Maria (UFSM), Santa Maria, Brazil

**Correspondence**

[a] Camilla Filippi dos Santos Alves, Department of Clinical and Toxicological Analysis, Mycobacteriology Laboratory.

Universidade Federal de Santa Maria (UFSM), Santa Maria, Brazil. Av. Roraima n° 1000 Cidade Universitária, Bairro Camobi, Zip Code: 97105-900

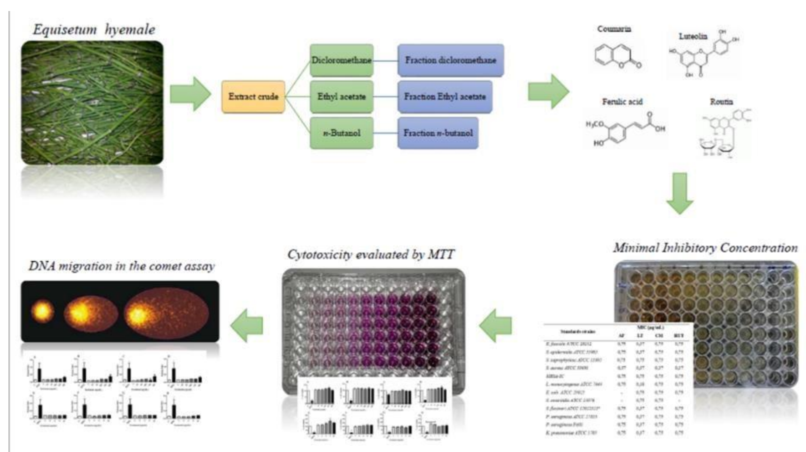
E-mail: camillafilippibio@gmail.com

## Abstract

The popular use of natural products has great benefits for the health of the human population. In this study we highlight *Equisetum hyemale*, belonging to the phylum *Sphenophyta* and the family *Equisetaceae*. Popularly, the stems of this plant are used for their diuretic, digestive, anti-anemic, and anti-inflammatory properties. Given this context, and the fact that the growth of antimicrobial resistance is a serious problem for global public health, this plant could be used as an alternative, to increase our therapeutic arsenal. Therefore, it is important to more clearly elucidate the complex structures present in plants, because these substances are mainly responsible for their beneficial and/or toxic effects. Thus, the objective of this study was evaluate the antimicrobial activity,cytotoxic and genotoxic potential of the phenolic compounds separately. The substances luteolin, ferulic acid and coumarin were identified at high concentrations by ultra high-performance chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS). Antimicrobial activity was elucidated through microdilution in broth; the phenolic compounds were able to inhibit the visible growth of the standard bacterial strains at low concentrations. Cytotoxicity was evaluated by MTT and genotoxicity was analysed through cellular damage using a Comet assay; the results showed that it did not present cytoxicity or genotoxicity at the corresponding concentrations. With this, we suggest that *E. hyemale* may be an alternative for the treatment of infections by microorganisms that are resistant to synthetic drugs.

**Keywords**: *Equisetum hyemale.* Phenolic compounds. Cytotoxicity. Antimicrobial activity

**Graphical abstract**



## INTRODUCTION

*Equisetum hyemale* is notable for demonstrating important benefits to human health. Its morphology includes greatly reduced leaves and air fistulous stems, which resemble a ponytail; for this reason, it has received the popular name of horsetail. It belongs to the phylum *Sphenophyta* and family *Equisetaceae.* It is an air plant commonly found in tropical and temperate climates (BRUNE; THIV; HAAS, 2008; SAPEI et al., 2007). Popularly, the stems of the plant are used for the production of an infusion that acts as a diuretic, digestive, anti-anemic, anti-inflammatory, and antimicrobial, and is also employed to treat gonorrhea, diarrhea, kidney infections, and bladder and eye diseases (GIERLER; SAPEI; PARIS, 2008; YAMANAKA et al., 2012). Although there are few reports of the antimicrobial activity of *E. hyemale* in the literature, a study carried out by our research group demonstrated the antimicrobial activity of crude extract and fractions, as well as the ability of these extracts to inhibit the formation of biofilm formed by *P. aeruginosa* (01). These results were justified by the presence of high levels of phenyl compounds in the plant structure (DE QUEIROZ et al., 2015; DOS SANTOS ALVES et al., 2016; FERRAZZANO et al., 2013).

The phenyl compounds are secondary metabolites produced by plant with the functions of protection and reproduction. These substances have important biological activities and are classified into groups, according to their chemical structure. Among these groups, phenolic compounds are present in a wide range of fruits and vegetables (CORRÊA; AGERTT; BOLIGON, 2012; PIANA et al., 2015). Their chemical structure is characterized by having a hydroxyl attached directly to an aromatic hydrocarbon group, and exhibit different biological effects, such as antitumor, antioxidant, anti-inflammatory, antiviral and antimicrobial activities (BOLIGON et al., 2014; CORCORAN; MCKAY; BLUMBERG, 2012; KHAN et al., 2005; WITAICENIS; SEITO; DI STASI, 2010).

Therefore, the phenyl compounds could be used as an alternative, to increase our therapeutic arsenal, because the disorderly use of antimicrobial drugs is contributing to the growth of micro-organisms that are resistant to treatments; namely, these strains are able to multiply in the presence of antimicrobial concentrations higher than the doses recommended for the treatment of the patient (CENTER FOR DISEASE CONTROL AND PREVENTION, 2015; FRIEDRICH et al., 2016; HIRSCH; TAM, 2010). After the discovery of the first antimicrobial drugs, it was evident that bacteria possessed resistance mechanisms; however, this scenario was livened up with the frequent introduction of new antibiotics. Currently, antibiotic production is greatly reduced and the prevalence of pathogens' resistance to

antibiotics has increased, which has become a serious public health problem (BILAL et al., 2017; PATRICK, 2009; WANNMACHER, 2004).

In spite of the benefits offered by natural products, there is a need to preserve their integrity and pharmacological chemistry when raw material is transformed into medicine (MARTIN et al., 2013; PATRICK, 2009). With this, one should ensure the constancy of the biological action and their own safety, as well as enhance the therapeutic potential. In order for this goal to be reached, herbal production requires, necessarily, previous studies concerning the botanical aspects, agronomic, phytochemical, pharmacological, toxicological, analytical methodologies and development technology (CORCORAN; MCKAY; BLUMBERG, 2012; HAN et al., 2015). Given this, the objective of this study was to define the chemical composition of *E. hyemale*, as well as to assess its antimicrobial activity, cytotoxicity and genotoxicity.

## MATERIALS AND METHODS

### *Plant sample collection*

The plant material used in the study was collected in Santa Maria (Rio Grande do Sul, Brazil) in the district of São Martinho da Serra (29° 32′ 16″ S, 53° 51′ 18″ W), between September and November 2017. The dried specimen is preserved in the herbarium at the Department of Environmental Engineering, Federal University of Santa Maria (UFSM), under register number SMBD 6756. The compounds luteolin, ferulic acid, coumarin and routin were commercially disclosed by Sigma-Aldrich®.

### *Preparation of plant extracts*

The plant stems were dried in a stove (temperature < 40 ºC) and chopped using a knife mill. The obtained material was macerated at room temperature with 70% ethanol for one week, with one daily shakeup, and the solvent was renewed for 4 weeks. After filtration, the hydroalcoholic extract was evaporated under reduced pressure in a rotary evaporator to remove the ethanol, thereby obtaining an aqueous extract. Part of the aqueous extract was dried in a stove (temperature above 40 °C) to obtain the crude extract (CE). After that, the remaining

aqueous extract was partitioned with dichloromethane (DCM), ethyl acetate (EA) and n-butanol (NB), successively (BOLIGON et al., 2014).

*Preparation of solid-phase extraction (SPE)*

Firstly, 3 mL of the diluted extract was added to 12 mL of ultrapure water (1:4). Then, 15 µL of the acetic acid was added, producing a final proportion of 0.1% (v/v). From the SPE cartridges of Strata C-18E, 500 mg was used, with a capacity of 3 mL (Phenomenex, Torrance, USA). The cartridges went through a conditioning stage with 6 mL of 0.2% (1:1; v/v) methanol/acetic acid, then with 6 mL of 0.1% (v/v) acetic acid solution in water. After obtaining a fixed volume of 2 mL of the extracts, with a composition ethanol/water/acetic acid (20:80:0.1; v/v), the solution was percolated to a leak of 2 mL $min^{-1}$, followed by a washing with 2 mL of 0.1% (v/v) acetic acid solution. Finally, the removed analytes were eluted with 2 mL of MeOH. Just prior to the chromatographic analysis, the eluate obtained by the SPE procedure was diluted to 0.5 g $L^{-1}$ with 0.1% (1:1; v/v) methanol/acetic acid solution (FACCIN et al., 2016).

*Ultra high performance chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS)*

Analysis was performed according to Faccin and collaborators (2016), at the Research Center and Analysis of Contaminant Residues of the UFSM. The UPLC-ESI-MS system consists of a chromatograph (ACQUITY UPLC) coupled to a mass spectrometer (XEVO TQ-S triploquadrupolo), Milford, USA). The column C18 Acquity UPLC BEH (100 mm, i.d. 21 µm, particle 1.7 µm; Waters) was used, and maintained under heat at 40 °C. The method uses a gradient elution, containing 0.1% acetic acid (A) and acetonitrile (B), at a constant flow rate of 800 µL·$min^{-1}$, according to the following conditions: 8.0% B (0.00–0.10 min); 8.0–25.8% B (0.10–3.45 min); 25.8–54.0% B (3.45–6.90 min); 54.0–100.0% B (6.90–7.00 min); 100.0% B (7.00–9.00 min); and an injection volume of 5 µl. The mass spectrometer was operated using the negative electrospray ESI (-) ionization mode, with the following parameters: gas flow (N2) at 11 L $min^{-1}$; nebulizer pressure equal to 30 psi; capillary voltage equal to ±2.4 kV and during gas temperature equal to 250 °C. The comps were analyzed in a mass spectrometer operating under “multiple reaction monitoring” (MRM). The analyzed data

were acquired using quantitative analysis using the MassLynx Mass Spectrometry software (Waters).

*Preparation of inoculums*

The bacterial inoculum sizes were standardized according to Clinical and Laboratory Standards Institute standards (CLSI). The bacterial strains American Type Culture Collection (ATCC) *E. faecalis* (ATCC 29212), *S. epidermidis* (ATCC 35985), *S. saprophyticus* (ATCC 15305), *S. aureus* (ATCC 33691), MRSA-IC (clinical isolate of methicillin-resistant *Staphylococcus aureus*), *L. monocytogenes* (ATCC 7644), *E. coli* (ATCC 25922), *S. enteritidis* (ATCC13076), *S. flexineri* (ATCC 12022323), *P. aeruginosa* (ATCC 27853), *P. aeruginosa* PA01, and *K. pneumoniae* (ATCC 1705) were used. Isolated colonies were grown for 18 and 24 h in Mueller–Hinton agar (Himedia, India) and the suspension was prepared in saline solution (0.85%) with NaCl density adjusted to 0.5 on the McFarland scale ($1.5x10^8$ CFU/mL) (CLSI, 2017).

*Determination of minimum inhibitory concentration (MIC)*

The antibacterial activity, according to Clinical and Laboratory Standards Institute standards, of the phenolic compounds was evaluated using the broth microdilution method. The assay was carried out in 96-well microtiter plates using Mueller–Hinton broth (Himedia®). Each extract was diluted in dimethyl sulfoxide (DMSO) and gave rise to serial concentrations ranging from 2 to 0.185 µg/mL (initial concentrations of the tests for the phenolic compounds were established from the equivalent concentrations identified in the extracts). The inoculum was mixed in the dilution of compounds. The plates were incubated at 37 °C and the minimal inhibitory concentration (MIC) was recorded after 24 h of incubation. The MICs were defined as the lowest concentration of extract that inhibited visible bacterial growth. This test was performed in triplicate, and 2, 3, 5-triphenyltetrazolium chloride was used as an indicator of microbial growth. A positive control growth culture medium and microorganism were used. For the control of sterility, only culture medium was used for negative growth. In the same way, a control of the solvent DMSO was carried out at the same concentration used for the dilution of the samples (CLSI, 2017).

*Mononuclear cells*

Blood was collected for toxicological tests. Peripheral blood samples were obtained through three discarded samples from the Laboratory of Clinical Analysis of the University Franciscan, under the approval of the Institution's Ethics Committee on Human Beings (CAAE: 31211214.4 .0000.5306), with the absence of identification data. The samples were obtained through venipuncture using tubes with Vacutainer®-type heparin, which were used to separate PBMCs and the subsequent treatments and cell cultures (SAGRILLO et al., 2015).

*Evaluation of cell viability by the MTT technique.*

In the MTT assay, the 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide reagent is water-soluble and yellowish in color, being readily incorporated by viable cells, which reduce this compound's mitochondrial activity by the enzyme succinate dehydrogenase. Upon being reduced, MTT is converted into formazan crystals, which are insoluble in water and bluish-purple in color and stored in the cell cytoplasm, and subsequently solubilized by the addition of DMSO (dimethyl sulfoxide); they were quantified calorimetrically by spectrophotometry over a 570 nm wave. Curves with different concentrations were calculated from the MIC values for both the extracts [14] and the compounds; these concentrations were added to the cell cultures. The absorbance value is proportional to the number of viable cells, in comparison to the negative control (BONEZ et al., 2016; SAGRILLO et al., 2015).

*Genotoxicity evaluation*

The genotoxicity evaluation was performed by a Comet assay. The cells were treated with different concentrations, which varied between 3 µg/mL and 300 µg/mL for the crude extract and fractions, and 2 µg/mL and 10 µg/mL for the phenolic compounds. These concentration ranges were defined on the basis of the minimum inhibitory concentrations (MICs) obtained in our first published study (Dos Santos Alves et al.,2016). The concentration,were suspended in low-melting agarose (deposited on a glass slide precoated with a layer of 1.5% agarose). The material was immersed in lysis solution (lysis solution: 89 and 10 mL of dimethyl sulfoxide and 1 mL of Triton X-100) for the removal of the membranes and cytoplasm. Subsequently, the slides were incubated in alkaline electrophoresis buffer at a pH of 13 (300 mmol $L^{-1}$ NaOH and 1 mmol $L^{-1}$ EDTA in distilled water) and subjected to

electrophoresis for 30 min at 25 V and 300 mA. Then, the processes of neutralization (neutralizing buffer pH 7.5), fixation (15% trichloroacetic acid) and coloration (acridine orange) were performed, so that the genetic material could be analyzed. One hundred cells were analyzed in an optical microscope and were classified according to their tail length. The cells received scores from 0 (no damage) to 4 (maximum damage). The test was performed in triplicate, and the data were transformed into a damage index, which was analysed statistically. The genotoxic potential was measured 48 h after exposure to the extracts and compound; they were considered genotoxic when the concentrations resulted in a damage index higher that 0.1 (BONEZ et al., 2016).

*Statistical analysis*

OD readings obtained from the assay were recorded as mean +/- SE and were submitted to a one-way ANOVA. A p value $\geq 0.05$ was considered to indicate statistical significance. Graphs were prepared using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA).

## RESULTS AND DISCUSSION

*Phytochemical characterization by UPLC-ESI-MS*

The major crude extract (CE) compounds found using the UPLC-ESI-MS system included luteolin (LUT; 3.7 µg/mL) and ferulic acid (FA; 1.7 µg mL) )( fig.**1**a), where as in the ethyl acetate (AE) fractions, coumarin (CM; 2.06 µg/mL) and ferulic acid (FA; 3.8 µg/mL) stood out (fig.**1**b). The main components of the dichloromethane (DCM) (fig. **1**c). and butanol (NB) fraction (fig.**1**d) were ferulic acid (FA; 3.5 µg/mL and 1.5 µg/mL, respectively) and routin (RUT; 2.8 µg/mL and 1.7 µg/mL) respectively.

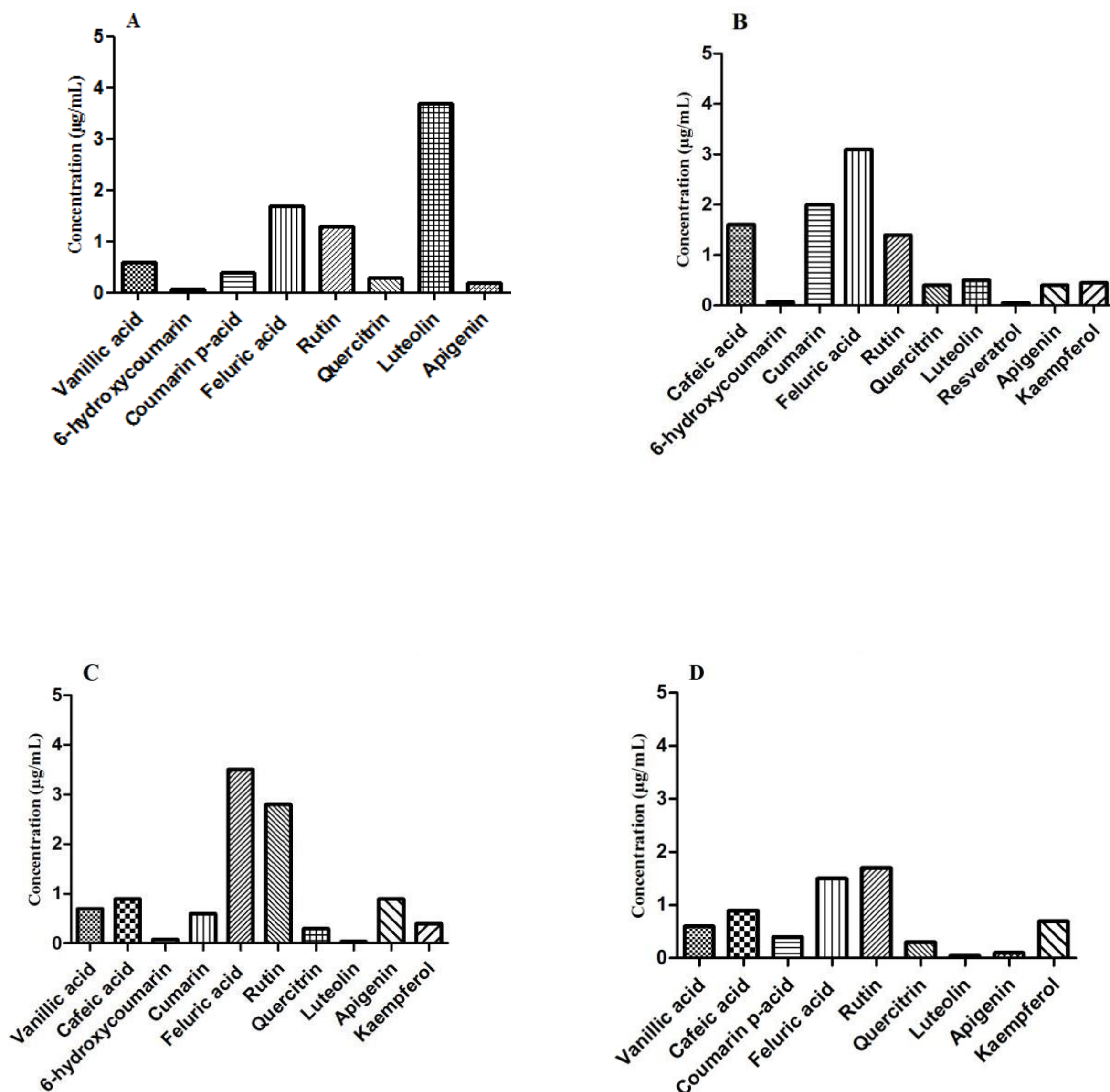



**Fig. 1.** Identification of major compounds presents in crude extract (A) acetate fraction (B), dichloromethane (C) and n-butanol fraction (D) of *E.hyemale* (B) by the LC-MS method in µg/ml.

These substances are part of the phenol compounds group and the antimicrobial activity this compounds is often reported in the literature. Luteolin has a potent antimicrobial action and can operate via different mechanisms of action, such as the inhibition of the production of proteins and peptidoglycan, the alteration of the permeability of the internal membrane of the

microorganism, and inhibition (EUMKEB; SIRIWONG; THUMANU, 2012; LOU et al., 2015). In the same way, ferulic acid has been shown to have moderate antimicrobial activity (BEN YAKOUB et al., 2018; KUMAR; PRUTHI, 2014). The for coumarin, this action is attributed to its lipophilic chemical structure, since the presence of hydroxyl groups and the size of the carbon chain facilitate their entry into the microbial cell (ZHU; JIANG, 2018). One study has made it clear that routin acts in synergy with other phenolic compounds, potentiating the antimicrobial activity of phytochemicals (ARIMA; ASHIDA; DANNO, 2002). All of these studies corroborate with the results obtained in our research, since we observed that the major substances of *E. hyemale* have significant antimicrobial activity alone and in adjusted concentrations at the values identified in the crude extract and fractions of the plant.

*Antimicrobial Activity*

We calculated the identified relative values of each major substance present in the crude extract and fractions, and we evaluated the activity of these substances alone. The results showed that the phenolic compounds were able to inhibit the visible growth of the *S. epidermidis* ATCC 35985, *S. saprophyticus* ATCC 15305, *S. aureus* ATCC 33691, *MRSA-IC* (clinical isolate of methicillin-resistant *Staphylococcus aureus)*, *E. coli* ATCC 25922, *S. enteritidis* ATCC 13076, *S. flexineri* ATCC 12022323[a], *P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01, *K. pneumoniae* ATCC 1705, *E. faecalis* ATCC 29212 and *L. monocytogenes* ATCC 7644. The MIC values were low and satisfactory, with concentrations ranging from 0.37 µg/mL at 0.75 µg/mL MIC, as shown in Table 1.

**Table 1** - Antibacterial activity of compounds phenolics identified in crude extract and fractions of *E. hyemale* against strains bacterial standards.

MIC: Minimal Inhibitory Concentration; ATCC: American Type Culture Collection; AF: Ferulic acid; LT: Luteolin; CM: Coumarin; RUT: Routin

In a previous study carried out by our research group, we demonstrated that crude extracts, the dichloromethane fraction, acetate fraction and N-butanol fraction of *E. hyemale* showed antimicrobial activity against *S. epidermidis* ATCC 35985, *S. saprophyticus* ATCC 15305, *S. aureus* ATCC 33691, *MRSA-IC* (clinical isolate of methicillin-resistant *Staphylococcus aureus)*, *E. coli* ATCC 25922, *S. enteritidis* ATCC 13076, *S. flexineri* ATCC 12022323[a], *P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01, *K. pneumoniae* ATCC 1705, *E. faecalis* ATCC 29212 and *L. monocytogenes* ATCC 7644, with minimal inhibitory concentrations (MIC) ranging from 3.270 at 5.2410 µg/mL (DOS SANTOS ALVES et al., 2016). Therefore, in this study, we clearly identify the presence of the major substances in the crude extract and fractions previously analyzed (routin, coumarin, ferulic acid and luteolin).

*Safety of using*

In order to prove the safety of using *E. hyemale*, we performed a cytotoxicity and genotoxicity test *in vitro*. The crude extracts, fractions and phenolic compounds were able to

maintain cell viability at all exposure times, and the concentration values used were adjusted with the value obtained for the MIC (fig. **2**). Similarly, in the comet assay shown in fig.**3**, the low obtained damage rates show that the extracts and compounds did not damage the genetic material of the cells.

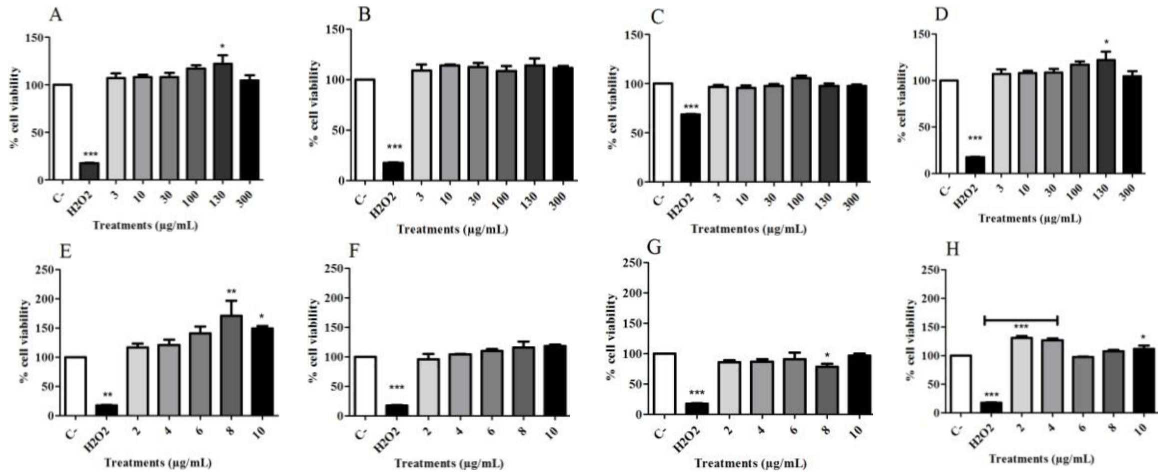



**Fig. 2** - Comparison of cytotoxicity evaluated by MTT reduction in mononuclear cells exposed to $H_2O_2$ and treated with crude extract, fractions and major compounds of *E. hyemale*. Extract crude (A), acetate fraction (B), Dichloromethane fraction (C), N- butanol fraction (D), Luteolin (E), Ferulic acid (F), Coumarin (G) and Routin (H). Results were expressed as percentage of the negative control (100%).

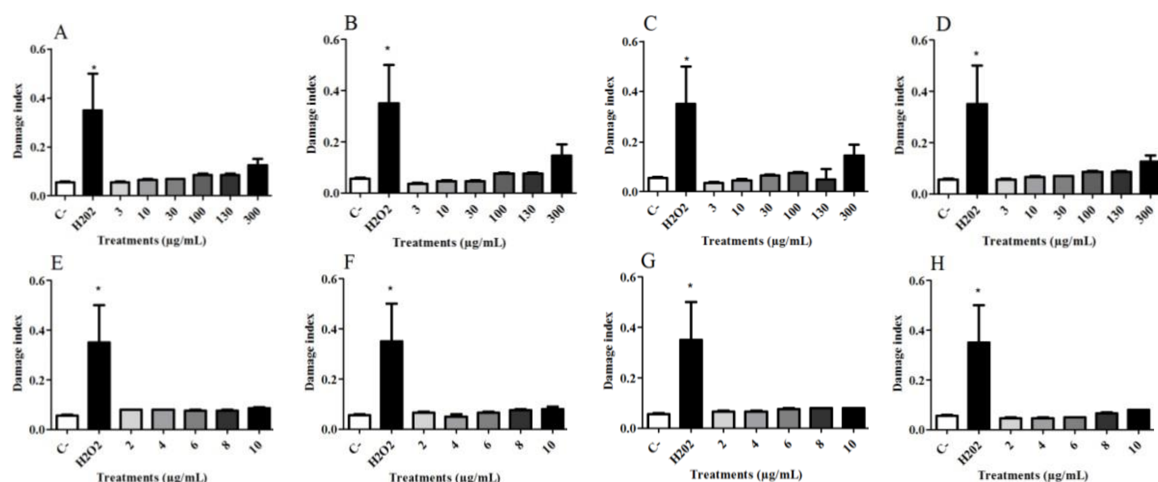



**Fig. 3-** DNA migration in the comet assay for assessment of genotoxicity of crude extract, frations and major compounds of *E. hyemale* exposed to peripheral blood mononuclear cell culture for 72h. Extract crude (A), acetate fraction (B), Dichloromethane fraction (C), N-butanol fraction (D), Luteolin (E), Ferulic acid (F), Coumarin (G) and Routin (H).

The presence of high levels of phenol compounds in the *Equisetum* genus have been demonstrated in the literature (BRUNE; HAAS, 2011; FRANCESCATO et al., 2013; GARCIA et al., 2013; GIERLER; SAPEI; PARIS, 2008; KUKRIC et al., 2013; PARK; TOMOHIKO, 2011). Therefore, few studies have evaluated the biological and toxicology activities of these substances alone. The results obtained in this study suggest that phenolic compounds were mainly responsible for the biological activities of the plant (DE OLIVEIRA et al., 2013; KUKRIC et al., 2013).

Although these plants are beneficial for health human, high doses may present toxic and deleterious effects (BARCELOUX, 2008; JOBIM et al., 2014). Most people still use this vegetable without previous knowledge of the composition of the products (MARGINĂ et al., 2015).Other studies report that, in the presence of metals and in high concentrations, phenolic compounds have pro-oxidant activity and can cause cellular damage (FRAGA et al., 2010). When the hydro-alcoholic extracts of five *Equisetum* species (*E. arvense*, *E. sylvaticum*, *E. fluviatile*, *E. palustre* and *E. telmateia Ehrh*) were evaluated for their genotoxicity, the results showed that high concentrations induce cellular damage (SPATZ; EMANNS, 2004). However, another study noted that the phytoextract of the genus *Equisetum* did not induce acute toxicity in *Drosophila* or in human lymphocytes in culture, at low concentrations (QUEVEDO; HORACIO; CASTA, 2007).

## CONCLUSION

Given this, we conclude it is extremely important that, along with studies of the biological activities of plants, evaluations of the cytotoxicity and genotoxicity of these plants should be conducted, in order to offer greater safety to humans. *Equisetum hyemale* has the potential for antimicrobial activity and does not show cytotoxic and genotoxic effects. Therefore, this plant may be an alternative treatment for microbial infections; these activities are attributed to the presence of phenolic compounds in the plant. From these results, studies can be carried out using models of infection in experimental animals that, in turn, will redefine the basis for therapeutic studies in humans.

## CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

## ACKNOWLEDGMENTS

This work was financially supported by FAPERGS, CNPq and CAPES.

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3.2 MANUSCRITO 1

**Antibiofilm activity of *Equisetum hyeamale*: interference with *quorum sensing***

Camilla Filippi dos Santos Alves[a], Pauline Cordenonsi Bonez[b], Camila Casagrande[c], Dariane Jornada Clerici[b], Camila Marina Verde[b], Carolina Gonzalez Urquhart[b], Marcos Vinicius Missel[b], Thais Barin[b], Roberto Christ Vianna Santos[b] e Marli Matiko Anraku de Campos[a].

*[a]Department of Clinical and Toxicological Analysis, Mycobacteriology Laboratory, Universidade Federal de Santa Maria (UFSM), Santa Maria, BRAZIL; [b]Oral Microbiology Research Laboratory, Microbiology and Parasitology Department, Universidade Federal de Santa Maria (UFSM), Santa Maria, BRAZIL.*

## Abstract

The biofilm formation results from successive stages of adhesion, coaggregation e auto aggregation, bacterial motility and quorum sensing (QS). Thus, it is important to look for alternatives capable of interfering with these parameters and, consequently, inhibit the formation of biofilms. Plants are a rich source of research, as they produce secondary metabolites that have important biological activities. In this study, we aimed to evaluate the antimicrobial, antibiofilm and anti-quorum sensing activities of a crude extract, fractions and major substances produced by *Equisetum hyeamale* against *P. aeruginosa*. The extracts and the fractions inhibited the bacterial growth of *P. aeruginosa* PAO1 with Minimal Inhibitory Concentration (MCI) ranging from 26.2 µg/mL to 13.1 µg/mL), as did the major substances, that haved MIC from 0.75 µg/mL to 0.37 µg/mL). Using the biofilm inhibition technique on polystyrene plates, we observed that the crude extract and fractions were able to inhibit biofilm formation by 29-64%, while the major substances inhibited biofilm formation by 67-79%. Atomic force microscopy (AFM) allowed to visualize the decrease in microbial mass. Colony counting also showed that there was a significant difference in the colony counts, in relation to the positive control. Moreover, all extracts and compounds were shown to influence motility (swimming, swarming and twitching). To evaluate QS inhibition, we assessed violacein, a mediator of QS induced by the *CviI* autoinducer synthase. The plant extract and the major compounds were able to significantly reduce the production of violacein at subinhibitory concentrations. In view of this, we have shown that this plant has relevant antibiofilm activity and interferes with essential biofilm formation factors such as motility and QS. Thus, *E. hyeamale* is a potential new therapeutic alternative against infections associated with biofilms.

**Keywords**: biofilm; plant; ferulic acid; gallic acid; motility; pathogenic bacteria

## 1. Introduction

Microorganisms live in planktonic or sessile form. In the sessile form, microbes attach to biotic or abiotic surfaces, forming a complex structure known as a biofilm. Biofilms are formed by microorganisms of one or multiple species. The motility of bacteria and coaggregation are very important factors for approximation of the microorganisms at surface and in biofilm maturation. This process is possible because microorganisms communicate with each other (DONLAN; COSTERTON, 2002). Communication between bacterial cells is crucial in establishing a biofilm; this is called quorum sensing (QS). Besides regulating collective behaviors, QS controls the production of virulence factors (SHARMA et al., 2014).

The formation of a biofilm is a favorable way of life for microorganism, as it favors the acquisition of nutrients and protects against aggressive external factors. However, biofilm formation has a considerable impact on health when these structures form on living tissues or medical devices. Biofilm infections are generally slower to produce overt symptoms than planktonic cells, and biofilm-related infections are rarely resolved by host defense mechanisms (MARCINKIEWICZ; STRUS; PASICH, 2013; PASICH et al., 2013). Moreover, the complex structure of biofilms is directly related to antimicrobial resistance, since antimicrobials and antiseptics are often unable to penetrate the structure, resulting in difficulty in treating infections (SELEZSKA et al., 2012).

*Pseudomonas aeruginosa* is among the microorganisms most frequently associated with biofilm formation on medical devices of permanent use, causing hospital infections that persist even after antimicrobial treatment (DAVIES, 2002; GIBSON; BURNS; RAMSEY, 2003; NEVES et al., 2011) Lister et al., 2009). It is one of the most commonly isolated bacteria in clinical laboratories, and is responsible for infections of the respiratory and urinary tract, as well as bloodstream infections (HIRSCH; TAM, 2010). In this context, its essential to investigate new effective alternatives for inhibiting biofilm formation, through strategies that may prevent initial formation or cellular communication, i.e. QS (BOSGELMEZ-TINAZ et al., 2007).

For thousands of years, plants have been used for therapeutic purposes and have therefore become a rich source of research in the discovery of new drugs (BONEZ et al., 2013; DE QUEIROZ et al., 2015; DE SOUZA et al., 2017). In this study, we highlight the plant *Equisetum hyemale*, popularly known as “cavalinha”. The stems of this plant are used to treat several pathologies, such hypertension (diuretic), digestive disorders, renal infections and ocular diseases. High levels of phenolic compounds and caffeic acid derived compounds have already been identified in *E. hyemale*, and the antimicrobial and antibiofilm activity of the crude

extract (CE) and fractions have been demonstrated (DOS SANTOS ALVES et al., 2016; FRANCESCATO et al., 2013; GIERLER; SAPEI; PARIS, 2008). Here, we evaluated the capacity of the CE and fractions of *E. hyemale*, as well as previously identified major components, to inhibit biofilm formation by *P. aeruginosa*, through the inhibition of QS signaling, motility and autoaggregation.

## 2. Methodology

### 2.1. Collection of plant samples

The plant material was collected in Santa Maria (Rio Grande do Sul, Brazil) in the district of São Martinho da Serra (29° 32′ 16″ S, 53° 51′ 18″ W), between September and November 2018. A dried voucher specimen was deposited in the herbarium at the Department of Environmental Engineering, Federal University of Santa Maria (UFSM), under register number SMBD 6756. The compounds luteolin, ferulic acid, coumarin and rutin were commercially obtained from Sigma-Aldrich®.

### 2.1.1 Preparation of plant extracts

The plant stems were dried in an oven (temperature < 40ºC) and chopped using a knife mill. The obtained material was macerated at room temperature with 70% ethanol for one week, with one daily shakeup, and the solvent was renewed after 4 weeks. After filtration, the hydroalcoholic extract was evaporated under reduced pressure in a rotary evaporator to remove the ethanol, thereby obtaining an aqueous extract. Part of the aqueous extract was dried in an oven (temperature < 40°C) to obtain the crude extract (CE). After that, the remaining aqueous extract was partitioned with dichloromethane (DCM), ethyl acetate (EA) and n-butanol (NB), successively (BOLIGON et al., 2014).

Phenolic compounds were previously identified by ultra high-performance chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS). Analysis was performed according to Faccin et al (2016) at the Research Center and Analysis of Contaminant Residues of the UFSM. In the crude extract (CE), the compounds found included luteolin (LT; 3.7 µg/mL) and ferulic acid (FA; 1.7 µg mL), whereas in the ethyl acetate (EA) fraction, coumarin (CM; 2.06 µg/mL) and ferulic acid (FA; 3.8 µg/mL) stood out. The main components of the dichloromethane (DCM) and butanol (NB) fractions were ferulic acid (FA; 3.5 µg/mL

and 1.5 µg/mL, respectively) and rutin (RT; 2.8 µg/mL and 1.7 µg/mL) respectively (FILIPPI et al., 2019).

### 2.3 Preparation of bacterial inoculum

The bacterial inoculum sizes were standardized according to the Clinical and Laboratory Standards Institute (2017). The *P. aeruginosa* PA01 strain was used. Isolated colonies were grown for 18 and 24 h in Mueller-Hinton agar (Himedia, India) and the suspension was prepared in saline solution (0.85%) with the NaCl density adjusted to 0.5 on the McFarland scale ($1.5 \times 10^8$ CFU/mL) (CLSI, 2017).

### 2.4 Determination of the minimum inhibitory concentration

The antibacterial activity of the phenolic compounds was evaluated according to the Clinical and Laboratory Standards Institute (2017) using the broth microdilution method. The assay was carried out in 96-well microtiter plates using Mueller-Hinton broth (Himedia®). Each extract was diluted in dimethyl sulfoxide (DMSO) and gave rise to serial concentrations ranging from 52.4 to 32.7 µg/mL. The major substances were diluted in DMSO and gave rise to serial concentrations ranging from 2 to 0.185 µg/mL (initial concentrations of the tests for the phenolic compounds were established from the equivalent concentrations identified in the extracts) (FILIPPI et al., 2019). The plates were incubated at 37°C and the minimal inhibitory concentration (MIC) was recorded after 24 h of incubation. The MICs were defined as the lowest concentration of extract that inhibited visible bacterial growth. This test was performed in triplicate, and 2,3,5-triphenyltetrazolium chloride was used as an indicator of microbial growth. A positive control growth culture medium and microorganism were used. For the control of sterility, only culture medium was used for negative growth. In the same way, a control of the solvent DMSO was carried out at the same concentration used for the dilution of the samples.

### 2.5 Biofilm inhibition

Bacterial were inoculated in 5 mL of trypticase soy broth (TSB) and grown up to the stationary phase. The cultures were diluted to 1:100 in TSB, and 100 µL of each dilution was pipetted into four wells in a sterile flat-bottomed microtiter plate together whit the extracts (13.1 µg/mL to 0.81 µg/mL) and major substances (0.37µg/mL to 0.18 µg/mL), i.e. at MIC and sub-inhibitory concentrations. After incubation for 24 h at 37°C, all of the planktonic

microorganisms were removed and washed with distilled water three times. Biofilm analysis was performed using 125 µL of 0.1% crystal violet solution (Sigma Chemical®) in each well. The microplates were washed with distilled water and air-dried. To solubilize the biofilm, 200 µL of 95% ethanol was added. The solution was transferred to a new microtiter plate and the biofilm formation was determined by measuring the absorbance at 570 nm in a microplate reader. For the control culture, broth was used for negative control and only *P. aeruginosa PA01* inoculum was used for the positive control (BONEZ et al., 2017).

**2.6 Sessile colony counting**

Biofilm was allowed to form in 96-well plates, then washed twice in PBS to remove loosely attached cells. The biofilm was then resuspended by repeated pipetting. The resuspended biofilm, after treatment with extracts and major substances in sub-inhibitory concentrations, was vigorously vortexed for 5 min to disrupt the biofilm matrix and serial decimal dilutions (in PBS) were plated onto Mueller Hinton agar (Sigma Chemical®). The plates were incubated for 24 h at 37°C, and the number of cultivable biofilm cells was determined by counting colony forming units (CFUs) following biofilm cell resuspension. The total CFUs per unit area (log CFU/mL) of the microtiter plate were enumerated. Experiments were repeated on three occasions with individual samples evaluated in triplicate (SILVA et al., 2010).

**2.5 Coaggregation**

*P. aeruginosa* PA01 and *E. coli* ATCC strain 25922 were grown at 37°C for 24 h in TPY broth. The cells were harvested by centrifugation and suspended in phosphate buffered saline (PBS) (adjusted optical density (O.D.) units at 600 nm). In each tube, 1 mL of *P. aeruginosa* PA01 inoculum and 1 mL of *E. coli* ATCC 25922 inoculum were added, and the major substances and extracts were added in subinhibitory concentrations. The cells were then resuspended in culture supernatant. After incubation at 37°C for 2 h, 1 mL of the upper suspension was transferred to another tube and the O.D. measured. Coaggregation was expressed as (O.D. upper suspension/O.D. total bacterial suspension)/100 (DEL RE et al., 2000).

**2.6 Motility assay**

Swimming, swarming and twitching motilities were analyzed by inoculating 40 µL of bacterial suspension of the *P. aeruginosa PAO1* standard strain into the center of plates containing culture media specific for each type of motility. After 24 h of incubation at 37°C, motility was evaluated by analyzing the circular area formed by bacterial cells migrating away from the inoculation site. The halos formed were measured and subjected to statistical analysis. The motility assay was performed on the culture media without treatment and on the media containing the subinhibitory concentration of extracts and compounds phenolics. The interpretation was performed by observing the variation in the colony growth (in mm) from the inoculation point, comparing the cultures on the plates with and without the compounds and extracts (BONEZ et al., 2017).

**2.7 Quorum sensing inhibition assay**

The inhibition of the quorum sensing was performed based on a protocol described by Burt and collaborators (2014), with some modifications. Was measured the amount of violacein, a violet pigment, produced by *Chromobacterium violaceum* as a result of QS activity. 1 mL of a subinhibitory concentration of the crude extract, fractions and major compounds were added to 2 mL portions of Brain Heart Infusion Broth (BHI) inoculated *C. violaceum* (adjusted to optical density (D.O) 600 nm) and incubated for 24 h. The CFU/mL in the original inoculate were confirmed and the CFU/mL after incubation in the presence of *E. hyemale* was determined by decimal dilution and plating out on agar. From each 24 h sample, a 2 mL portion was centrifuged for 5 min at 13000 g and the supernatant was discarded. Violacein was then extracted from the pellet was resuspended in 150 µL of BHI, to which 150 µL 10% SDS was added to lyse the bacteria. After 5 min at room temperature, 675 µL of water-saturated n-butanol was added, vortexed to dissolve the violacein, and centrifuged 5 min at 13000 g. Supernatants were transferred to a 96-well plate and the OD was measured at 540 nm on a plate reader. A blank reading (sterile BHI) was subtracted from the measurements. The assay was carried out three times.

**2.8 Biofilm observation by AFM**

The three-dimensional analysis of the biofilm structure was performed by AFM. Images of the biofilms formed on f high density polyethylene (HDP) substrates, with and without extracts and compounds of the *E. hyemale* at subinhibitory concentrations, were captured using

an Agilent Technologies 5500 microscope (California, USA). The images (1 mm x 1 mm) were collected in non-contact mode using PPP-NCL tips (Nanosensors, Neuchatel, Switzerland; force constant ¼ 48 N/m) and were analyzed using PicoView software (Keysight Technologies, Sao Paulo, Brazil) (CHATTERJEE et al., 2014b).

## 3 Results and Discussion

In this study, we demonstrated the antibiofilm activity of the extracts and major components present in *E. hyemale* against *P. aeruginosa* PA01, a microorganism of great clinical relevance, mainly because it is capable of forming biofilms. The ability of microorganisms to form biofilms is closely related to increased resistance to antimicrobials, as this complex structure does not allow drug penetration, and consequently infections persist causing high costs to public health, in many cases leading the patient to death (DAVIES, 2002; MARCINKIEWICZ; STRUS; PASICH, 2013; SELEZSKA et al., 2012). Given this, it is of great importance to search for new drugs that are capable of inhibiting the formation of biofilms. In this context, medicinal plants are highlighted as rich sources of research because they produce substances known as secondary metabolites (ALBUQUERQUE et al., 2013; BOLIGON et al., 2013). In the plant, secondary metabolites have the function of protection and reproduction, but they also confer biological activities to plants (SLOBODNÍKOVÁ et al., 2016; WOJNICZ et al., 2012).

To get the extracts, we used the polarity separation technique; thus, each extract has a different chemical composition, which confers different biological activities. (BOLIGON et al., 2013; PIANA et al., 2015). In previous work done by our research group, we identified through the UPLC-ESI-MS system the major components of each of the *E. hyemale* extracts. In the CE, luteolin (LT) (3.7 μg/mL) and ferulic acid (FA) (1.7 μg mL) were identified. Coumarin (CM) (2.06 μg/mL) and ferulic acid (FA) (3.8 μg/mL) were present in the ethyl acetate (EA) fraction. The dichloromethane (DCM) and n-butanol (NB) fractions contained ferulic acid (FA) (3.5 μg/mL and 1.5 μg/mL, respectively) and rutin (RT) (2.8 μg/mL and 1.7 μg/mL, respectively) (FILIPPI et al., 2019). FA, CM, RT and LT are substances that are classified in the group of phenolic compounds, due to the presence of a hydroxyl linked directly to an aromatic hydrocarbon group. Some studies report that phenolic compounds have relevant antimicrobial activity through mechanisms of action such as the inhibition of protein and peptidoglycan production, alterations to the permeability of the internal membrane of the microorganism and

the inhibition of β-lactamase enzymes (BEN YAKOUB et al., 2018; FRANCESCATO et al., 2013; KUMAR; PRUTHI, 2014).

The CE and the fractions were able to inhibit the visible bacterial growth of *P. aeruginosa* PAO1 at low and satisfactory concentrations, with MICs ranging from 26.2 μg/mL to 13.1 μg/mL (Table 1). According to some authors, the ideal MIC for plant extracts should be less than 100 μg/mL (DE QUEIROZ et al., 2015; GIBBONS, 2008). Antimicrobial activity has been attributed to the presence of significant levels of flavonoids found in the same extracts (DE QUEIROZ et al., 2015; FERRAZZANO et al., 2013; GIBBONS, 2008).

The major substances showed MIC values between 0.75 μg/mL and 0.37μg/mL, as shown in Table 1. LT has potent antimicrobial action and can operate through different mechanisms, such as the inhibition of protein and peptidoglycan production, alterations to the permeability of the internal membrane of the microorganism and the inhibition of β-lactamase enzymes (EUMKEB; SIRIWONG; THUMANU, 2012). In the same way, it has been shown that FA has moderate antimicrobial activity against *Citrobacter sakazakii*, according to Shi et al. (2016), thus suggesting that the presence of flavonoids is a determinant of antibacterial activity. Zhu & Jiang (2018), in determining the antimicrobial activity of CM, attributed this to the lipophilic chemical structure of CM, since the presence of hydroxyl groups and the size of the carbon chain facilitate entry into the microbial cell. As for RT, it can potentially inhibit *Escherichia coli*, *P. aeruginosa* and *Bacillus subtilis* (GANESHPURKAR; SALUJA, 2017). A study carried out by Arima et al. (2002) makes it clear that RT acts in synergism with other phenolic compounds, thereby potentiating the antimicrobial activity of phytochemicals.

By assessing biofilm inhibition on polystyrene plates, CE was able to inhibit biofilm formation by 63% and 64% in subinhibitory concentrations (6.55 μg/mL and 3.27 μg/mL, respectively) (Figure 1A). The AE fraction provided 29% inhibition when used at half of the MIC (1.63 μg/mL) (Figure 1B). For the DCM and NB fractions, the inhibition percentages for subinhibitory concentration (13.1 μg/mL) were, respectively, 37% and 63% (Figures 1C and 1D). Although the extracts inhibited biofilm formation, when phenolic compounds were tested separately, there was an increase in inhibition. AF and CM provided 67% and 79% inhibition, respectively at the MIC (0.37 μg/mL) (Figures 1E and 1F). RT inhibited biofilm formation by 78% at the MIC (0.37 μg/mL) and LT inhibited biofilm formation by 75% at 0.37 μg/mL (Figures 1G and 1H).

These results were confirmed by AFM, which allowed us to visualize, through images, the decrease in microbial mass. The three-dimensional images were analyzed in an adhesion profile analysis and compared with the positive control image (PA01 only), where a peak of 6

µm was observed. In Figures 2A and 2B, the inhibition of the biofilm treated with CE and AC, provided peaks of 0.5 and 1.6 µm, respectively. Similarly, DCM and NB showed peaks of 0.3 and 0.8 µm, respectively (Figures 2C and 2D). However, the major substances, AF and CM provided peaks of 1 and 1.2 µm, respectively (Figures 2E and 2F). RT and LT provided peaks of 0.8 and 0.4 µm, respectively (Figures 2G and 2H). Even at subinhibitory concentrations, the tests show low peak values that approximate the value of the negative control, which means low biofilm mass formation, as well as a darker color, covering a range of values, which is also similar to the negative control.

We performed colony counting in order to evaluate the CFU of microorganisms that adhered to polystyrene plates after treatment. It was observed that there was a significant difference in the colony counts of the tests, in relation to the positive control, both for the extracts and for the isolated compounds; the CE, AC, DCM and NB fractions provided the best results (Figure 3A, 3B, 3C and 3D). Likewise, the major substances AF, CM, RT and LT also showed a significant reduction in the colony counts (Figure 3E, 3F, 3G and 3H), thus corroborating the previous tests.

Other studies have already related phenolic compounds to antibacterial activity, such as Shen et al. (2014), where LT was able to significantly inhibit uropathogenic *E. coli* (UPEC) biofilm formation at a concentration of 0.05 µg/mL. The importance of the results obtained in this work rests on the fact that of *P. aeruginosa* can colonize and adhere to the surfaces of instruments and medical implants, causing serious and chronic infections. FA is able to reduce the biofilm mass of Gram-negative bacteria by up to 70% (BORGES; SAAVEDRA; SIMÕES, 2012). The same compound prevented *E. coli* biofilms from forming on urinary catheters at a concentration of 0.5% (PANWAR et al., 2016). A recent study on RT, carried out by Peng et al. (2018), reported its antibiofilm activity in *E. coli* (APEC-O78) at concentrations of 12.25 and 50 µg/mL. Therefore, we can suggest that the presence of these substances in *E. hyemale* is a determinant of its antibiofilm activity and can be used separately for the minimization of biofilm formation.

Biofilm formation depends on several factors to initiate, one of which is the redistribution of cells linked by surface motility, where the contraction of the cells plays a role in surface aggregation. Therefore, we evaluated the inhibition capacity of the three different types of motility presented by PA01: *swarming, swimming* and *twitching*. After analysis of all compounds against bacterial motility, all extracts and compounds were shown to influence motility, but the CE, AC and NB fractions inhibited *swimming*, *swarming* and *twitching* in P. *aeruginosa* strain PAO1 altogether (Figure 4). Swarming motility is a continuous movement in

groups (KEARNS; LOSICK, 2003) in viscous liquids. Unlike swarming, where cells move individually and randomly and only occur when the surface is sufficiently thick. In twitching, cells move predominantly in an isolated manner, although moving as aggregates may occur (COPELAND; WEIBEL, 2009; DUAN et al., 2013; REMIS; COSTERTON; AUER, 2010).

The major substances influenced the three different types of microbial motility (Figure 5). Figure 6A, 6B and 6C demonstrate the significant differences in motility halos compared to the positive control. Therefore, only the extract and fractions were able to totally inhibit microbial motility, which can be explained by synergistic effects between the substances in plant (VAN VUUREN; VILJOEN, 2008). Corroborating our results, some studies evaluated the ability of phenolic compounds to inhibit microbial motility. FA is cited for inhibiting swarming and swimming motility in *L. monocytogenes* and *E. coli* (BORGES et al., 2014b). Culture supplemented with FA inhibit swarming motility in *P. aeruginosa* (UGURLU et al., 2016). Uropathogenic strains of *E. coli*, when tested against LT, showed a decrease in the expression of the (fimbrias) *fimh* type 1 adhesin fimbriae gene and also lower bacterial surface hydrophobicity and *swimming motility* (SHEN et al., 2014).

The biofilm structure may be formed by microorganisms of the same and/or different species. In this way, secondary colonizers begin the process of self-aggregation (genetically the same microorganisms) and coaggregation (genetically different microorganisms), and then the clusters become organized and complex structures (HSU et al., 1990; RICKARD et al., 2002, 2003). This assay allowed us to infer that the CM, FA and RT as well as the AE and NB fractions had a substantial influence on the coaggregation of P. *aeruginosa* PA01 cells, classified as a moderate effect (Table 2). On the other hand, CE, DCM and LT interfered less with coaggregation. Our results demonstrate that the major substances interferes with the coaggregation of *PAO1* and *E. coli*.

All aspects previously evaluated in this study are fundamental to biofilm formation and are controlled by the QS system, a type of cell-to-cell communication that bacteria use to regulate collective behaviors, thereby controlling the production of virulence factors, such as the ability of some bacterial species to form biofilms. This process depends on the production, detection and response of a group of extracellular signaling molecules called autoinducers (AHLs). These molecules are constantly produced and received at the basal level in the bacterial cell, such that when the population density of the bacteria increases, consequently the concentration of AHLs also increase. They diffuse and accumulate in the surrounding environment when they reach a threshold (QS level), then these molecules diffuse back into the microbial cell and induce the expression of a set of target genes that alter the behavior of the

microorganism (PACZKOWSKI et al., 2017a; PÉREZ-PÉREZ et al., 2017; RASMUSSEN et al., 2005; REMIS; COSTERTON; AUER, 2010).

Given this, many studies are looking for a way to inhibit this signaling and consequently prevent the formation of biofilms (BORGES et al., 2014a; O'LOUGHLIN et al., 2013; PACZKOWSKI et al., 2017b). To evaluate QS inhibition, we used the violacein assay, which mediates QS induced by the *CviI* autoinducer synthase; violacein is released to the environment and diffuses back into the bacterium when quorum has been reached. The autoinducer then binds to the transcriptional regulator *CviR* and participates in the expression of specific genes such as violacein produced by *C. violaceum*, a Gram-negative soilborne bacterium that resides in the tropical and subtropical areas (BURT et al., 2014; RIVERA et al., 2019; VASAVI et al., 2017). In this study, both extracts of the plant and their major compounds were able to significantly reduce the production of violacein at subinhibitory concentrations. The compound CE (Figure 7A) and LT (Figure 7C), which reduced more than half of the production of violacein. The cell viability curves performed from aliquots of *C. violaceum* cultures in contact with subinhibitory concentrations of extracts and compounds show that the microorganism presents logarithmic growth in 24 h due to the ability of the compounds and extracts to inhibit QS, and due to antimicrobial activity itself (Figure 7B and 7D).

Coumarin is described as inhibiting QS signaling (KOT et al., 2015; SLOBODNÍKOVÁ et al., 2016; ZHANG et al., 2017). Reen et al. (2015) showed that, in the presence of increasing coumarin concentrations, there was a significant reduction in pigments produced by QS signaling by *S. marcescens* SP15, *C. violaceum* DSM 30191 and *A. tumefaciens* NTL4 strains. In addition, they performed biosensor signal competition assays, where they described coumarin-specific interference with AHL. Given the clinical importance, these researchers observed that CM decreases QS-related gene expression in *P. aeruginosa* (PA14 strain) and also inhibits biofilm formation and the motility of this pathogen. RT interferes with QS by reducing the secretion of autoinducers, thereby reducing the production of *E.coli* virulence genes (APEC) (PENG et al., 2018). In addition, the administration of phenolic compounds to *P. aeruginosa* alters the transcription of QS-controlled target promoters and suppresses virulence factor production, confirming its potential anti-QS activity (PACZKOWSKI et al., 2017a).

In a recent study by our research group, it was observed that both extracts and isolated substances do not present cytotoxicity or genotoxicity against human mononuclear blood cells, suggesting that they offer safety in the possible use of the plant for therapeutic purposes (Santos et al. 2019). In the present study, we showed that the phenolic compounds present in the plant

are mainly responsible for antibiofilm activity. Given this, we can conclude that *E. hyemale* represents a new alternative for the mitigation of damage caused by biofilms, as it was able to inhibit important factors that are involved in the process of microbial mass formation.

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**Table 1**. Minimal Inhibitory Concentrations (MIC) (µg/mL) of extracts and majority substances of *E. hyemale*.

| | MIC *P.aeruginosa PA01*(µg/ml) |
|---|---|
| Crude extract | 13.1 |
| Acetate fraction | 3.27 |
| Dichloromethane fraction | 26.2 |
| Butanol Fraction | 26.2 |
| Feluric acid | 0.75 |
| Routin | 0.75 |
| Coumarin | 0.75 |
| Luteolin | 0.37 |

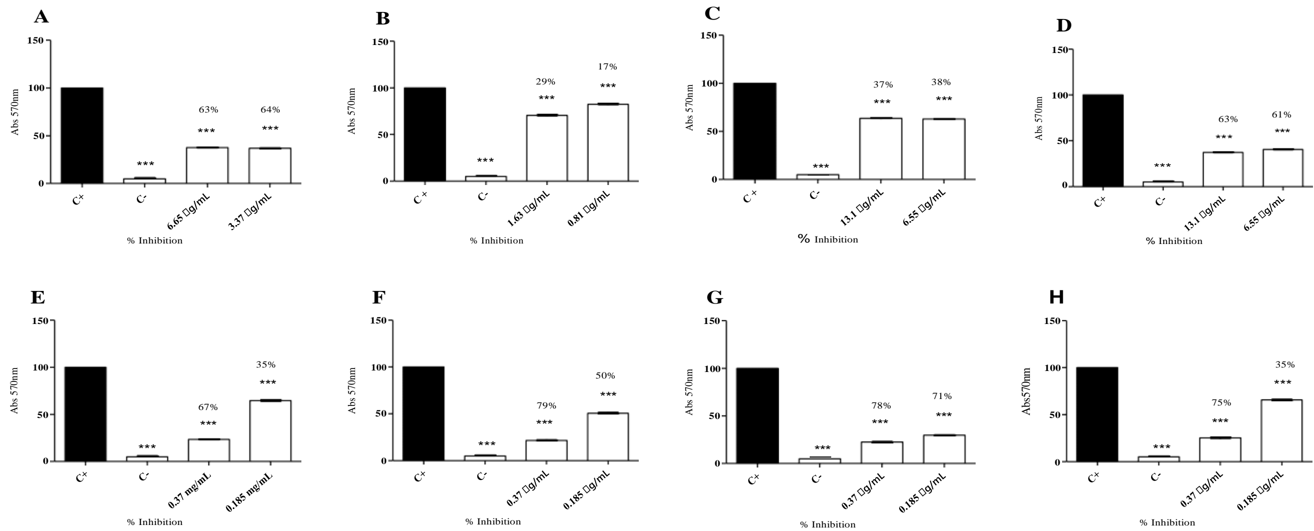


**Fig 1**. Percentage of PAO1 biofilm inhibition against crude extract (A), acetate fraction (B), dichloromethane fraction (C), butanol fraction (D), ferulic acid (E), Coumarin (F), Routin (G), Luteolin (H). In sub-inhibitory concentration.

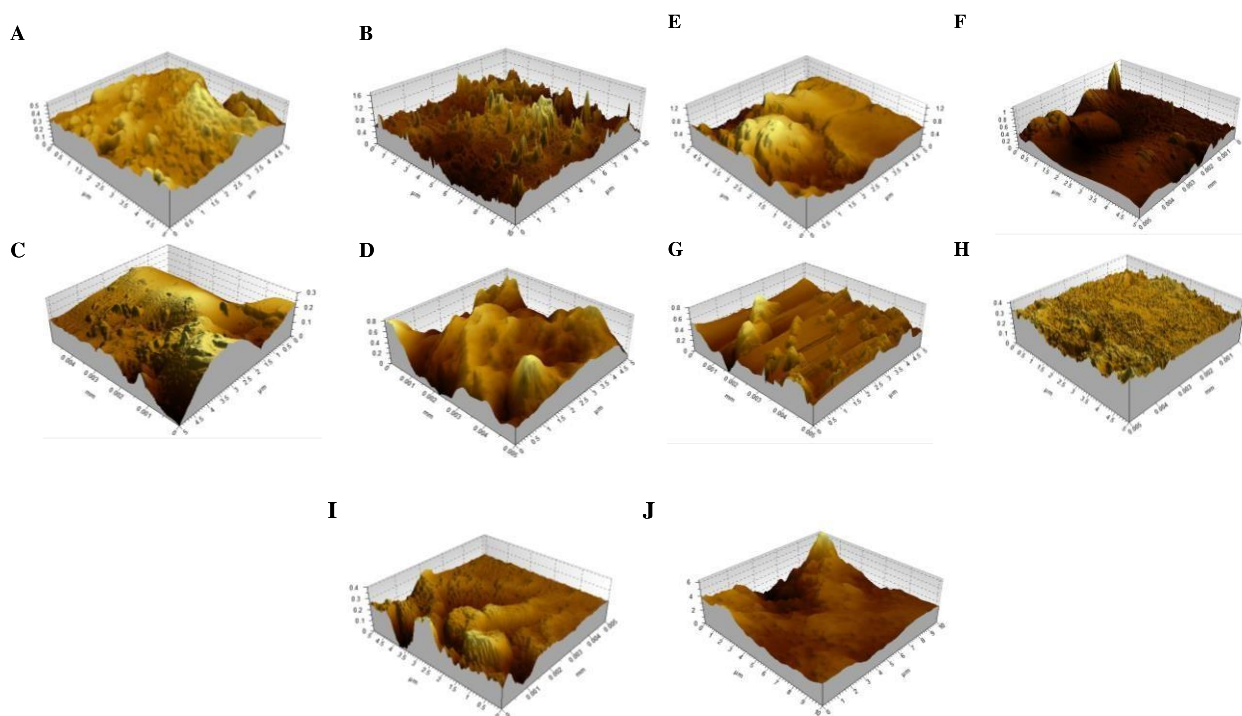


**Fig 2.** AFM of that inhibition of biofilm formation crude extract (A), acetate fraction (B), dichloromethane (C) fraction, butanol (D) fraction, ferulic acid (E), coumarin (F), Routin (G) and Luteolin negative control (I), and positive control (J). Subinhibitory concentrations were used for the test.

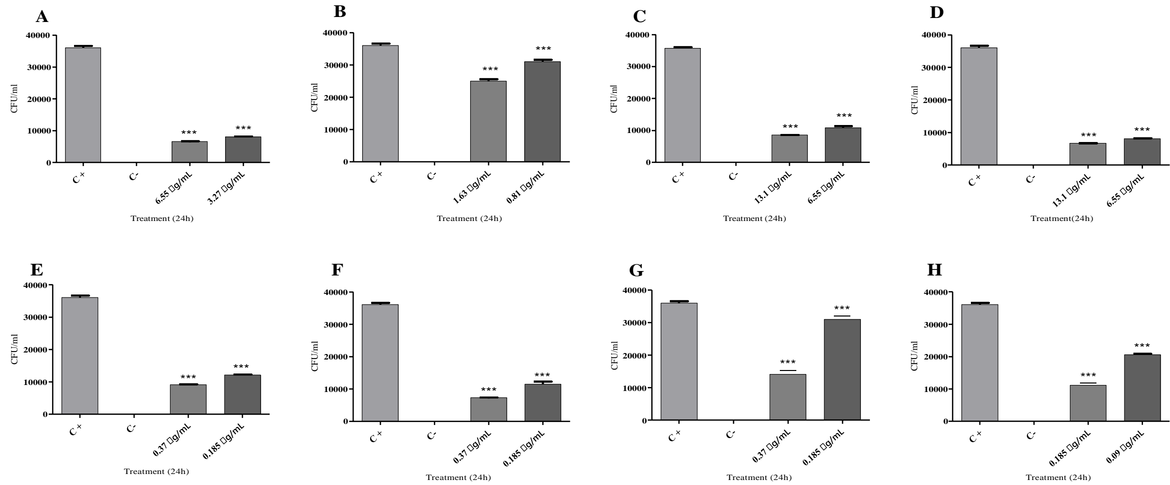


**Fig 3.** Sessile colonies of *P. aeruginosa* PA01 against crude extract (A), acetate fraction (B), dichloromethane fraction (C), butanol fraction (D), ferulic acid (E), coumarin (F) routin (G), and luteolin (H). Were used one-half (1/2) and one-quarter (1/4) of the minimal inhibitory concentrations (all subinhibitory concentrations). O que signofica *** $p < 0.05$.

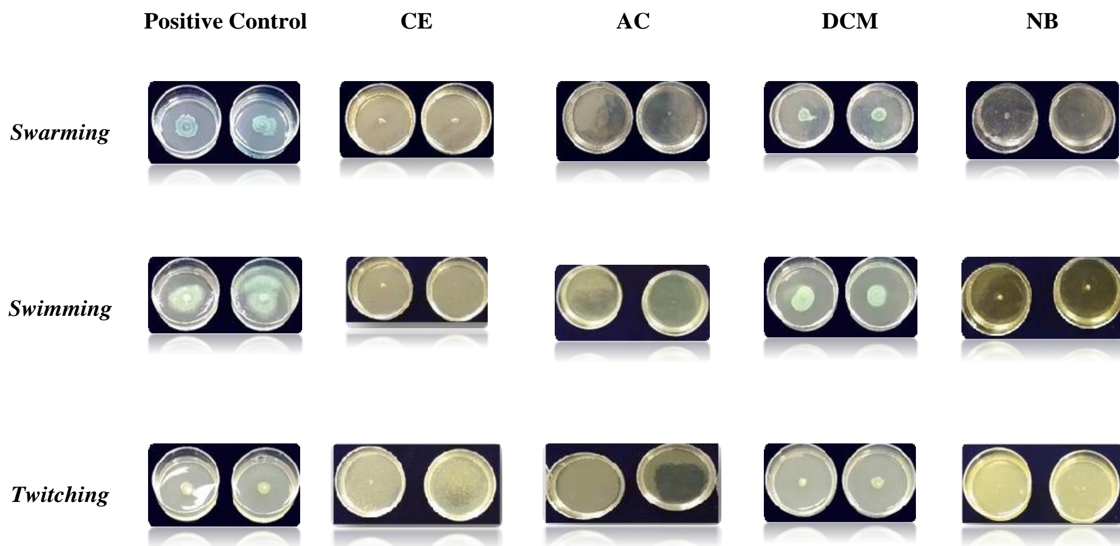


**Fig 4**. Figures of the *Swimming*, *swarming* and *twitching* motility of *P. aeruginosa* PAO

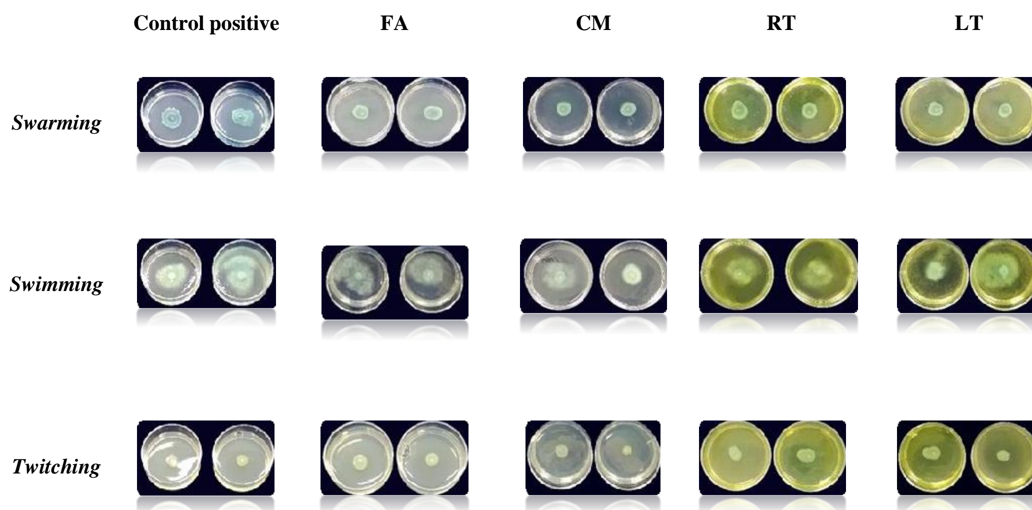


**Fig 5.** Figures of the Swimming, swarming and twitching motility of. *P. aeruginosa* PAO1.

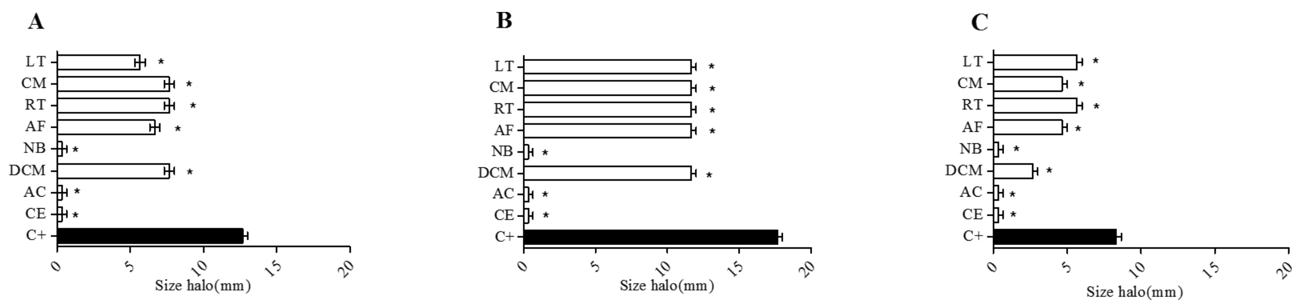


**Fig 6.** Graphic *Swimming* (A), *swarming*(B) and *twitching* (C) motility of *P. aeruginosa* PAO1 without extract crude, fractions and majority substance in subinhibitory concentrations. Growth halos were measured and subjected to the statistical t-test.

**Table 2**. Co-aggregation profile of *P. aeruginosa* PA01with *E.coli* ATCC 25922 (positive control), in the presence of crude extracts, fractions and major compounds of *E. hyemale*. Coaggregation was expressed as (O.D. upper suspension/O.D. total bacterial suspension)/100. Where percentages ≥ 70% are classified as high coaggregation, < 70% ≥ 20% mean aggregation and < 20% low coaggregation.

| | **High** | **Average** | **Low** |
|---|---|---|---|
| **Positive Control** | 140% | - | - |
| **CE** | 81,28% | - | - |
| **EA** | | 68,75% | - |
| **DCM** | 71,42% | - | - |
| **NB** | - | 68,42% | - |
| **FA** | - | 63,95% | - |
| **CM** | - | 63,90% | - |
| **RT** | - | 64,45% | - |
| **LT** | 100% | - | - |

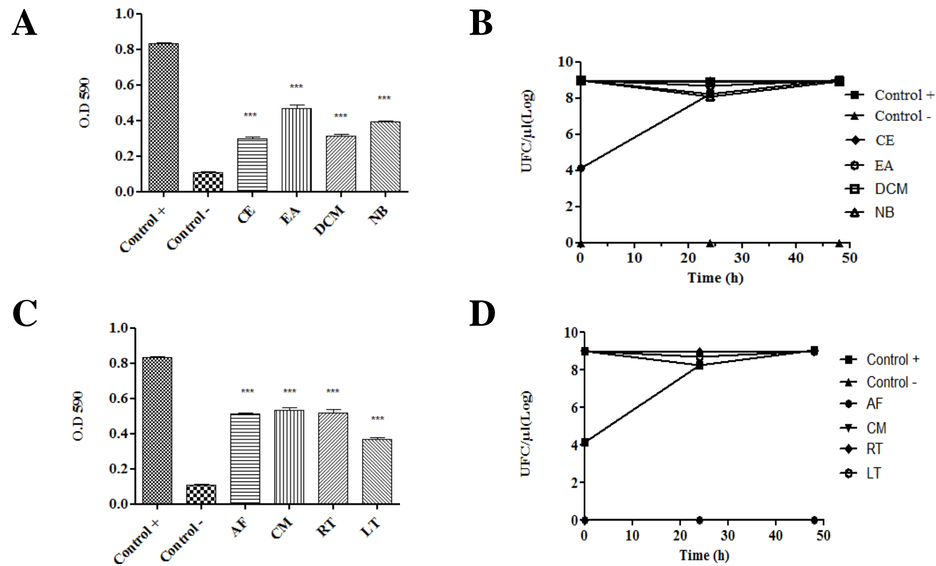


**Fig 7.** (A) Inhibition of violacein production by crude extract and fractions of the *E.hyemale* (measured as OD 590). (B) Cell viability of *C. violaceum* (log cfu / µl) against crude extract and fractions of the *E.hyemale* . (C) Inhibition of violacein, by majority substance of *E. hyemale* (measured as OD 590) (C) Cell viability of *C. violaceum* (log cfu / µl) (D). They were considered statistically significant when $p < 0.05$.

## 4 DISCUSSÃO

A arquitetura dos biofilmes e suas características fisiológicas atribuem a essas estruturas, resistência antimicrobiana até 1000 vezes maior do em células planctônicas. Constantemente, milhões de pacientes são afetados por infecções hospitalares relacionadas à formação de biofilmes, em todo o mundo (WALKER; MOORE, 2014). O resultado disso, são elevadas taxas de morbidade e mortalidade, bem como, o encarecimento dos tratamentos no âmbito da saúde pública. Especula-se, que não exista um material que não possa ser colonizado por microrganismo, em muitos casos os biofilmes se instalam em dispositivos médicos (cateteres, próteses, tubos de ventilação mecânica, matérias cirúrgicas, entre outros) (COSTERTON; VEEH, 2003). Além disso, existe a preocupação acerca da colonização bacteriana em tecidos vivos, como por exemplo, biofilmes formados no pulmão relacionado com a piora de pacientes portadores de fibrose cística. Bem como, a presença dessas estruturas em feridas, que resultam em atraso cicatricial e complicações no tratamento (WALKER; MOORE, 2014)

A resistência dos biofilmes frente aos antimicrobianos convencionais, envolvem vários fatores. Dentre eles, são citados - acelerado crescimento celular, presença da camada de SPE (dificulta a entrada dos fármacos no interior do biofilme) e troca de informações genéticas incluindo genes de resistência. Além disso, a terapia antimicrobiana é baseada nos testes de susceptibilidade realizados em bactérias planctônicas, que demonstram comportamento distinto daquelas que compõe o biofilme (sésseis), com isso ocorrem falhas no tratamento e recorrências de infecções (BONEZ et al., 2013; STICKLER, 2008).

Diversos microrganismos possuem a capacidade de formar biofilmes, incluindo bactérias Gram negativas, Gram positivas, fungos e leveduras, entre outros. Porém as espécies que são citadas com mais frequência são – *S. epidermidis, S. aureus, E. coli, K. pneumoniae, P. aeruginosa, C. albicans e C. parapisilosis* (PASICH et al., 2013). Neste estudo, utilizamos como cepa padrão a *P. aeruginosa,* bactéria Gram negativa, bastante reportada por formar biofilmes em dispositivos médicos hospitalares. É o agente etiológico em grande parte dos casos de infecções nosocomiais crônicas (pneumonias, sepse e infecções pós cirúrgicos) (GIBSON; BURNS; RAMSEY, 2003; VASAVI et al., 2017).

Diante de toda a problemática que envolve a formação de biofilmes, pesquisadores buscam alternativas que impeçam e/ou destruam essas estruturas. O foco de muitos estudos é bloquear mecanismos determinantes no processo de adesão microbiana(ALBUQUERQUE et al., 2013; DE SOUZA et al., 2017; SAMPATHKUMAR et al., 2019; WILMS et al., 2005; WOJNICZ et al., 2012). Os fatores de virulência que conferem aos microrganismos capacidade

de formação de biofilme, são todos orquestrados por uma cascata de sinalização química, denominado *quorum sensing* (QS). São essas sinas que determinam, por exemplo, a expressão de genes que formam os flagelos e por consequência a motilidade microbiana. Além disso, o QS também determina a formação da camada de SPE e o recrutamento de células planctônicas (NADAL JIMENEZ et al., 2012; REMIS; COSTERTON; AUER, 2010).

As alternativas mais estudas acerca de mitigação dos efeitos da formação do biofilme, são as plantas e seus derivados (BOLIGON et al., 2013; REEN et al., 2018; SAMPATHKUMAR et al., 2019). Neste estudo, destacamos *E. hyemale*, planta popularmente utilizada como diurético e tratamento de infecções urinárias. Alguns estudos, relatam a presença de altos teores de metabólitos secundários no gênero *Equisetum.* Porém, poucos estudos avaliaram as atividades antimicrobiana, antibiofime e toxicológicas da espécie *hyemale* (DE OLIVEIRA et al., 2013; FRANCESCATO et al., 2013; MELLO; BUDEL, 2013). Diante disso, em nosso trabalho identificamos a presença dos compostos majoritários de *E. hyemale* (através do sistema UPLC-ESI-MS). No extrato bruto foram reconhecidos a luteolina e ácido ferúlico. Na fração acetato de etila observou-se a presença de cumarina e ácido ferúlico novamente. Já nas frações diclorometano e N-butanol além do ácido ferúlico, constatamos a presença da rutina.

Esses metabólitos secundários, fazem parte do grupo de compostos fenólicos e suas atividades antimicrobianas são frequentemente relatadas na literatura. A luteolina possui potente ação antimicrobiana e pode operar por diferentes mecanismos como por exemplo - a inibição da produção de proteínas e peptidoglicano e alteração da permeabilidade da membrana interna do microrganismo (EUMKEB; SIRIWONG; THUMANU, 2012; RIVERA et al., 2019). Alguns estudos relatam que o ácido fenólico apresenta moderada atividade antimicrobiana frente a células planctónicas, porém, a atividade antibiofilme é bastante significativa(BEN YAKOUB et al., 2018; HAJJI et al., 2010). A ação antimicrobiana da cumarina, é atribuída à sua estrutura química lipofílica, uma vez que a presença de grupos hidroxila e o tamanho da cadeia carbônica facilitam sua entrada na célula microbiana. Um estudo deixou claro que a rutina atua em sinergia com outras substâncias fenólicas. compostos, potencializando a atividade antimicrobiana de fitoquímicos (GANESHPURKAR; SALUJA, 2017; LOU et al., 2015)

Os resultados obtidos em nossa pesquisa corroboram com a literatura, uma vez que demonstramos que as principais componentes (isolados) da *E. hyemale* apresentam significativa atividade antimicrobiana frente a *S. epidermidis* ATCC 35985, *S. saprophyticus* ATCC 15305, *S. aureus* ATCC 33691, *MRSA-IC* (isolado clínico de *S. aureus* resistente à meticilina), *E. coli*

ATCC 25922, *S. enteritidis* ATCC 13076, *S. flexineri* ATCC 12022323ª, *P. aeruginosa* ATCC 27853, *P. aeruginosa* PA01, *K. pneumoniae* ATCC 1705, *E. faecalis* ATCC 29212 e *L. monocytogenes* ATCC 7644. As concentrações ajustadas nos valores identificados no extrato bruto e frações. Uma precedente pesquisa realidade pelo nosso grupo demonstrou que o extrato bruto e frações da planta apresentaram atividade antimicrobiana frente aos mesmos microrganismos supracitados. Com isso, podemos sugerir que ocorre sinergismo entre os metabólitos, potencializando a atividade biológicas dos fitoquímicos (DOS SANTOS ALVES et al., 2016).

A inibição da formação do biofilme ocorre através do impedimento da adesão microbiana a uma superfície ou então por bloqueio de sinalizações celulares. Atualmente, estudos que envolvem biofilme, seguem, principalmente por dois caminhos, o revestimento de superfícies com agentes inibidores de biofilme ou/e bloqueadores de QS (BONEZ et al., 2013, 2017; CHATTERJEE et al., 2014b). Diante disso, nós avaliamos a capacidade dos extratos e compostos fenólicos em inibir a formação de biofilme de PAO1 (*in vitro*) em placas de poliestireno. O EB foi capaz de inibir a formação de biofilme em 63% e 64% (6,55 µg/mL e 3,27 µg/mL, respectivamente). A fração AE forneceu 29% de inibição (1,63 µg/mL). Para as frações DCM e NB, as porcentagens de inibição foram, respectivamente, 37% e 63% (13,1 µg/mL). Posteriormente, os compostos fenólicos foram testados separadamente, houve um aumento na porcentagem de inibição. AF e CM forneceram 67% e 79% de inibição, respectivamente (0,37 µg/mL). A RT inibiu a formação de biofilme em 78% (0,37 µg/mL) e a LT inibiu a formação de biofilme em 75% a 0,37 µg / mL.

Esses resultados foram confirmados através de microscopia de força atômica (MFA), que permitiu visualizar, através de imagens, a diminuição da massa microbiana. As imagens tridimensionais foram analisadas em uma análise de perfil de adesão e comparadas com a imagem de controle positivo (somente PA01). Realizamos contagem de colônias para avaliar a UFC de microrganismos que aderiram às placas de poliestireno após o tratamento. Observou-se que houve uma diminuição significativa nas contagens de colônias dos testes, em relação ao controle positivo, as frações EB, AC, DCM e NB apresentaram os melhores resultados. Da mesma forma, as principais substâncias AF, CM, RT e LT também apresentaram uma redução significativa na contagem de colônias, o que confirma os resultados da inibição do biofilme.

Outros estudos já relacionaram compostos fenólicos à atividade antibiofilme, como Shen e colaboradores (2014), onde o LT foi capaz de inibir significativamente a formação de biofilme de *E. coli* uropatogênico (UPEC) a uma concentração de 0,05 µg / mL. A importância dos resultados obtidos neste trabalho baseia-se no fato de *P. aeruginosa* poder colonizar e aderir

à superfície de instrumentos e implantes médicos, causando infecções graves e crônicas. A FA é capaz de reduzir a massa de biofilme de bactérias Gram-negativas em até 70% (BORGES; SAAVEDRA; SIMÕES, 2012). O mesmo composto impediu a formação de biofilmes de *E. coli* em cateteres urinários a uma concentração de 0,5% (PANWAR et al., 2016). Um estudo recente sobre TR, realizado por Peng e colaboradores (2018), relataram sua atividade antibiofilme em *E. coli* (APEC-O78) em concentrações de 12,25 e 50 µg/mL. Portanto, podemos sugerir que a presença dessas substâncias em *E. hyemale* é um determinante de sua atividade antibiofilme e pode ser usada separadamente para minimizar a formação de biofilme.

Tendo em vista que um dos fatores cruciais para a formação do biofilme é a motilidade, onde a contração das células desempenha um papel na agregação da superfície. Bactérias que possuem conseguem se locomover através de pilis e flagelos, facilmente colonizam as superfícies. Além disso, facilitam os processos de auto-agregação, co-agreção e recolonização (DUAN et al., 2013). A *P. aeruginosa* consegue desenvolver três diferentes tipos de motilidade (*Swimming*, *swarming* e *twitching*) (SHROUT et al., 2006). Neste contexto, a expressão de genes que codificam pili e flagelos, é essencial, por outro lado, a supressão dos mesmos pode influenciar na motilidade e consequentemente no processo de adesão. Em nossos resultados, todos os extratos e compostos demonstraram influenciar a motilidade, apenas as frações CE, AC e NB inibiram totalmente as três formas de motilidade da *P. aeruginosa PA01.*

Fundamentando nossos resultados, alguns estudos avaliaram a capacidade de compostos fenólicos em inibir a motilidade microbiana. A cultura suplementada com ácido ferulico inibiu a motilidade do tipo *swarming* em *P. aeruginosa* (UGURLU et al., 2016). Além disso, o ácido ferulico foi citado por inibir a mobilidade dos tipos *swarming* e *swimming* de *L. monocytogenes* e *E. coli* (BORGES; SAAVEDRA; SIMÕES, 2012) . As cepas uropatogênicas de *E. coli*, quando testadas contra a luteolina, mostraram uma diminuição na expressão do gene da *adesina fimbriae* (fimbrias) *fimh* tipo 1 e também menor hidrofobicidade da superfície bacteriana e motilidade *swimming* (SHEN et al., 2014).

A estrutura do biofilme pode ser formada por microrganismos da mesma e / ou espécies diferentes. Dessa maneira, os colonizadores secundários iniciam o processo de auto-agregação (geneticamente os mesmos microrganismos) e co-agregação (microrganismos geneticamente diferentes), e então os agrupamentos se tornam estruturas organizadas e complexas(HSU et al., 1990; RICKARD et al., 2002, 2003). Esse ensaio permitiu inferir que as frações CM, FA e RT, bem como as frações AE e NB, tiveram uma influência substancial na co-agregação das células *de P. aeruginosa PA01*, classificadas como efeito moderado. Por outro lado, CE, DCM e LT interferiram menos na co-agregação.

Todos os aspectos previamente avaliados neste estudo são fundamentais para a formação de biofilme e são controlados pelo sistema QS, um tipo de comunicação célula a célula que as bactérias utilizam para regular comportamentos coletivos, controlando a produção de fatores de virulência, como a capacidade de algumas espécies bacterianas para formar biofilmes. Esse processo depende da produção, detecção e resposta de um grupo de moléculas de sinalização extracelular chamadas auto-indutores (AHLs) (NADAL JIMENEZ et al., 2012; RASMUSSEN et al., 2005; SINGH et al., 2017).

Essas moléculas são constantemente produzidas e recebidas no nível basal da célula bacteriana, de modo que, quando a densidade populacional da bactéria aumenta, consequentemente a concentração de AHLs também aumenta. Eles se difundem e se acumulam no ambiente circundante quando atingem um limiar (nível de QS), então essas moléculas retornam para a célula microbiana e induzem a expressão de um conjunto de genes-alvo que alteram o comportamento do microrganismo (PACZKOWSKI et al., 2017b; PÉREZ-PÉREZ et al., 2017; REMIS; COSTERTON; AUER, 2010).

Para avaliar a inibição da QS, utilizamos o ensaio de violaceína, que medeia a QS induzida pela auto-indução da sintase *CviI*. O auto-indutor se liga ao regulador transcricional *CviR* e participa da expressão de genes específicos, como a violaceína, produzida por *C. violaceum* (uma bactéria Gram-negativa transmitida pelo solo que reside nas áreas tropicais e subtropicais). A violaceína é liberada no ambiente e difunde-se de volta à bactéria quando o QS é atingido (BURT et al., 2014; RIVERA et al., 2019; VASAVI et al., 2017). Neste estudo, os extratos da planta e seus principais compostos foram capazes de reduzir significativamente a produção de violaceína em concentrações sub-inibitórias. O composto EB e LT, que reduziram mais da metade da produção de violaceína. As curvas de viabilidade celular realizadas a partir de alíquotas de culturas de *C. violaceum* em contato com concentrações sub-inibitórias de extratos e compostos mostram que o microrganismo apresenta crescimento logarítmico em 24 h demonstrando que a interferência ocorre somente na sinalização QS.

Ainda neste contexto, a cumarina é descrita como inibidora da sinalização QS. Reen e colaboradores (2015) mostraram que na presença de concentrações crescentes de cumarina, houve uma redução significativa nos pigmentos produzidos pela sinalização QS nas cepas de *S. marcescens* SP15, *C. violaceum* DSM 30191 e *A. tumefaciens* NTL4. Além disso, eles realizaram ensaios de competição de sinal de biossensor, onde descreveram a interferência específica da cumarina na AHL. Dada a importância clínica, esses pesquisadores observaram que o CM diminui a expressão gênica relacionada a QS em *P. aeruginosa* (cepa PA14) e

também inibe a formação de biofilme e a motilidade desse patógeno. A RT interfere no QS, reduzindo a secreção de auto-indutores, reduzindo assim a produção dos genes de virulência de *E.coli* (APEC) (PENG et al., 2018). Além disso, a administração de compostos fenólicos a *P. aeruginosa* altera a transcrição de promotores-alvo controlados por QS e suprime a produção de fatores de virulência, confirmando seu potencial anti-QS (PACZKOWSKI et al., 2017a).

No presente estudo, mostramos que os compostos fenólicos identificados na planta são os principais responsáveis pela atividade do antibiofilme. Apesar, de apresentarem significativos efeitos biológicos, poucos estudos avaliaram as atividades toxicológicas dos extratos e compostos fenólicos presentes na espécie E. *hyemale*. Extratos hidro-alcoólicos de outras cinco espécies de *Equisetum* (*E. arvense, E. sylvaticum, E. fluviatile, E. palustre e E. telmateia*) foram avaliados quanto à sua genotoxicidade, os resultados mostraram que altas concentrações induzem danos celulares (MILOVANOVI; RADULOVI; TODOROVI, 2007). No entanto, outro estudo observou que o extrato do gênero *Equisetum* não induziu toxicidade aguda em *Drosophila* ou em linfócitos humanos em cultura, em baixas concentrações (QUEVEDO; HORACIO; CASTA, 2007). Nossos resultados, observamos que extratos e substâncias isoladas não apresentam citotoxicidade ou genotoxicidade frente a células sanguíneas mononucleares humanas, sugerindo que elas oferecem segurança no possível uso da planta para fins terapêuticos.

Por fim, diante de todo o conjunto de questões acerca da formação de biofilmes, e considerando a ampla utilização de produtos naturais ou derivados como opção terapêutica, podemos sugerir que o estudo da *E. hyemale* representa um novo caminho a ser explorado com a finalidade de mitigar os danos causados por biofilmes. Pois, apesar de não elucidar os mecanismos de ação, a planta e seus compostos majoritários foram capazes de inibir e/ou bloquear fatores importantes envolvidos no processo de formação de massa microbiana.

# 5 CONCLUSÕES

Fundamentando os objetivos propostos neste estudo e os resultados experimentais obtidos, podemos concluir que:

- ✓ Os compostos fenólicos rutina, ácido ferúlico, luteolina e cumarina foram identificados como majoritários no extrato bruto e frações da *E.hyemale* ;

- ✓ Os compostos fenólicos desmontaram atividade antimicrobiana frente as células planctónicas de *E. faecalis* (ATCC 29212), *S. epidermidis* (ATCC 35985), *S. saprophyticus* (ATCC 15305), *S. aureus* (ATCC 33691), MRSA-IC (clinical isolate of methicillin-resistant *Staphylococcus aureus*), *L. monocytogenes* (ATCC 7644), *E. coli* (ATCC 25922), *S. enteritidis* (ATCC13076), *S. flexineri* (ATCC 12022323), *P. aeruginosa* (ATCC 27853), *P. aeruginosa* PA01, and *K. pneumoniae* (ATCC 1705);

- ✓ O extrato bruto, frações e compostos majoritários exibiram efeito inibitório acerca da formação de biofilme de PAO1 em placas de poliestireno;

- ✓ A inibição do biofilme, pode ser observado nas imagens obtidas através da MFA (Microscopia de Força Atômica);

- ✓ O extrato bruto, frações e compostos majoritários, proporcionaram a diminuição da contagem de colônias sésseis do biofilme formado por PAO1 em placas de poliestireno;

- ✓ O processo de co-agregação da PAO1 e *E. coli*, sofreu influência do extrato bruto, frações e compostos majoritários;

- ✓ A motilidade da PAO1 foi afetada e/ou inibida pelo extrato bruto, frações e compostos majoritários;

- ✓ A sinalização QS em *C. violaceum* foi inibida extrato bruto, frações e compostos majoritários;

- ✓ O extrato bruto, frações e compostos majoritários não causaram danos em células sanguíneas mononucleares em concentrações ajustadas ao CIM.

## 6 PERSPECTIVAS

A capacidade dos microrganismos de formar biofilmes é um preocupante problema de saúde pública, pois as bactérias na forma séssil tornaram-se muito mais resistentes aos fármacos. Com isso ocorre o aumento da morbidade e mortalidade de pacientes acometidos por esse tipo de infecção. Por esta razão, torna-se de extrema importância a busca por novas alternativas efetivas de tratamento.

Tendo em vista que as plantas são potentes fontes de compostos bioativos, o presente trabalho concentrou-se, até o momento, em identificar os compostos bioativos presentes na *Equisetum Hyemale*, bem como demonstrou a efetividade desses compostos em inibir a formação de biofilme de PA01 *in vitro* e influenciar em fatores essenciais para esse processo como inibição de motilidade e inibição de co-agregação. Além disso, a capacidade dos compostos e extratos da planta em inibir o *quorum sensing* foi avaliado, assim como ficaram evidenciados os seus baixos efeitos cito e genotóxicos frente às células sanguíneas mononucleares.

Considerando que diversos microrganismos possuem a capacidade de formar biofilmes, torna-se importante mais testes que explorem a atividade antibiofilme dos compostos e extratos, também frente a outros microrganismos, como por exemplo as *Micobactérias*. A micobacteriose é um tipo de infecção causada por micobactérias de crescimento rápido (RGM), que pode variar desde doenças localizadas, como doenças de pele até doenças mais disseminadas. No entanto, o tratamento disponível não funciona nesses pacientes, uma vez que algumas cepas clínicas apresentam capacidade de formar biofilmes e assim adquirem resistência aos antimicrobianos existentes.

Além disso, apesar de os resultados obtidos neste estudo se demonstrarem promissores, eles permitem apenas um entendimento preliminar acerca das ações da planta e seus compostos bioativos sobre a inibição de biofilme. Diante disso, são necessários estudos mais aprofundados que objetivem elucidar os mecanismos de ação da planta frente os biofilmes e conhecimentos acerca da diversidade genética dos microrganismos para que possa se conhecer seus potenciais patogênicos e assim estabelecer alternativas terapêuticas.

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# ANEXOS

## ANEXO A - CARTA DE ACEITE DO ARTIGO 1 NA REVISTA *PHARMACOGNOSY JOURNAL*

**Pharmacognosy Journal**

An Open Access, Peer Reviewed Journal in the field of Pharmacognosy

**ACCEPTANCE OF MANUSCRIPT**

Title: **Antimicrobial, Cyto and Genotoxic Activities of *Equisetum hyemale***

**Corresponding Author(s): Camilla Filippi dos Santos Alves, Pauline Cordenonsi Bonez, Marcia de Souza Ebling, Camila Casagrande, Litiane Freitas, Carolina Dolwitsch, Fernanda Pires, Michele Rorato Sagrillo, Gerson Fernandes de Brum, Marli Matiko Anraku de Campos, Roberto Christ Vianna Santos**

Dear Respected Author,

I am pleased to inform you that Editors and Reviewer have given valuable time for your article and recommended your manuscript as **"Accepted" for publication** in the Volume 11 of Pharmacognosy Journal ISSN: 0975-3575.

Thank you for your valuable contribution with us. We hope to have your next quality publication(s) with us.

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