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TRIAGEM ETNO-DIRIGIDA DE PLANTAS MEDICINAIS DA CAATINGA BRASILEIRA CONTRA BIOFILMES DE BACTÉRIAS PATOGÊNICAS

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Trabalho de Conclusão de Curso na forma de artigo científico a ser submetido à revista Pharmaceutical Biology como requisito para obtenção de Grau de Bacharel em Ciências Biológicas pela Universidade Federal do Rio Grande do Sul

Orientador: Prof. Alexandre José Macedo

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Apresentação

Optou-se por apresentar este trabalho em forma de artigo cientifico, conforme possibilitado pela Decisão 02/2013 da Comissão de Graduação em Ciências Biológicas (http://www.ufrgs.br/comgradbio/index.php). O presente trabalho será submetido na forma de Short Communication à revista Pharmaceutical Biology, sendo que obedece aos padrões de apresentação exigidos pela mesma, devidamente anexados ao final do artigo. No entanto, decidiu-se por apresentar as figuras e tabelas no decorrer do texto devido à praticidade de leitura e correção pelos membros da Banca Examinadora. Algumas figuras apresentam a formatação original em inglês.

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Palavras-chaves: antibiofilme, antibiótico, plantas medicinais, citotoxicidade, Caatinga, etnofarmacologia.

Sumário

Contexto: As comunidades locais que habitam a região da Caatinga brasileira possuem um conjunto significativo de conhecimento tradicional e um número considerável de plantas medicinais usadas para curar várias doenças.

Objetivos: Este estudo teve como objetivo rastrear 23 extratos vegetais aquosos contra dois modelos bem conhecidos e estudados de formação de biofilme de bactérias: *Staphylococcus epidermidis* e *Pseudomonas aeruginosa*.

Materiais e métodos: Para avaliar o efeito de extratos na formação de biofilme, utilizou-se o ensaio de cristal violeta e imagens de Microscopia Eletrônica de Varredura, assim como medições da absorbância a 600 nm para avaliar o crescimento bacteriano. Os extratos que apresentaram atividades promissoras foram investigados quanto à sua citotoxicidade por MTT e o seu perfil fitoquímico qualitativo foi avaliado por CCD.

Resultados: Dez extratos apresentaram atividade importante contra o biofilme de *S. epidermidis*, enquanto apenas 3 extratos reduziram o crescimento bacteriano. Em relação a *P. aeruginosa*, os extratos exibiram um perfil diferente: 3 extratos inibiram 100 % do crescimento bacteriano, prevenindo a formação de biofilme por meio da inibição de crescimento bacteriano. As imagens de MEV confirmam que a adesão bacteriana e a estrutura do biofilme foram fortemente inibidas. Além disso, foram encontradas respostas diferentes para viabilidade celular. A análise fitoquímica preliminar dos extratos mais ativos revelaram a presença de flavonóides, terpenóides, esteróides, aminas e polifenóis.

Discussão e conclusão: Este trabalho indica que os frutos de *Apuleia leiocarpa* e *Poincianella pyramidalis*, bem como folhas de *Harpochilus neesianus* possuem limitada citotoxicidade in vitro, e têm um alto potencial como fonte de protótipos no desenvolvimento de drogas antibiofilme.

1.0 Introdução

Há muito tempo, as plantas vêm sendo utilizadas historicamente como agentes medicinais, sendo que várias abordagens têm sido realizadas, ao longo dos anos, a fim de selecionar plantas como candidatas para a descoberta de novas drogas (Balunas e Kinghorn, 2005). Neste contexto, a etnofarmacologia contribui com uma visão interdisciplinar na busca para a saúde humana, e têm provado ser uma ferramenta poderosa na descoberta de produtos naturais com ação terapêutica (Reyes-Garcia, 2010).

A Caatinga brasileira, localizada na região nordeste do Brasil, esta exposta a um clima semiárido quente e seco, resultando em vegetação xerofítica com uma diversidade florística significativa (Rodal e Nascimento, 2006) . Este bioma semiárido é a única grande região totalmente inserida no território nacional, e, por este motivo, têm se discutido cada vez mais mecanismos e estratégias para a conservação da sua biodiversidade (Albuquerque et al., 2012). A região da Caatinga possui uma riqueza de conhecimento tradicional acumulado pelos habitantes locais onde as plantas medicinais são uma componente social e cultural importante, e às vezes são as únicas alternativas disponíveis para o tratamento de doenças. Estudos etnofarmacológicos recentes demonstraram que as plantas da Caatinga têm potencial de prevenir a adesão de bactérias e seu crescimento (Trentin et al., 2011; Trentin et al., 2013), reduzir significativamente a viabilidade de protozoários flagelados (Frasson et al., 2012), e de reduzir a inflamação, lipoperoxidação e hiperalgesia em ratos (Santana et al., 2012).

Nos últimos anos, os biofilmes têm atraído muita atenção especialmente pelo seu enorme impacto na medicina e na saúde pública. As bactérias, sob a forma de biofilme contribuem para a cronicidade e persistência das infecções, tais como aquelas associadas com dispositivos médicos implantados (Francolini e Donelli, 2010). Este

importante estilo de vida permite aos patógenos escapar das defesas imunes do hospedeiro e resistir aos tratamentos antibacterianos (Høiby et al., 2010).

Como parte de nossa investigação etnodirigida, o presente estudo teve como objetivo avaliar 23 extratos vegetais aquosos contra *Staphylococcus epidermidis* e *Pseudomonas aeruginosa*. Além disso, os extratos mais ativos foram submetidos a uma avaliação citotóxica e um perfil fitoquímico preliminar.

2.0 Métodos

2.1 Material vegetal e extratos

As plantas utilizadas neste estudo tem aplicações medicinais pelas comunidades da Caatinga (Agra et al., 2008 e informação da comunidade local) conforme resumido na Tabela 1. As plantas foram coletadas no Parque Nacional do Catimbau (PARNA do Catimbau), Pernambuco, Brasil, em 2010. As espécies foram identificadas no herbário do Instituto Agronômico de Pernambuco (IPA), onde um *voucher* de cada espécie foi depositada. Os extratos aquosos foram preparados para reproduzir o seu uso tradicional, de acordo com Trentin et al. (2011). Os ensaios foram feitos com uma concentração final de 4 mg/mL.

2.2 Condições de cultura das cepas bacterianas

Pseudomonas aeruginosa ATCC 27853 e *Staphylococcus epidermidis* ATCC 35984 foram cultivadas em caldo Mueller Hinton (MH) (Oxoid Ltd., Inglaterra) durante a noite, a 37 °C, e uma suspensão bacteriana em solução salina estéril a 0,9 %, correspondente a 1 escala de McFarland (3×10^8 UFC / mL), foi usada nos ensaios.

2.3 Atividade antibiofilme e antibiótica

O ensaio da atividade antibiofilme foi realizado como estabelecido por Trentin et al. (2011) empregando a técnica de cristal violeta. O crescimento bacteriano foi avaliado pela diferença entre os valores de absorbância inicial (t = 0) e final (t = 6 h para *P. aeruginosa* e de 24 h para o *S. epidermidis*) a 600 nm, em placas de microtitulação de 96 poços (Costar 3599, Corning, Inc., EUA). Valores superiores a 100% representam uma estimulo do crescimento bacteriano ou formação de biofilme em comparação com a amostra controle (não tratada). As amostras controle (sem tratamento) foram substituídas por água.

2.4 Microscopia Eletrônica de Varredura (MEV)

Biofilmes de *Staphylococcus epidermidis* foram cultivados em microplacasplacas de 96 poços com um pedaço de lâmina de Permanox ™ (NalgeNunc International, EUA). Após as 24 horas de tratamento, as amostras foram processadas e as lâminas foram secas usando a técnica de ponto crítico de CO_2, conforme descrito por Trentin et al. (2011), e por fim examinadas em um microscópio de varredura JEOL JSM-6060.

2.5 Perfil fitoquímico

Os extratos que apresentaram atividade antibiofilme promissora contra os microrganismos testados foram aplicados em placas de cromatografia em camada fina (CCD) (gel de sílica Merck 60 F254) utilizando butanol, ácido acético e água (5:1:4) como fase eluente. As placas foram visualizadas sob luz UV (254 e 365 nm, Handheld lâmpada UV modelo 9403E, BioAmerica Inc., EUA) e reveladas com diferentes sprays químicos. Reagente natural seguido de polietilenoglicol foi utilizado para detectar os

flavonóides; cloreto férrico para polifenóis; ninidrina para aminas e aminoácidos; reagente anisaldeído sulfúrico para esteróides, terpenos e saponinas e reagente de Dragendorff para alcalóides e compostos de nitrogenados heterocíclicos(Wagner e Bladt, 1996).

2.6 Estudo da citotoxicidade

A toxicidade dos extratos selecionados (em 0,4, 2,0 e 4,0 mg/mL) foi investigada usando a linhagem de células Vero de mamífero no ensaio de MTT (Tiazolil Azul Tetrazolium Brometo, Sigma-Aldrich)(Mosmann, 1983). Nas amostras controle (células não tratadas), os extratos foram substituídos por água (100 % de viabilidade), enquanto que 1 % de solução de Triton X-100 foi usada como controle positivo.

2.7 Análise estatística

Todos os ensaios foram realizados pelo menos em triplicata, e os dados estão apresentados como percentagem média ± desvio padrão. As diferenças entre os grupos foram avaliadas pelo teste t de Student ($p \leq 0,05$ foi considerado significativo).

3.0 Resultados e Discussão

Vinte e três dos 14 extratos de plantas diferentes foram testados a fim de determinar a sua atividade antibiofilme e antibacteriana contra duas importantes espécies bacterianas (Tabela 1). Considerando *S. epidermidis*, o mais importante causador de infecções associadas a dispositivos médicos (Otto, 2009), os resultados revelaram que 10 extratos têm atividade potencial antibiofilme (que permitiu ≤ 50% da formação de biofilme): frutos de *Apuleia leiocarpa* (47%), folhas de *Byrsonima gardneriana* (16%) , mistura de *Harpochilus neesianus* (19%) , folhas de *Harpochilus*

neesianus (31%) , folhas de *Jacaranda rugosa* (37%) , raízes de *Piriqueta guaianensis* (26%) , frutos de *Poincianella pyramidalis* (36%) , ramos (13%) e folhas de *Sideroxylon obstusifolium* (15%) e folhas de *Turnera* melochioides (26%). Já o crescimento de *S. epidermidis* foi inibido por apenas três extratos: folhas de *Byrsonima gardneriana* (16%), ramos (57%) e folhas de S. *obstusifolium* (52%). Em relação a *P. aeruginosa*, espécie intrinsecamente resistente a vários tipos de antibióticos(Strateva e Yordanov, 2009) , os nossos resultados demonstram que os extratos exibiram um perfil de atividade diferente, em que a prevenção do biofilme parece estar relacionada com a inibição do crescimento bacteriano, como observado para: folhas de *Byrsonima gardneriana*, folhas de *J. rugosa*, folhas de *M. lewessia*, frutos de *P. pyramidalis*, raízes de *P. guianensis* e ramos e folhas de *S. obtusifolium*. Estes resultados podem explicar por que todas as plantas descritas acima, com exceção de *P. guianensis* e *T. melochioides*, são utilizadas pelas comunidades da Caatinga contra as doenças que se assemelham a inflamações e infecções (Tabela 1).

Table 1 – Dados etnofarmacológicos e atividade biológica de 23 extratos aquosos (4 mg/mL) de 14 plantas medicinais da Caatinga contra a formação de biofilme e crescimento de *Staphylococcus epidermidis* ATCC 35984 e *Pseudomonas aeruginosa* ATCC27853.

Nome científico da espécie e da família	Voucher	Formas de uso, preparação e indicação terapêutica	Parte usada	*S. epidermidis*		*P. aeruginosa*	
				Formação de biofilme (%)	Crescimento bacteriano (%)	Formação de biofilme (%)	Crescimento bacteriano (%)
Apuleia leiocarpa (Vogel) J.F.Macbr. – Fabaceae	IPA 87902	A decocção é usada contra úlceras externas. É consumida como tónico (CP).	Folhas	185.7 ± 3.3*	121.4 ± 6.4*	99.6 ± 16.0	134.9 ± 2.5*
			Frutos	47.9 ± 2.0%*	209.3 ± 5.5*	82.4 ± 4.8*	83.4 ± 1.3
Byrsonima gardneriana A.Juss. – Malpighiaceae	IPA 85917	Decocções são usadas contra úlceras externas e inflamações (Agra et al., 2008).	Folhas	16.0 ± 3.8*	162.0 ± 34.0*	31.7 ± 5.08*	44.5 ± 2.3*
Croton heliotropiifolius Kunth -Euphorbiaceae	IPA 85697	A decocção é usada contra dor intestinal, gripe, asma e bronquite. (Agra et al., 2008).	Folhas	85.4 ± 11.3	91.8 ± 2.4	87.2 ± 2.5*	125.9 ± 3.6
Harpochilus neesianus Mart. ex Nees. - Acanthaceae	IPA 85766	O xarope é usado contra asmas, tosse, bronquite e como expectorante (CP).	Mistura de folhas, frutos e ramos	19,6 ± 3.2*	46.2 ± 7.1*	78.9 ± 5.4*	118.2 ± 2.3
			Folhas	31.3 ± 12.1*	143.2 ± 3.2*	103.1 ± 13.8	109.6 ± 3.4
Ipomea brasiliana Choisy Meisn – Convolvulaceae	IPA 85701	Contra dermatite, sarna, sífilis e úlceras de pele. Usada para tomar banho ou lavar as partes afetadas (CP).	Mistura de ramos e folhas	150.1 ± 14.3*	217.3 ± 5.6*	60.7 ± 17.1*	103.3 ± 1.4
Jacaranda rugosa A.H.Gentry - Bignoniaceae	IPA 85710	Uma infusão em água ou maceração em álcool. Possui uso contra sífilis e úlceras (CP).	Folhas	37.2 ± 3.8*	198.9 ± 26.5*	26.6 ± 10.0*	0 ± 0*
Mimosa lewisii Barneby – Fabaceae	IPA 85902	Xarope é usado como expectorante e contra doenças respiratórias (CP).	Folhas	129.0 ± 10.7*	199.9 ± 28.4*	45.2 ± 8.3*	0 ± 0*
Piriqueta guianensis N.E.Br. – Turneraceae	IPA 84869	A decocção é usada contra amenorréia e como abortivo (CP).	Folhas	156.8 ± 2.4*	285.2 ± 7.0*	80.7 ± 10.4*	161.0 ± 2.5*
			Raízes	26.6 ± 1.6*	70.5 ± 0.7*	12.8 ± 8.8*	9.9 ± 0.2*
			Ramos	86.3 ± 3.2*	118.7 ± 1.9	78.4 ± 8.3*	88.4 ± 2.4

Tabela 1 (continuação)

Nome científico da espécie e da família	Voucher	Formas de uso, preparação e indicação terapêutica	Parte usada	*S. epidermidis*		*P. aeruginosa*	
				Formação de biofilme (%)	Crescimento bacteriano (%)	Formação de biofilme (%)	Crescimento bacteriano (%)
Poincionella pyramidallis (Tul.) L.P.Queiroz – Fabaceae	IPA 85911	Contra disenterias, diarreias e dor de estômago. Como um expectorante, é usado contra infecções respiratórias como bronquite e tosse. Uma decocção com açúcar é utilizado como um xarope (Agra et al., 2008).	Frutos	36.7 ± 6.3*	163.3 ± 1.6*	1.6 ± 4.8*	0 ± 0*
			Folhas	237.0 ± 7.4*	243.7 ± 3.3*	111.0 ± 1.7*	202.5 ± 7.2*
Sideroxylon obtusifolium (Roem. & Schult.) T.D. Penn – Sapotaceae	IPA 85873	Contra inflamações de ovário e diabetes. Uma decocção ou maceração de um punhado é preparada num litro de água (Agra et al., 2008).	Ramos	13.7 ± 1.3*	57.7 ± 6.7*	30.6 ± 7.6*	57.0 ± 2.9*
			Folhas	15.0 ± 1.5*	51.6 ± 1.7*	41.9 ± 3.6*	58.0 ± 2.8*
Stylosanthes viscosa Sw. – Fabaceae	IPA 85698	Infusões de uma colher em um copo de água. Extratos consumidos como solução estomacal após as refeições (CP).	Planta inteira	118.0 ± 1.0*	170.8 ± 5,2*	114.1 ± 2.4	164.0 ± 1.7*
Turnera hermannioides Cambess. – Turneraceae	IPA 84962	Contra amenorréia e dismenorréia. Uma decocção de um punhado é preparada num litro de água. É consumida como chá (CP).	Raízes	98.1 ± 2.1	161.5 ± 4.4*	72.7 ± 1.6*	81.4 ± 0.2
			Ramos	241.8 ± 5.6*	202.0 ± 2.6*	94.5 ± 3.4*	106.4 ± 1.8
			Folhas	91.4 ± 13.8	82.5 ± 1.6	78.5 ± 7.1*	119.3 ± 0.8*
Turnera melochioides Cambess. – Turneraceae	IPA 84959	Contra amenorréia e dismenorréia. Uma decocção de um punhado é preparada num litro de água. É consumida como chá (CP).	Folhas	26.5 ± 5.7*	190.4 ± 34.2*	73.1 ± 8.7*	107.5 ± 2.1
			Ramos	152.5 ± 14.3*	252.2 ± 11.3*	104.8 ± 8.7	121.2 ± 0.7
Turnera subulata Sm. – Turneraceae	IPA 84965	Contra amenorréia e dismenorréia. Uma decocção de um punhado é preparada num litro de água. É consumida como chá (Agra et al., 2008) .	Ramos	182.0 ± 30.8*	249.6 ± 2.4*	82.5 ± 15.9*	86.7 ± 1.0

Os resultados representam a média ± desvio padrão de 3 experiências. CP – Comunicação pessoal. * Representa diferença significativa em relação ao controle ($p < 0,05$).

Três extratos ativos foram selecionados para uma investigação mais aprofundada: folhas de *H. neesianus*, frutos de *A. leiocarpa* e *P. pyramidalis*. *Harpochilus neesianus* e *P. pyramidalis* são espécies arbóreas endêmicas amplamente distribuídas na Caatinga (Santana et al., 2012; Vogel et al., 2004), enquanto *A. leiocarpa* é uma leguminosa amplamente distribuída no Brasil. No entanto, a floresta da Caatinga tem se tornado cada vez mais escassa devido à sua devastação e a extração de madeira, pondo em risco a riqueza da sua biodiversidade (Albuquerque et al., 2012). Estas espécies foram capazes de impedir a formação de biofilme por *S. epidermidis*, sem inibir o crescimento bacteriano (Tabela 1). A inibição da formação de biofilme onde o crescimento bacteriano não é afetado negativamente compreende uma abordagem alternativa e atraente, pois pode dificultar o rápido desenvolvimento da pressão seletiva para a resistência bacteriana (Rasko e Sperandio, 2010). As imagens de MEV corroboram os resultados obtidos pelo ensaio colorimétrico, mostrando que os extratos selecionados impediram fortemente a formação de biofilme de *S. epidermidis* como também induziram superprodução da matriz e/ou a modificação da morfologia bacteriana (Fig. 1, C e D).

A fim de avaliar os extratos em relação à sua citotoxicidade contra células de mamíferos e correlacionar com a sua capacidade de prevenir a formação de biofilme de *S. epidermidis*, duas concentrações menores do extrato foram incluídas (2,0 e 0,4 mg/ mL). Observou-se que o extrato de folhas de *H. neesianus* não mostrou citotoxicidade, o extrato de frutos de *A. leiocarpa* apresentou citotoxicidade somente na maior concentração testada e o extrato de frutos de *P. pyramidalis* diminuiu significativamente a viabilidade em células de mamífero em 4,0 e 2,0 mg/ml, mas não era citotóxico a 0,4 mg/mL (figura 1, painel E). Até o nosso conhecimento não há nenhum estudo na literatura avaliando a toxicidade de *H. neesianus* e *A. leiocarpa*. No estudo de Alviano

et al. (2008), os autores relataram que o extrato aquoso de *Caesalpinia pyramidalis* apresentou baixa toxicidade *in vivo*, embora em nosso estudo *in vitro* os frutos da planta sinônima *P. pyramidalis* demonstrou uma citotoxicidade dose- dependente. Em relação à atividade antibiofilme dos extratos de *H. neesianus* e *A. leiocarpa*, nenhuma variação importante foi observada mesmo quando a concentração inferior a 10 vezes foi usada. Diferentemente, o extrato de *P. pyramidalis* demonstrou um perfil dose-dependente da inibição da formação de biofilme, sendo ativo na maior concentração e não significativamente ativo nas concentrações menores (Fig. 1, painel E). O ensaio fitoquímico qualitativo destes extratos indicou a presença de flavonóides, terpenóides, aminas, esteróides, enquanto os polifenóis foram detectados apenas em *P. pyramidalis* (dados não mostrados). Estes resultados conduzirão nossos esforços futuros na purificação dos compostos bioativos e em estudos sobre suas vias de ação.

Esta triagem *in vitro* utilizando abordando o conhecimento etnofarmacológico é importante para a validação do uso tradicional de ervas medicinais por comunidades da Caatinga, para estimular uma ativa política de preservação das plantas e também para orientar novos estudos de bioprospecção. O estudo destaca o conhecimento etnofarmacológico valioso preservado nesta região, uma vez que todas as plantas utilizadas pelas comunidades contra as doenças que se assemelham a inflamações e infecções corroboram com a nossa investigação anti-infecciosa. Além disso, revelamos plantas com alto potencial de drogas antibiofilme com limitada citotoxicidade *in vitro* contra um importante patógeno formador de biofilme, *S. epidermidis*.

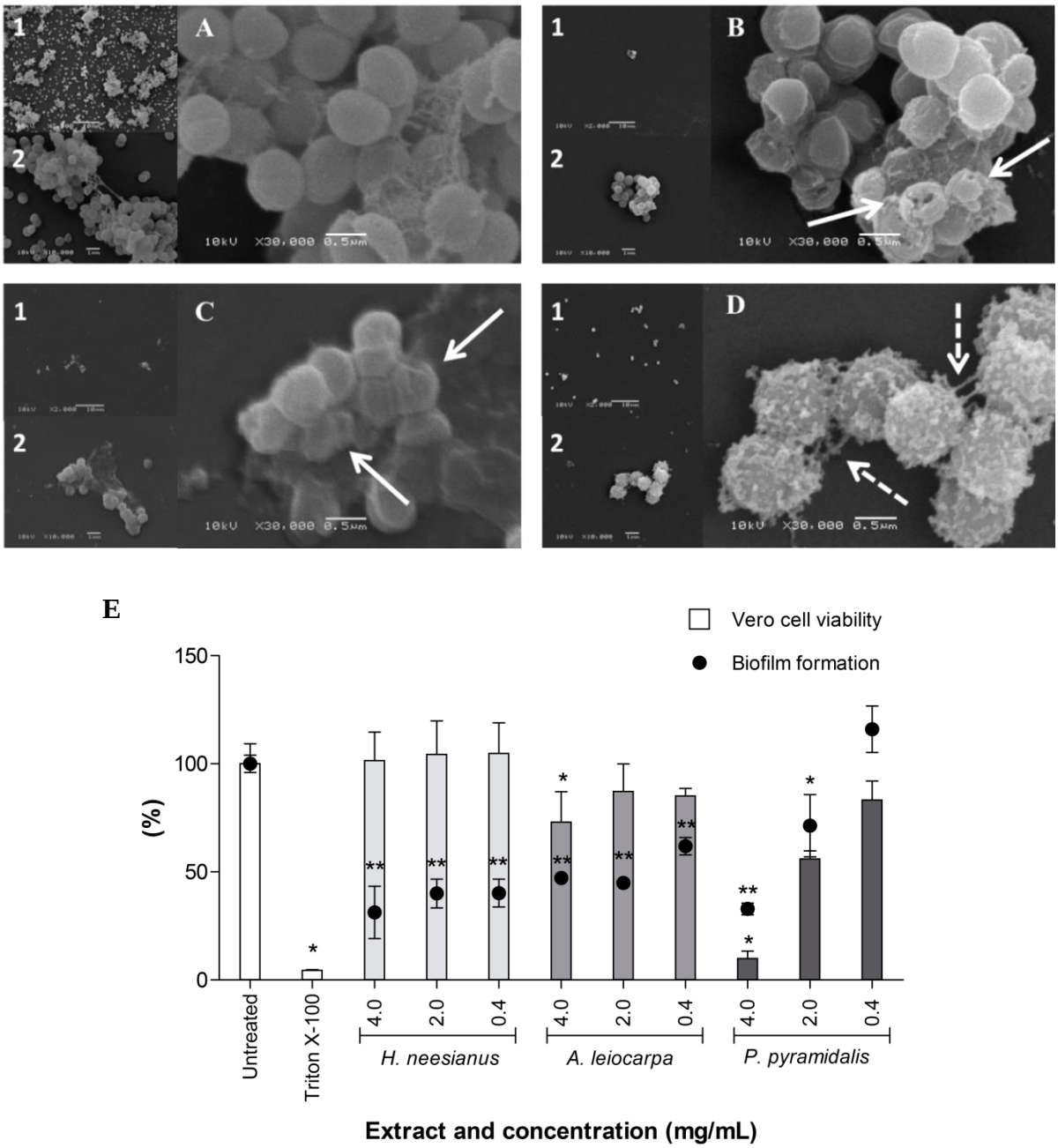

Fig.1 - (A a D) Imagens de microscopia eletrônica de varredura do biofilme de estafilococos sobre Permanox ™ . (A) *S. epidermidis* não tratado (controle) e bactérias tratadas com os três extratos selecionados: (B) folhas de *H. neesianus*, (C) frutos de *A. leiocarpa*, e (D) frutos de *P. pyramidalis*. Escala: ampliação 30.000x (nas imagens: quadro 1 - ampliação 2.000x e quadro 2 - 10.000x ampliação). Setas sólidas: deformação celular. Setas pontilhadas: superprodução de matriz. (E) Viabilidade das células de mamífero Vero e a formação de biofilme por *S. epidermidis* de acordo com

diferentes concentrações dos extratos. Células e bactérias não tratadas foram consideradas como tendo 100% de viabilidade e formação de biofilmes, respectivamente. * Indica diferença significativa em relação às amostras não tratadas em relação aos resultados sobre a viabilidade das células de mamífero ** sobre os resultados de formação de biofilme.

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Anexo

Pharmaceutical Biology

Instructions for Authors

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Pharmaceutical Biology will publish manuscripts describing the discovery, methods for discovery, description, analysis characterization, and production/isolation (including sources and surveys) of biologically-active chemicals or other substances, drugs, pharmaceutical products, or preparations utilized in systems of traditional medicine.
Topics may generally encompass any facet of natural product research related to pharmaceutical biology. Papers dealing with agents or topics related to natural product drugs are also appropriate (e.g., semi-synthetic derivatives). Manuscripts will be

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Book: Vyas SP, Khar RK. (2001). *Targeted and Controlled Drug Delivery*. New Delhi, India: CBS Publisher and Distributor.

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Electronic Resources: Lin A-S, Shibano M, Nakagawa-Goto K, Tokuda H, Itokawa H, Morris-Natschke SL, Lee K-H. (2007). Cancer Preventive Agents. 7. Antitumor-Promoting Effects of Seven Active Flavonolignans from Milk Thistle (*Silybum marianum*) on Epstein-Barr Virus Activation. *Pharm Biol* [Online]. Available at: http://www.informapharmascience.com/doi/abs/10.1080/13880200701585592. Accessed on 12 April 2009.

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