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ANGELO DE OLIVEIRA CALEARE

NOVAS OPÇÕES DE TRATAMENTO PARA INFECÇÕES CAUSADAS POR *Helicobacter pylori* E *Porphyromonas gingivalis*

Maringá
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas (Área de Concentração – Biologia Celular e Molecular) da Universidade Estadual de Maringá, para a obtenção do grau de Doutor em Ciências Biológicas.

Orientador: Prof. Dr. Celso Vataru Nakamura

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BIOGRAFIA

Angelo de Oliveira Caleare, nasceu em Maringá/PR em 09/09/1986. Possui graduação em Ciências Biológicas pela Universidade Estadual de Maringá. Realizou estágio no Laboratório de Análise de Água do setor de Microbiologia Básica da UEM, o qual abriu as portas para duas iniciações científicas, onde trabalhou avaliando o perfil de susceptibilidade de bactérias isoladas de efluentes do Hospital Universitário de Maringá. Após trabalhar com atividade anti-*Trypanosoma cruzi* no mestrado, voltou a trabalhar com atividade antibacteriana no doutorado, realizando também um estágio de doutorado sanduíche no Instituto de Biologia Farmacêutica e Fitoquímica da Universidade de Münster – Alemanha. Tem experiência na área de microbiologia aplicada.

Dedico este trabalho a Cintia Caroline Gomes Caleare e Leonardo Gomes Caleare

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APRESENTAÇÃO

Essa tese é composta de dois artigos científicos. No primeiro capítulo (artigo 1) está a descrição da atividade anti-virulência de um extrato acetona:água da espécie *Limonium brasiliense* contra a bactéria *Porphyromonas gingivalis*. O segundo capítulo (artigo 2) é uma revisão da atividade anti-*Helicobacter pylori* de novos compostos sintéticos. O capítulo 3 se refere ao porquê da escolha do extrato utilizado no primeiro artigo e outras informações importantes. Em consonância com as normas vigentes do Programa de Pós-Graduação em Ciências Biológicas, eles foram redigidos conforme as regras das revistas para as quais eles serão submetidos:

1. Artigo:

Limonium brasiliense polyphenols as inhibitors of the virulence factors of *Porphyromonas gingivalis*

Phytotherapy Research (ISSN Online: 1099-1573)
Fator de impacto (2015/2016): 2.694 – Qualis B1 (2014).

2. Artigo:

Helicobacter pylori infection therapy: Promising new synthetic compounds

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RESUMO GERAL

INTRODUÇÃO

O bacilo Gram-negativo *Porphyromonas gingivalis* é uma bactéria anaeróbia obrigatória, que desempenha um importante papel nas formas crônicas e agressivas da periodontite. Esta doença inflamatória do tecido gengival, que sem um tratamento adequado pode levar até mesmo a perda dos dentes. Como novas formas de tratamento, os produtos naturais, principalmente polifenóis, têm sido descritos para periodontite com o objetivo de reduzir a inflamação, a adesão bacteriana de *P. gingivalis* às células hospedeiras, bem como a inibição das gingipaínas, enzimas do tipo cisteína-proteases, diretamente relacionadas à virulência desta bactéria.

Helicobacter pylori é uma bactéria de formato bacilar, Gram-negativa e microaerófila que coloniza cerca de 50% da população mundial. Ela tem a capacidade de se instalar no ambiente hostil do estômago, promovendo reações inflamatórias que podem levar até mesmo ao câncer. A terapia contra a infecção por *H. pylori* está tornando-se menos eficiente devido ao desenvolvimento de resistência frente a antimicrobianos por este micro-organismo. Novas opções para o tratamento são necessárias para vencer a batalha contra esta bactéria. Novos compostos sintéticos com atividade anti-*Helicobacter pylori* são apresentados nesta revisão.

MATERIAIS E MÉTODOS

No estudo com a bactéria *P. gingivalis*, o extrato acetona:água (7:3 – v/v) de *Limonium brasiliense* foi testado para atividade citotóxica contra células de mamíferos empregando a linhagem celular KB e para a ação antimicrobiana contra *P. gingivalis*. Para analisar o efeito do extrato sobre a adesão bacterina, foram utilizados dois métodos, fluorimetria por leitor de placa e citometria de fluxo. Também foi analisado o efeito do extrato sobre a atividade das gingipaínas. Em todos os métodos aqui descritos foi utilizada a clorexidina para fins de comparação. Finalmente, o extrato foi analisado por cromatografia líquida acoplada à espectrometria de massa (CLAE-MS) e ressonância magnética nuclear (RMN) para a caracterização dos compostos presentes.

Com referência ao artigo de revisão, levantamentos foram realizados na base PUBMED no período de 2001 até o presente ano com os seguintes termos: *Helicobacter pylori*; synthetic compounds; treatments e anti-*Helicobacter pylori* activity.

RESULTADOS E DISCUSSÃO

O extrato acetona:água (7:3 – v/v) de *L. brasiliense* não mostrou atividade contra a bactéria *P. gingivalis* (MIC> 1000 µg/mL) e apresentou um baixo efeito nocivo para as células KB (100 µg/mL = cerca de 30% de redução da viabilidade) após 24 h de tratamento. A clorexidina apresentou alta redução da viabilidade frente as células KB em baixas concentrações (<0,002%), até mesmo por apenas duas horas de tratamento. Na concentração de 100 µg/mL, *L. brasiliense* reduziu a adesão de células KB em cerca de 80% para ambas as metodologias e a clorexidina teve apenas um efeito em doses mais elevadas. O extrato reduziu as atividades das gingipaínas, principalmente a específica para as cadeias peptídicas, nas quais a arginina está presente (Rgp) em 75%. Sobre a caracterização do extrato por CLAE-EM e RMN, os dados obtidos foram comparados com a literatura, mostrando que o ácido gálico, epigalocatequina galato e samarangeninas A e B, foram as substâncias detectadas no extrato.

A busca no PUBMED pelos termos utilizados no artigo de revisão resultou em vários artigos, dos quais a grande maioria trata das classes já conhecidas de antibióticos, *e.g.* (quinolonas, macrolídeos, entre outras). Relatou-se também aqueles que se incluem em novas classes. Dentre elas podemos destacar as que tiveram melhores resultados: quinoxalinas, oligômeros de lisinas aciladas, quinonas, pirazolopirimidinedionas, pirazolinas, cumarina-3-carboxamidas e xantonas sintéticas.

CONCLUSÕES

Estes resultados fazem do extrato hidroacetônico obtido da planta *Limonium brasiliense*, um agente promissor para combater a periodontite, pois inibiu o principal fator de virulência de *P. gingivalis*, as gingipaínas. Estudos clínicos são necessários para o desenvolvimento de um produto para terapias orais contra esta doença. Novas moléculas também são necessárias para o tratamento da infecção causada por *H. pylori* e estudos adicionais são de importância fundamental para o desenvolvimento de agentes efetivos frente a esta bactéria.

GENERAL ABSTRACT

INTRODUCTION

The Gram negative bacillus *Porphyromonas gingivalis* is an obligatory anaerobic bacteria, this micro-organism plays an important role in chronic and aggressive forms of periodontitis, which is an inflammatory disease of the gum tissue that without proper treatment may even lead to teeth's loss. As new treatments, natural products, especially polyphenols, have been described for periodontitis in order to reduce inflammation, bacterial adhesion of *P. gingivalis* to host cells as well as inhibition of gingipains, a type of cysteine protease, the main virulence factor of the bacteria.

Helicobacter pylori is a Gram-negative rod and microaerophilic bacterium, that infects about 50% of the world population. It has the ability to settle in the hostile environment of the stomach and reside there, promoting inflammatory reactions that can even lead to stomach cancer. The therapy against *H. pylori* infection is becoming less effective due to the development of resistance against antimicrobials by this micro-organism. New options for treatment are necessary to overcome the battle against this bacterium. New synthetic compounds with anti-*Helicobacter pylori* activity are presented in this review.

MATERIAL AND METHODS

In the study with the bacterium *P. gingivalis*, the acetone:water extract of *Limonium brasiliense* was tested for cytotoxicity against mammalian cells using the cell line KB and for antimicrobial activity against *P. gingivalis*. In order to analyze the anti-adhesive effect, two methods were used, by plate reader fluorimetry and flow cytometry. Inhibition of gingipains was also tested. In any method described herein chlorhexidine was used as control for comparison. Finally, the extract was analyzed by liquid chromatography coupled with mass spectrometry.

With reference to the review article, surveys were conducted in PUBMED database in the period 2001 to the present year with the following terms: *Helicobacter pylori*; synthetic compounds; treatments and anti-*Helicobacter pylori*.

RESULTS & DISCUSSION

The extract showed no cytotoxicity against the bacteria (MIC > 1000 µg/mL) and lower noxious effect to KB cells (100 µg/mL = nearly 30% of reduction of viability) after 24 h of treatment. Chlorhexidine showed high reduction of cell viability at low concentrations (< 0.002%) even for just 2 hour of treatment. At 100 µg/mL, *L. brasiliense* reduced the adhesion to KB cells to nearly 20% for both methodologies and chlorhexidine had an effect just in higher doses. Notably, the extract reduced the gingipain activities, mainly the argine-specific (Rgp gingipains). About the chemical characterization, a putative identification showed that the galloylated proanthocyanidins are present in this extract. These findings make this plant as a promising agent to combat periodontitis and clinical studies are needed for developing an agent for oral therapeutics against periodontitis.

A PubMed search for the terms used in the review article resulted in several articles, however the great majority discusses the previously known classes of antibiotics, e.g., (quinolones, macrolides, among others). We reported new classes also. Among them we can highlight which had better results: quinoxaline, oligomers acylated lysines, quinones, pirazolopirimidinediones, pyrazolines, coumarin-3-carboxamides and synthetic xanthones.

CONCLUSIONS

These results make hydroacetonic extract obtained from the plant *Limonium brasiliense*, a promising agent for combating periodontal disease and clinical trials are needed to develop a product for oral therapies for this disease. New molecules are also needed for the treatment of infection caused by *H. pylori* and additional studies are of fundamental importance for the development of effective agents to forward this bacterium.

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INTRODUÇÃO

INTRODUÇÃO

Periodontite e *Porphyromonas gingivalis*

A periodontite é uma doença inflamatória crônica induzida por bactérias, que compromete a integridade dos tecidos de suporte do dente, os quais incluem gengiva, ligamento periodontal e osso alveolar, conjuntamente conhecido como periodonto. Ela difere-se da gengivite por ser um estágio avançado da doença periodontal (HAJISHENGALLIS, 2015). A gengivite é o passo inicial das doenças periodontais, sendo mais branda e caracterizada pelo biofilme bacteriano que se instala no dente adjacente a gengiva, levando a uma inflamação da mucosa e tendo como sintomas o inchaço, vermelhidão e sangramento. Caso não ocorra o tratamento adequado, ela pode levar ao quadro de periodontite (PIHLSTROM *et al.*, 2005).

O isolamento de bactérias Gram-negativas anaeróbias provenientes da cavidade bucal, produtoras de pigmento preto, levou os pesquisadores a primeiramente classificar estes micro-organismos como pertencentes ao gênero *Bacteroides* (COYKENDALL *et al.*, 1980). Estudos posteriores mostraram que estas bactérias possuíam muitas características diferentes do referido gênero, dentre elas a ausência de glicose-6-fosfato desidrogenase e 6-fosfogluconato desidrogenase e o metabolismo do tipo fermentativo, que justificaram a transferência para um novo gênero *Porphyromonas* (SHAH & COLLINS, 1988).

O principal patógeno associado a periodontite é a bactéria *Porphyromonas gingivalis*, um cocobacilo não móvel, assacarolítico e anaeróbio obrigatório. Em ágar sangue apresenta-se como pequenas colônias brancas em um tempo de incubação curto

e após 4 dias tornam-se pretas devido a formação de protoheme (GIBSON & GENCO, 2006).

Este micro-organismo possui diferentes fatores associados à superfície celular e também ao meio extracelular, como adesinas, enzimas associadas à célula ou secretadas, toxinas e hemolisinas que contribuem para a sua virulência (SUMMANEN & FINEGOLD, 2015).

Na progressão das doenças periodontais há uma sucessão microbiológica (fig. 01), primeiramente estreptococos colonizam a superfície do dente, promovendo uma superfície específica e reduzindo a concentração de oxigênio para a colonização de *Fusobacterium nucleatum*, *Tannerella forsythia*, *Treponema denticola* e *P. gingivalis*, todas estas bactérias importantes no desenvolvimento da periodontite (WRIGHT *et al.*, 2013).

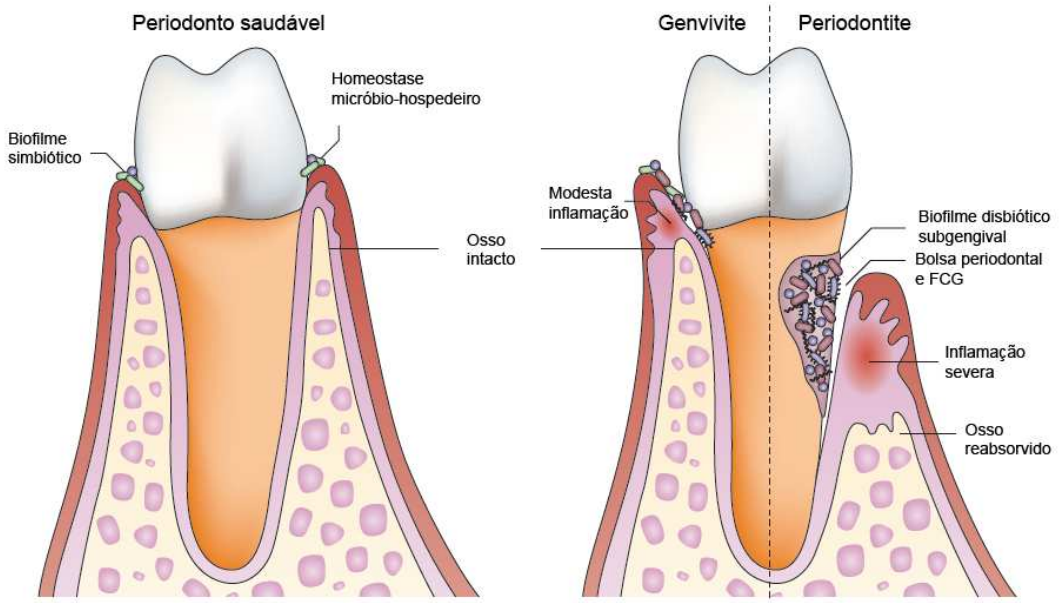

Fig. 01: Comparação entre o periodonto saudável, gengivite e periodontite. FCG: fluído crevicular gengival. Modificado de Hajishengallis, 2015.

A transmissão da bactéria *P. gingivalis* se dá principalmente entre indivíduos adultos via horizontal, sendo a transmissão vertical rara. A adoção de um alto padrão de

higiene oral contribui para a diminuição da sua disseminação (VAN WINKELHOFF & BOUTAGA, 2005).

Para avaliar a presença das doenças periodontais, estabeleceu o *índice periodontal comunitário* (CPI) que verifica a presença de sangramento, cálculo e presença de bolsa periodontal (rasa e profunda) tendo como referência o exame por sextante (grupos de 6 dentes entre os 32 da arcada dentária) conforme preconizado pela Organização Mundial da Saúde (OMS) (PETERSEN & OGAWA, 2005). No Brasil, o Ministério da Saúde publicou um documento onde mostra a prevalência destas doenças sobre sua população (MINISTÉRIO DA SAÚDE, 2012). Em se tratando da bolsa periodontal, no grupo de 15 a 19 anos a prevalência de bolsas rasas (3 a 5 mm) foi de 9% e de bolsas profunda 0,7%. No grupo etário de 35 a 44 anos, a presença foi de 15,2% rasas e 4,2%, profundas e nos idosos de 65 a 74 anos foi de 2,5% de bolsas rasas e 0,8% de bolsas profundas (MINISTÉRIO DA SAÚDE, 2012).

Como a periodontite é uma doença crônica, que pode levar a perda até mesmo dos dentes, é mais do que necessário a pesquisa por novos tipos de tratamento que previnam esta enfermidade.

Os produtos derivados de plantas têm-se revelado uma fecunda fonte de novos agentes terapêuticos (CALIXTO, 2000). O emprego das plantas no tratamento das doenças dava-se de maneira empírica, de tal forma que os conhecimentos adquiridos foram sendo passados ao longo do tempo, de geração para geração (CUNHA et al., 2003). Com as limitações dos fármacos existentes, o emprego de conhecimentos calcados no método empírico para a síntese de drogas derivadas de plantas tornou-se uma alternativa promissora. Com efeito, as plantas podem ser fontes potenciais de novas drogas seguras e efetivas de inestimável valor no tratamento de doenças infecciosas. Nesse contexto, a busca por novos princípios isolados de plantas tem

fomentado o investimento de muitas indústrias farmacêuticas em estudos envolvendo plantas (SIMÕES et al., 2004).

Dentro desta perspectiva, pode-se afirmar que o Brasil é um país privilegiado, uma vez que detém extensa e diversificada flora, com destaque para as formas de vegetação encontradas por todo o território nacional – Floresta Amazônia, Mata Atlântica, Cerrado, Catinga e Pantanal (CALIXTO, 2000); (YUNES et al., 2001). Em todo o mundo, inúmeros grupos de pesquisa investigam as possíveis atividades biológicas das plantas, baseando-se em informações etnobotânicas. Os dados obtidos atestam cada vez mais, e de maneira científica, as propriedades terapêuticas das plantas, em detrimento dos resultados empíricos de outrora.

Uma das classes mais estudadas de compostos oriundos de plantas é a dos taninos. Formam um grupo complexo de polímeros e uma definição química é de difícil resolução. A mais aceita é que são compostos polifenólicos com peso molecular maior que 500 e se ligam a proteínas em solução podendo precipita-lás. Sua interação com proteínas pode ocorrer via ligação covalente, ligação iônica, pontes de hidrogênio ou interação hidrofóbica, esta a mais estudada e observada (BENNICK, 2002).

Taninos isolados de *Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke, uma árvore distribuída principalmente na região amazônica, sendo suas sementes muito utilizadas como estimulante físico e mental na medicina popular (KLEIN et al., 2012), mostraram atividade anti-adesão contra *Streptococcus mutans*, uma das bactérias responsáveis pelas cáries (YAMAGUTI-SASAKI et al., 2007).

O baicuru é uma planta herbácea que ocorre na Argentina, Uruguai e na costa sul do Brasil. Ela é utilizada popularmente no tratamento da síndrome pré-menstrual, problemas menstruais e infecções urinárias. Não existem muitos estudos sobre esta planta. No que concerne a respeito da atividade biológica, ela demonstrou uma atividade

bacteriostática, anti-inflamatória e antioxidante, a qual pode ser devida a presença de taninos hidrolisáveis, saponinas, cumarinas entre outros (BLAINSKI et al., 2013).

Helicobacter pylori

Bactérias espiraladas que habitam a mucosa gástrica já foram documentadas na literatura científica há mais de 100 anos, por exemplo, o trabalho de Krienitz em humanos no ano 1906 e o de Bizzozero com gatos em 1892 (KUSTERS et al., 2006). Estes trabalhos foram praticamente esquecidos até que Marshall e Warren tiveram sucesso no isolamento de uma bactéria Gram-negativa, microaerofílica das biópsias gástricas humanas e propuseram que ela poderia ser a causa da gastrite crônica e da úlcera péptica (MARSHALL & WARREN, 1984).

Como a infecção em animais na época não foi positiva, Marshall resolveu tomar uma cultura de *Helicobacter pylori* e em um pouco mais de uma semana manifestou sintomas de gastrite e biópsias revelaram a presença de bactérias espiraladas. Tal fato, fez com que em 2005 o prêmio Nobel da medicina fosse para Marshall e Warren (COPELAND & STAHLFELD, 2012).

Para viver no ambiente hostil do estômago, o *H. pylori* conta com mecanismos que o protegem das agressões deste órgão. Para burlar o baixo pH do estômago, esta bactéria conta com a enzima urease, a qual hidrolisa ureia em amônia, criando uma camada com pH neutro que envolve a bactéria (fica claro aqui, que o *H. pylori* não é um micro-organismo acidofílico). Ela precisa também vencer a camada de muco que recobre o estômago, para chegar até as células epiteliais gástricas e estabelecer a infecção. Como possui flagelos polares e a forma helicoidal, ela consegue penetrar facilmente esta barreira e chega a superfícies das células gástricas (MONTECUCCO & RAPPUOLI, 2001).

A adesão de bactérias à superfície de células hospedeiras é o primeiro passo para o estabelecimento de infecções (BOYLE & FINLAY, 2003). No caso do *H. pylori*,

diversas proteínas da membrana externa, tem a capacidade de ligar a receptores do epitélio gástrico do hospedeiro, sendo esta proteínas classificadas como adesinas. A primeira descrita foi a BabA, do inglês *blood group antigen-binding adhesin* e se liga à os antígenos dos grupos sanguineos H-tipo 1 e Lewis b expressos na células gástricas de indivíduos secretores, contribuindo para os passo iniciais da infecção (MAGALHÃES & REIS, 2010). Após essa etapa inicial, o *H. pylori* induz a inflamação e aumenta a expressão de antígenos contendo ácido siálico no epitélio gástrico (sialil-*Lewis X* ou sialil-*Lewis A*), esta bactéria então passa a expressar uma adesina que tem afinidade por estes antígenos (TORRES & BACKERT, 2008). Ela foi denominada *sialic acid-binding adhesin* (SabA), e contribui severamente para a cronicidade da infecção por este micro-organismo (ODENBREIT, 2005). Diversas outras adesinas já foram identificadas em *H. pylori*, no entanto seus receptores permanecem desconhecidos, além destas proteínas, o lipopolissacarídeo (LPS) e também as integrinas desempenham um papel importante na adesão (SHEU et al., 2010).

Diversos componentes do genoma do *H. pylori* estão relacionados com a carcinogênese. A ilha de patogenicidade *cag* (do inglês, *cytotoxin-associated gene*), o fator de virulência mais estudado desta bactéria, possui o gene *cagA*, que codifica diversos tipos de proteína para formar o sistema de secreção tipo IV, o qual injeta dentro das células epiteliais gástricas, a proteína CagA (PIAZUELO et al., 2010). Dentro destas células esta oncoproteína, pode ser fosforilada por tirosina-cinases ou seguir uma via independente de fosforilação, estas vias induzem a alterações na sinalização da célula hospedeira, que pode causar uma resposta mitogênica, levando ao desenvolvimento de neoplasias. O exato mecanismo pelo qual esta proteína promove o câncer ainda não está claro (SIBONY & JONES, 2012).

Um outro fator de virulência importante na manifestação do câncer gástrico causado pelo *H. pylori* é a citotoxina vacuolizante (*VacA*, do inglês *vacuolating cytotoxin*), a que ficou conhecida por causar vacuolização citoplasmática em células epiteliais em cultura. Dentre as alterações estruturais e funcionais que ela pode causar podemos destacar, o rompimento da maturação endosomal, resultando em vacuolização, perda da permeabilidade seletiva das células epiteliais polarizadas, devido à disrupção das barreiras proporcionadas pelas junções tight e indução de danos as mitocôndrias, levando ao escape da citocromo c para o citosol, dando início a apoptose (WEN & MOSS, 2009). Outros fatores de virulência tem um papel importante na progressão para o câncer gástrico, dentre eles podemos destacar a proteína inflamatória externa (OipA – do inglês, *outer inflammatory protein*), o gene promotor de úlcera duodenal A (DupA – sigla *duodenal ulcer promoting gene A*) (YAMAOKA, 2010) e também as adesinas, BabA e SabA (WROBLEWSKI et al., 2010).

Não somente os fatores de virulência do *H. pylori* atuam no desenvolvimento do câncer gástricos, mas também a resposta do hospedeiro, como inflamação gástrica e a redução na secreção de ácidos, além de fatores ambientais (WROBLEWSKI et al., 2010). Na inflamação, os macrófagos desempenham um papel importante na progressão para o câncer gástrico, pois geram espécies reativas de oxigênio que podem causar danos ao DNA das células epiteliais gástricas (MENAKER et al., 2004). O *H. pylori* pode também inibir ou estimular a secreção de ácidos dependendo do tipo de infecção, na aguda geral ocorre uma redução na capacidade de secreção e na crônica ambas as possibilidades podem ocorrer, o que leva a um aumento na inflamação.

Fatores ambientais podem aumentar os riscos do desenvolvimento do câncer gástrico, como dieta com elevado teor de sal, infecções por helmintos e consumo de cigarro (WROBLEWSKI et al., 2010).

Aproximadamente mais da metade da população mundial está colonizada por este micro-organismo (WROBLEWSKI et al., 2010). A incidência desta bactéria nos países em desenvolvimento é notavelmente maior do que nos desenvolvidos, onde pode chegar à 50% nas crianças infectadas e mais de 90% nos adultos (KHALIFA et al., 2010). Este micro-organismo causa várias doenças gastrointestinais, como úlcera péptica em 10% dos infectados e adenocarcinoma gástrico em torno de 1-2%. Tem sido evidente por mais de 20 anos, que esta bactéria está envolvida com o desenvolvimento de adenocarcinoma gástrico e em 1994 a Organização Mundial de Saúde, definiu ele como agente cancerígeno da classe I em humanos (WEN & MOSS, 2009). Não se sabe por qual modo *H. pylori* é transmitida e novas infecções são provavelmente disseminadas via oral-oral, fecal-oral ou gastro-oral (KUSTERS *et al.*, 2006).

O tratamento para a erradicação consiste em combinações de fármacos, geralmente se usa dois antibióticos, amoxicilina mais claritromicina ou amoxicilina mais metronidazol, combinados com inibidores da bomba de prótons, esta é chamada de tripla terapia, o tratamento de primeira escolha (SELGRAD & MALFERTHEINER, 2011). A eficácia deste tratamento era em torno de 90% ou mais de cura, no entanto, devido a prevalência da resistência antimicrobiana, o sucesso deste tratamento tem caído a níveis não aceitáveis, 80% ou menos em muitos países (CHUAH et al., 2011).

Um estudo recente em âmbito nacional demonstrou taxas preocupantes de resistência frente à claritromicina (16,9%) e fluoroquinolonas (13,5%) por meio de testes moleculares em 490 pacientes adultos de cinco regiões do Brasil (SANCHES et al., 2016).

A importância de *H. pylori* no surgimento de doenças gastroduodenais severas como câncer gástrico, úlcera péptica e outros, levaram à pesquisa em estratégias de tratamento. Os gastos com saúde pública diminuíram muito com a descoberta de *H.*

pylori porque os antibióticos são mais baratos do que os antiácidos, os quais os pacientes devem toma-los por um longo período (cerca de 10 vezes mais caro) e mais baratos que o procedimento extremo, vagotomia (remoção do nervo vago). Normalmente, o regime de antibioticoterapia de duas semanas é suficiente para eliminar a infecção (SONNENBERG & TOWNSEND, 1995).

Estes fatos tornam a busca por novas substâncias frente a *H. pylori* de suma importância para a pesquisa acadêmica.

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CAPÍTULO 01

Research paper

Limonium brasiliense polyphenols as inhibitors of the virulence factors of *Porphyromonas gingivalis*

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Abstract

Porphyromonas gingivalis is a pathogen strongly involved in chronic and aggressive forms of periodontitis. Natural products, mainly polyphenols, have been described for advanced treatment of periodontitis by inhibition of the bacterial adhesion of *P. gingivalis* to the epithelial host cells. An aceton:water extract (LBE) from the rhizomes of *Limonium brasiliense* was tested under *in vitro* conditions for potential antiadhesive effects against *P. gingivalis* to human KB cells and for inhibition of the proteolytic activity of gingipains, the main virulence factor of *P. gingivalis*. LBE ≤ 100 µg/mL had no cytotoxicity against the bacteria and did not influence the cell physiology of KB cells. At 100 µg/mL LBE reduced the adhesion of *P. gingivalis* to KB cells significantly to about 80%. LBE at 20 µg/mL reduced the proteolytic activity of the arginin-specific *Rgp* gingipain by about 75%. Chemical profiling of LBE indicated the presence of gallic acid, epigallocatechin-3-O-gallate and samarangenins A and B.

Keywords: Phenolic compounds, oral anaerobe bacterium, Baicuru, cysteine proteases, periodontal diseases.

Abbrevations: BAPNA: N_{α}-benzoyl-D,L-arginine 4-nitroanilide hydrochloride; BCR: bacteria cell ratio; CHX: chlorhexidine digluconate; DMSO: dimethysulfoxide; EC_{50}: half-maximal toxicity; *fimA*: gene coding for fimbrillinA; FITC: fluorescein isothiocyanate; *kgp*: gene coding for lysin-specific gingipain; LBE: *Limonium*

49 *brasiliense* extract; LEU: leupeptin; MIC: minimum inhibitory concentration; OMP:
50 outer membrane protein; *rgpA*: gene coding for arginine-specific gingipain; TLC: thin
51 layer chromatography; TLCK: N_{α}-Tosyl-L-lysinchlormethylketone hydrochlorid; TTC:
52 2,3,5-triphenyltetrazolium chloride.
53

1. Introduction

Periodontal diseases are characterized by a complex microorganism-induced inflammation of periodontal tissue which is clinically characterized by swelling and bleeding of the gums, and if left untreated leads to the destruction of the tooth supporting system and eventually tooth loss. Amongst the opportunistic pathogens which are strongly related with periodontitis *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are the most virulent bacteria (Teles et al. 2013). The Gram-negative anaerobic bacterium *P. gingivalis* plays a dominant role in chronic and aggressive forms of periodontitis (Rescala et al. 2010). Furthermore, subgingival colonization with high rates of this bacterium in the infected tissue has been demonstrated to increase the risk of disease progression significantly (Haffajee et al. 1991). Additionally *P. gingivalis* has emerged as a potential mediator in the etiology of presumably unrelated chronic diseases, such as rheumatoid arthritis [for review see (Koziel, Mydel, and Potempa 2014)], cardiovascular diseases, diabetes and, more recently, different types of oral cancers [for review see (Atanasova and Yilmaz 2014)].

P. gingivalis colonizes the subgingival region, in a process that involves several steps. Initially the bacteria must pass between oral fluids to reach the gingiva and the periodont (Lamont and Jenkinson 2000). For the establishment of infections, the adhesion of bacteria to the host cells is an initial key step (Boyle and Finlay 2003). *P. gingivalis* has multiple adhesins including fimbriae, haemagglutinins, and proteinases, which allow the specific adhesion to epithelial cells, fibroblasts, erythrocytes and extracellular matrix components such as laminin, elastin, fibronectins, among others (Lamont and Jenkinson 1998). The multi-target adhesion of *P. gingivalis* to epithelial cells is complex. While fimbriae mediate adherence not only to the periodontal cell

surface(Feldman and Grenier 2012), but also to other oral bacterial species, five different hemagglutinins, associated with lipopolysaccharides and lipids on the cell surface, and a secreted *exo*-hemagglutinin are responsible for effective binding to erythrocytes as well as to epithelial cells (Bonifait and Grenier 2010).

The most potent adhesins and virulence factors of *P. gingivalis* located within the fimbriae are the gingipains, three cysteine proteases that bind and cleave a wide range of host proteins Gingipains are trypsin-like cysteine proteases and are classified into two groups based on the respective substrate specificity (Fitzpatrick, Wijeyewickrema, and Pike 2009). The arginine-specific cysteine protease (Arg-gingipain, Rgp) is encoded by genes *rgp*A and *rgp*B, while the lysine-specific cysteine protease (Lys-gingipain, Kgp) is encoded by *kgp* gene. The gingipains are located on the surface of *P. gingivalis* from where subfractions are secreted into the extracellular fluid (Amano 2003). Due to their proteolytic activity, gingipains are capable of degrading host proteins, such as collagen, fibronectin, immunoglobulin G and TNFα (Cutler, Kalmar, and Genco 1995), thus leading to a specific immune escape and destruction of the host tissue. Due to the adhesin function, the gingipains bind directly to extracellular matrix proteins (Fitzpatrick, Wijeyewickrema, and Pike 2009) or indirectly contribute to bacterial adhesion by processing the fimbrillin subunit (Chen et al. 2001).

Classical treatment for periodontitis consists of mechanical debridement and surgical procedures and local and systemic antibacterial treatment. Treatment with antibiotics alone is not effective due to pharmacokinetic problems as low concentrations of the active compounds in the gingival sulcus fluid, the fast elimination of antibiotics by salivary secretion and also by diminished penetration of the antibiotics into the plaques and the bacterial biofilm (Löhr et al. 2011).

Recent approaches for new therapeutic strategies against *P. gingivalis* focus on blocking the very early steps of the bacterial adhesion to the host cells. Over the past few years plant derived natural products, mainly polyphenols and polysaccharides (Löhr, Beikler, and Hensel 2015) have been described as a putative adjunctive therapy that aims to reduce inflammation as well as interaction with the bacterial adhesion of *P. gingivalis*.

Limonium brasiliense (Boiss.) Kuntze (Plumbaginaceae) is an herbaceous plant that occurs in Argentina, Uruguay and southern coast of Brazil. The rhizomes are traditionally used in the treatment of premenstrual syndrome, menstrual disorders and urinary tract infections (Fenner et al. 2006). Recent i*n vitro* studies indicate bacteriostatic, antiinflammatory and antioxidant activity of hydrophilic extracts from this plant (Fenner et al. 2006). The herbal material is characterized by the presence of hydrolyzable tannins, flavan-3-ols with trihydroxylated B-ring, A- and B-type prodelphinidins, leucoanthocyanidins, flavonoids, β-sitosterol, saponins and coumarins (Blainski, Lopes, and de Mello 2013).

As in previous studies on antiadhesive natural products it has been shown that galloylated gallotannins (Löhr, Beikler, and Hensel 2015) and oligomeric proanthocyanidins with at least dihydroxylated B-ring are strong inhibitors of gingipains from *P. gingivalis* (Schmuch et al. 2015) it seems interesting to investigate a proanthocyanidin-enriched extract of *L. brasiliense* due to its high content of oligomeric prodelphinidins and other proanthocyanidins against the adhesion of the bacterium.

2. Materials and methods

2.1. Chemicals and Reagents

Chlorhexidine digluconate (CHX) 20 % (w/w) solution, fluorescein isothiocyanate (FITC) and N_{α}-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) were purchased from Sigma-Aldrich, Steinheim, Germany. Leupeptin (LEU) and N_{α}-Tosyl-L-lysinchlormethylketone hydrochlorid (TLCK) were obtained from AppliChem, Darmstadt, Germany. Ac-Lys-pNA was obtained from Bachem, Bubendorf, Switzerland. All reagents and solvents were in analytical grade.

2.2. Plant material, preparation of test extract for functional testing

Rhizomes of *L. brasiliense* (Boiss.) Kuntze were collected in Rio Grande, in the state of Rio Grande do Sul, Brazil (S 31° 59' 33"/W 52° 10' 43") in February 2013. A voucher specimen is deposited at the Herbarium of the State University of Maringá (No. HUEM-27725). The plant material was collected under a permit from IBAMA-SISBIO, No. 11995-3, granted 02.11.2010, authentication code 46367613, under the responsibility of J. C. P. Mello. Access to the botanical material was authorized and licensed by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), registered under No. 010252/2015-0. The plant material was authenticated by Prof. Dr. Lilian A. Mentz, UFRG, cleaned with water to remove soil, dried in a circulating-air oven (37 ± 2 °C) and was powdered and sieved by use of a hammer mill (Tigre ASN-5; mean particle diameter 0.42 mm).

One gram of the plant material was homogenized with 10 mL of acetone:water (7:3, v/v) for 30 min on a rotating shaker. Subsequently, the resulting suspension was extracted in an ultrasonic bath (Sonorex RK 100, Bandelin, Berlin, Germany) for 30

147 min at room temperature, followed by centrifugation (5,000 × g, 5 min). The clear
148 supernatant was dried in a SpeedVac® (RVC 2-25 plus, Martin Christ, Osterode,
149 Germany) overnight at 40 °C and 1,000 rpm. The yield of this extract (named in the
150 following as LBE) was 26.3%, related to the starting material. For functional
151 investigations the dry extract was solubilized in dimethylsulfoxide (DMSO) and the
152 final concentration in each test was set to 0.5%.
153
154 *2.3. Cell culture*
155 *P. gingivalis* strain (ATCC 33277) was cultivated on solid medium (per 1 L: 16 g agar,
156 15 g trypticase peptone, 5 g neutralized soja peptone, 5 g sodium chloride, 5 g yeast
157 extract, 0.5 g L-cysteine, 50 ml sheep blood, enriched with 10 mg vitamin K and 5 mg
158 hemin) at 37 °C under anaerobic conditions, generated by Anaerogen™, Oxoid-Thermo
159 Scientific chamber (Cambridge, UK). Passaging was performed after 3 days. Liquid
160 culture media were composed as described above, but without agar and blood. Bacteria
161 were inoculated in a 25 cm^2 cell culture flask at OD_{660} = 0.1 and incubated for 24 h
162 (Schmuch et al. 2015).
163 Human epithelial cells line KB (KB cells - ATCC CCL-17 - HeLa) were
164 obtained by Dr. S. Eick (University of Jena, Germany). Despite the fact that this cell
165 line might be contaminated with HeLa cells (Capes-Davis et al. 2010), a plenty of
166 publications support this cell line as model for study the interaction with *P. gingivalis*
167 (Schmuch et al. 2015). KB cells were cultivated in Earl's minimum essential medium
168 (EMEM) (Biochrom, Berlin, Germany), supplemented with 8% fetal bovine serum
169 (FBS Superior, Biochrom, Berlin, Germany), 1% minimum essential medium (MEM)
170 non-essential amino acids (100 × solution) (GE Health Care Products, Freiburg,

Germany) and 50 µg/mL gentamicin (Sigma-Aldrich, Steinheim, Germany) at 5% CO_2/37 °C. Passaging was performed twice a week.

2.4. Influence of LBE on proliferation of P. gingivalis

Cytotoxicity test of LBE in liquid medium was performed by using a bacterial suspension (OD_{660} 0.1) and incubation for 24 h. Subsequently, the OD_{660} was adjusted to 0.2 and 100 µL of this suspension were added to 96 well plates (Sarstedt, Nümbrecht, Germany). Test solutions (100 µL) of LBE, positive control (CHX) or the respective control solutions were added and the plate was incubated for 24 h under anaerobic conditions. Twenty microliters of a solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 40 mg/mL were added (final concentration 0.36%) and the plate was incubated for 1 h at 5% CO_2/37 °C. The concentration at which no visible growth, i.e the reduction of TCC by the bacteria was absent (in other words: there is no detectable viable cells by this method) was regarded as minimum inhibitory concentration (MIC).

2.5. Influence of LBE on viability of KB cells

Influence of LBE on cell viability of KB cells was monitored by MTT assay (Mosmann 1983) by using a 24 h incubation time of KB cells (5×10^4 cells/well) with the test solutions. Positive control: medium, supplemented with fetal bovine serum 10 %. Additionally to LBE also CHX was tested to compare a potential cell toxicity of LBE with the gold standard in periodontitis therapy, CHX. Relative viability is expressed as percentage of MTT reduction, being the untreated control normalized to 100%.

2.6. Influence of LBE on adhesion of P. gingivalis to KB cells: plate fluorimetric

assay

KB cells were seeded in 96 well plates and incubated for 24 h. Bacterial liquid culture of *P. gingivalis* was centrifuged at 5,000 ×g for 10 min. The bacterial pellet was washed with PBS once, transferred to a 1.5 mL centrifuge tube and centrifuged at 7,500 rpm for 5 min (Centrifuge Hettich Mikro 120, Hettich, Tuttlingen, Germany). Subsequently, the pellet was resuspended in $NaHCO_3$ buffer 0.5 Mol/L, pH 8.0, the OD_{660} was adjusted to 0.4/100 µL and a solution of fluorescein isothiocyanate (FITC) (5 mg/mL) was added (10 µL) to obtain a final concentration of 50 µg/mL. The mixture was incubated at 37 °C for 30 min. FITC-labelled bacteria were washed 3 × and resuspended in EMEM without any supplementation at OD_{660} = 0.3.

Two hours before adding the FITC-labelled bacteria, the KB cells were washed two times with PBS, to remove any antibiotic. EMEM without supplementation was added and the plate was incubated.

Test solutions were added to the KB cells at a bacteria cell ratio (BCR) of 100:1. The plate was incubated for 90 min. The cell monolayer was washed twice with PBS and 100 µL of PBS was added. Readout was performed at $\lambda_{exc.}$ = 485 nm and λ_{em} = 538 nm in a Fluoroskan Ascent plate fluorometer (Thermo Fisher Scientific, Waltham, USA) (Messing 2013).

2.7 Influence of LBE on adhesion of P. gingivalis to KB cells: flow cytometric assay

1.5×10^6 KB cells/well were incubated for 24 h at 5% CO_2 /37 °C, washed and incubated with medium. FITC labelled *P. gingivalis* were used at OD_{660} 0.9 at a BCR of 100:1 in the coincubation assay together with the test solutions. The incubation mixture was incubated for 90 min at 5% CO_2/ 37 °C. Non-adherent bacteria were removed and

219 the remaining KB cell monolayer was washed three times with PBS. Cells were
220 detached with trypsin (10 × diluted) for 5 min in a CO_2 incubator. Trypsin activity was
221 stopped by addition of EMEM, containing 10% of FBS. Cells were transferred to a 15
222 mL tube and centrifuged for 400 ×g for 3 min in a refrigerated centrifuge (Eppendorf
223 5810R, Hamburg, Germany). The supernatant was discarded and 1 mL of PBS was
224 added. The samples were analyzed in a flow cytometer (FACScalibur, Becton,
225 Dickinson, Heidelberg, Germany) with the following parameters: FCS (Detector): E-1
226 (Voltage), 3.07 (Amp Gain), Lin (Mode); SSC: 332, 1.00, Lin; FL1: 360, 1.00, Log;
227 FL2: 350, 1.00, Log; FL2-A:-, 1.00, Lin; FL3: 570, 1.00, Log; FL4: 568, -, Log (Löhr et
228 al. 2011).
229
230 *2.8. Influence of LBE on gingipain activity*
231 A 3 day agar culture of *P. gingivalis* was harvested and resuspended in buffer (100 mM
232 Tris·Cl, pH 7.6; 75 mM NaCl; 2.5 mM $CaCl_2$; 10 mM L-cysteine). The OD_{660} was
233 adjusted to 0.03 and 0.006 for Kgp and Rgp activity respectively and the suspensions
234 were transferred to a 96-well plate. Solutions, containing the respective test compounds
235 (LBE, CHX, leupeptin, a specific inhibitor for Rgp, TLCK, a specific inhibitor for Kgp)
236 were added and the plate was incubated at room temperature for 10 min. The substrates
237 BAPNA for determination of Rgp activity and Ac-Lys-pNA for Kgp activity were added
238 both at 0.5 M and the absorbance was read at 405 nm over 30 min in each minute in a
239 microplate reader SpectraMax Plus 384 (Molecular Devices, München, Germany).
240 Protease activities were related to the untreated control (= 100 %) (Cronan et al. 2006,
241 Potempa and Nguyen 2007, Schmuch et al. 2015).
242

243 *2.9. Fractionation of LBE_2*

244 Powdered herbal material (300 g) from rhizomes of *L. brasiliense* were extracted by use
245 of a VDI-25 disperser (VWR, Darmstadt, Germany) at 9,500 rpm and 3 L of a mixture
246 of acetone: water (7: 3 v/v) in a cycle of 5 min of agitation and 5 min of maceration.
247 After centrifugation of the suspension the residual pellet was reextracted twice under the
248 same conditions. In total 9 l of solvent had been used. The organic solvent was removed
249 by rotary evaporation at 40 ° C and the remaining aqueous phase was lyophilized. The
250 yield of the extract (named LBE_2 in the following) was determined with 38.6 % (w/w),
251 related to the dried the starting material.

252 Fifty grams of LBE were partitioned between water and ethyl acetate (1: 1 v/v, 1
253 L) for five times. After separation of the two phases, the organic solvents were removed
254 by rotary evaporation; water was removed by lyophilization. The yield of the ethyl
255 acetate fraction (LBE_{2EA}) was 8.7 % (w/w) and that of the aqueous fraction (LBE_{2w})
256 was 86.2 % (w/w), related to LBE_2.

257 1.90 g of the ethyl acetate fraction LBE_{2EA} was dissolved in ethanol 96 % (v/v)
258 and fractionated on Sephadex® LH-20 stationary phase (General Electric, Munich,
259 Germany). Column dimension: 690 × 28 mm; mobile phase: step gradient: ethanol 96 %
260 (8,750 mL), methanol 100 % (3,000 mL) and acetone: water 7: 3 (1.000 mL). Flow
261 rate: 0.75 mL/min; fraction size: 15 mL. All fractions were investigated by TLC (silica
262 gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany), mobile phase ethylacetate:
263 water:formic acid (90:5:5; v/v/v), detection by vanillin-HCl spray reagent). Fractions
264 with similar composition were combined, the solvent was evaporated and the fractions
265 lyophilized. This chromatographic separation yielded 17 fractions (I to XVII). Fractions
266 VI (62 mg, 157.5 mL after 560 mL [from beginning], 3.26%), X (243 mg, 570 mL after

2,300 mL [from beginning], 12.8%), XII (75.6 mg, 720 mL after 5,045 mL [from beginning], 3.9%) and XIII (206.7 mg, 765 mL after 6,165 mL [from beginning], 10.9%) were submitted to mass spectrometry and NMR analysis.

2.10. ESI – MS/MS and ^{1}H NMR

Electrospray ionisation mass spectrometry (ESI-MS/MS) analysis was carried out using a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (MS) (Waters, Milford, MA, U.S.A.). Electrospray ionization (ESI) source was operated in the positive and negative mode. Data were acquired and processed using MassLynx™ version 4.0 software (Waters Corp., Milford, MA, U.S.A.). MS parameters: capillary voltage 2.5 kV, extraction cone 4 V, desolvation temperature 450 ºC, cone 25 V, source temperature 120 ºC, cone gas flow 40 L/h, desolvation gas flow 700 L/h and collision energy of 10-35 eV were used. Test samples (1 mg/mL in water-acetonitrile 1:1, v/v) were directly injected into the MS via syringe pump at a flow rate of 40 µL/min. For collision-induced dissociation (CID) experiments, argon was used as the collision gas. The identity of compounds was assessed by the product-ion spectrum (MS/MS spectrum) in comparison with published data.

^{1}H-NMR spectra were recorded in CD_3OD (Cambridge Isotope Laboratories Inc., Andover, MA, USA) methanol-D4 (D, 99.8%) in a Bruker Avance III at 400 MHz.

2.11. Statistical analysis

Results are presented as means ± standard deviation of the mean (SD) of 3 independent experiments. Data were analyzed by one way analysis of variance (ANOVA) and Tukey post-test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

3. Results and Discussion

3.1. LBE does not influence cell vitality of P. gingivalis and KB cells

From the dried rhizomes of *L. brasiliense* an acetone/water extract was prepared from which after removal of the solvents and lyophilization the dry extract LBE was obtained in a yield of 26.3 % (w/w) related to the starting material. LBE up to 1000 µg/mL did not influence the bacterial proliferation *P. gingivalis* in liquid culture over 24 h incubation time (data not shown).

LBE had no influence on the cell viability of KB cells as shown by MTT assay (Mosmann 1983) in a concentration range from 50 to 500 µg/mL over a 2 h incubation interval (Fig. 1A). Prolonged incubation to 24 h resulted in significantly reduced mitochondrial dehydrogenase activity at higher concentrations (Fig. 1B). In contrast these investigations indicated also the quite high toxicity potential of CHX, the gold standard medication in clinical treatment of periodontitis, which has been used as positive control in the experiments (Fig. 1). It is well know that CLX exerts problems in regard to toxicity against mammalian cells *in vitro* and periodontal tissues (Hidalgo and Dominguez 2001), (Giannelli et al. 2008). By comparison of the minimal inhibition concentration (MIC) of CHX against *P. gingivalis* with the toxicity of CHX against KB cells a selectivity index of 0.7 can be calculated which indicates this common antimicrobial agent is even more toxic for eukaryotic cells than to prokaryotic cells.

From these data it was concluded that LBE will not exert toxic effects against the pro- and eukaryotic cells when short incubation intervals and low concentrations of ≤ 100 µg/mL are used for *in vitro* investigations. These *in vitro* data are in congruence with *in vivo* animal experiments in which an acetone:water extract from *L. brasiliense* showed low or no toxicity against male and female Swiss mice in an acute toxicity test

315 (Antonelli-Ushirobira et al. 2015). Although during chronic toxicity tests no toxicity in
316 Wistar rats no toxic effects of this extract has been detected (Antonelli-Ushirobira et al.
317 2015). The same authors proved absence of mutagenic potential and absence of
318 hepatotoxic activity by the extract (Antonelli-Ushirobira et al. 2015). These findings
319 define this acetone:water extract to be suitable for further investigations concerning
320 potential use of LBE for treatment of periodontitis.
321
322 3.2. *LBE inhibits adhesion of P. gingivalis to KB cells*
323 The influence of LBE on the adhesion of *P. gingivalis* to KB cells was analysed
324 quantitatively by two different assays, by use of a fluorimetric 96-well plate assay as
325 well as within a flow cytometric assay. In principle, FITC-labelled bacteria are
326 coincubated together with a monolayer of KB cells in the presence or absence of LBE.
327 Subsequently, non-adhering bacteria are removed by washing. Within the 96-well plate
328 assay the resulting fluorescence of the KB cell monolayer with attached FITC-labelled
329 bacteria was measured in total. For the more sensitive flow cytometric assay the cell
330 monolayer with adhering or invaded FITC-bacteria was trypsinised and the fluorescence
331 of individualized KB cells was quantified by flow cytometry.
332 LBE showed a strong antiadhesive activity within the plate fluorimetric assay
333 (Fig. 2A) as well as in the flow cytometric evaluation (Fig. 2B). Interestingly,
334 significant inhibition of bacterial adhesion (residual adhesion of 79 ± 11 %) was
335 obvious in the 96-well assay only at a LBE concentration of 100 µg/mL. Higher extract
336 concentrations were ineffective, which was probably due to absorbed polyphenolic
337 compounds to the plate material and leading to typical quenching effects of fluorescence
338 (Fig. 2A). This problem was easily overcome by establishing the flow cytometric assay,

339 which showed concentrations-dependent and significant inhibition of the adhesion of *P.*
340 *gingivalis* to KB cells (Fig. 2B).
341
342 *3.3. LBE inhibits Arg-gingipain*
343 In order to investigate the influence of LBE on the major adhesins of *P. gingivalis*, the
344 arginin- and lysine-specific gingipain activities were monitored during incubation with
345 the test extract. For differentiation of the effects on the Arg- and Lys-gingipain
346 activities, peptide substrates with a colorimetric moiety (nitroanilide) were used for
347 protease assays after incubating the bacteria with LBE, namely Bz-Arg-pNA (N_α-
348 Acetyl-L-lysine-4-nitroanilide) for Arg-gingipain and Ac-Lys-pNA (N_α-Acetyl-L-lysine-
349 4-nitroanilide) for Lys-gingipain (Pike et al. 1996). The tripeptide leupeptin (5 µM), a
350 specific inhibitor of Arg-gingipain, served as positive control (Baba et al. 2001). LBE
351 inhibited Arg-gingipain in a concentration-dependent manner (Fig. 3A). In contrast Lys-
352 gingipain activity was only influenced to a lower extend (Fig. 3B). In further
353 experiments also CHX was investigated if this compound also interacts with the
354 bacterial adhesion, but up to 500 µg/mL no inhibitory activity was found (data not
355 shown).
356
357 *3.4. Fractionation of LBE_2 and phytochemical characterization of selected fractions*
358 LBE_2 was subjected to a fractionation and preliminary phytochemical characterization.
359 The extract was portioned between ethylacetate and water, yielding the EtOAc extract
360 LBE_{2EA} and the water extract LBE_{2W}. According TLC investigations LBE_{2EA} contained
361 quite high amounts of flavan-3-ols and oligomeric proanthocyanidins up to the degree
362 of polymerization of 3 to 4.

Both fractions, LBE_{2EA} and LBE_{2w}, were tested on potential antiadhesive activity at a concentration of 50 µg/mL by flow cytometric adhesion assay. Significantly reduced bacterial adhesion of *P. gingivalis* was observed for both fractions: LBE_{2EA} reduced the adhesion by 26.9 ± 6.7% while LBE_{2w} diminished the adhesion by 28.9 ± 15.1%.

LBE_{EA} was subjected to fractionation on Sephadex® LH 20 stationary phase using a step gradient of ethanol, methanol and acetone-water (7:3, v/v). This chromatographic separation yielded 17 fractions I to XVII. Fractions VI, X, XII and XIII were submitted to analytical investigations by ESI-MS/MS and ^{1}H-NMR.

The ESI-MS/MS spectra in the negative mode of fraction VI indicated *m/z* of 169 $[M-H]^-$ with fragmentation to *m/z* of 125. Chemical shifts of this fraction within ^{1}H-NMR showed a singlet at 7.08. These data are in congruence with the presence of gallic acid (Fig. 4; compound **1**) (Fracassetti et al. 2013) (Braca et al. 2003).

MS analysis of fraction X revealed *m/z* of 457 $[M-H]^-$ and fragments of *m/z* 331, 305, 169. The chemical shifts in ^{1}H-NMR (δ ppm) [4.99 (1H, *s*, H-2), 5.55 (1H, m, H-3), 2.78-2.94 (2H, *m*, H-4), 5.99 (2H, *s*, H-6 and H-8), 6.54 (2H, *s*, H-2' and H-6'), 6.98 (2H, *s*, H-2'' and H-6'')] indicated this compound to be epigallocatechin-3-O-gallate (Fig. 4, compound **2**). The respective data are in congruence with published data (Baselga-Escudero et al. 2014). The presence of epigallocatechin 3-O-gallate and gallic acid in *L. brasiliense* has been documented already, together with strong anti-oxidant activity (Murray et al. 2004).

In fraction XII a signal *m/z* 759 $[M-H]^-$ was detected besides the fragments of *m/z* 607, 589, 481, 463, 423. The ^{1}H-NMR (δ ppm) spectrum showed a typical chemical shift for the ether linkage between the C-2' and the C-3'''' position at a potential galloyl

387 residue [6.75 (1H, *s*, H-6') and 7.04 (1H, *d*, *J* = 1.96 Hz, H-6"") 7.51 (1H, *d*, *J* = 1.2 Hz,
388 H-2"")] Additionally the presence of the signal δ 4.32 (1H, *s*, H-3) relative to the
389 hydroxyl group at the C-3 group is strong indicator for the presence of samarangenin A
390 (Fig. 4, compound **3**), a compound typical for the rhizomes of *L. brasiliens* (Andressa
391 Blainski Pinha, personal communication, 2016), (Lin, Kuo, and Chou 2000), (Nonaka et
392 al. 1992). A differentiation to samaragenin B is possible by using the signals for H-3,
393 the position were both compounds differ from each other. In our investigations a shift of
394 a singlet at 4.32 ppm was founds, while reported a 4.33 ppm doublet for samaragenin A
395 with a coupling of 2 Hz (Nonaka et al. 1992). This difference might be related to the
396 different solvents (CD_3OD vs. acetone-d_6 + D_2O used by (Nonaka et al. 1992).
397 The signal at *m/z* 911 [M- H] in the fraction XIII, its fragmentation pattern *m/z*
398 of 759, 741, 589, 571, 445, 423 and the ^{1}H-NMR data are similar to those found for
399 samarangenin A, except for the difference in the chemical shifts at δ 5.73 ppm (1H, *m*,
400 H-3) and 6.92 ppm (2H, *s*, galloyl). These data are interpreted to prove the presence of
401 samarangenin B (Fig. 4, compound **4**) (Andressa Blainski Pinha, personal
402 communication, 2016), (Lin, Kuo, and Chou 2000).
403 From these phytochemical data the antiadhesive effect of LBE can be explained
404 by the flavan-3-ols and oligomeric derivatives. Especially the compounds with
405 trihydroxylated B-ring are known for a strong interaction with the bacterial adhesion of
406 *P. gingivalis* and gingipains (Schmuch et al. 2015). Also green tea polyphenols have
407 been studied as a tool for combating *P. gingivalis* with epigallocatechin 3-O-gallate as
408 the major active compound with the galloyl part being essential for the anti-adhesive
409 effect (Sakanaka et al. 1996).
410 The here described finding pinpoint *L. brasiliense* extract as a valuable and

potent source for the development of oral care products against periodontitis. Detailed clinical infection studies are needed to clarify the antiperiodontal effect of this extract and the respective polyphenols.

Conclusion

Polyphenol-enriched acetone:water extract from *L. brasiliense* could have an impact for the development of oral care products against periodontitis because of its specific inhibitory activity against the virulence factors of *P. gingivalis*. More studies are needed in order clarify the anti-virulence effects of the extract and the isolated compounds, especially the unusual samaragenins, within clinical trials in humans.

Conflict of interest

The authors declare no conflicts of interest.

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Figures:

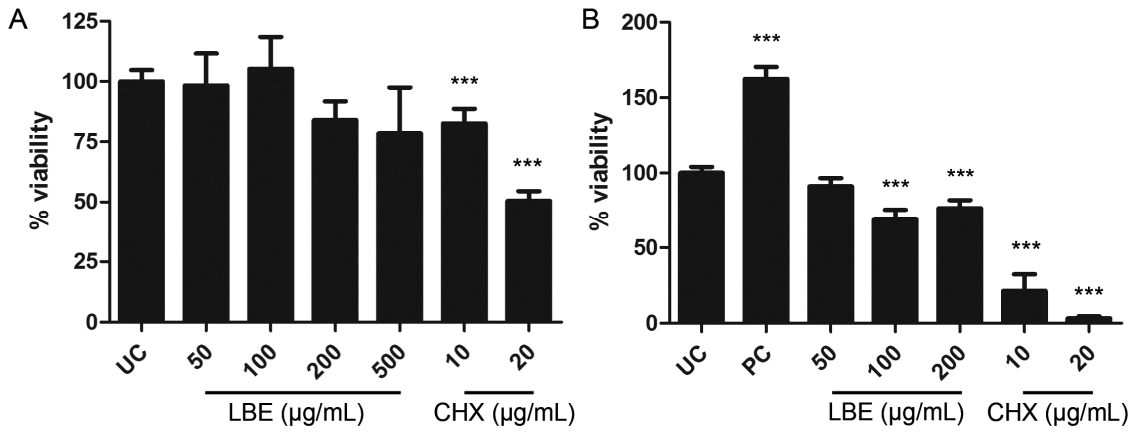
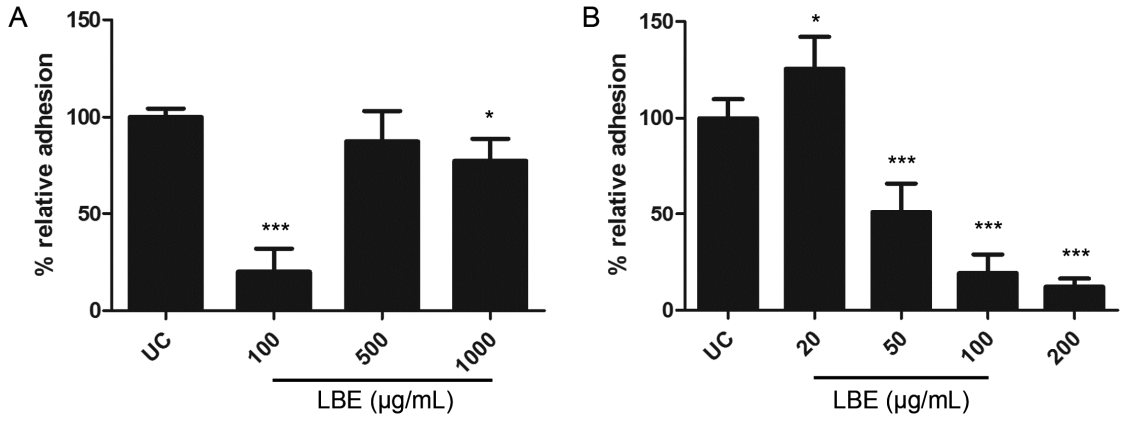


Figure 1: Relative viability (MTT assay) of KB cells after 2 h (**A**) and 24 h (**B**) treatment with different concentrations of extract LBE from *L. brasiliense* and Chlorhexidine (CHX). Relative viability is related to the untreated control UC (= 100 %). FBS supplemented medium (10 %) served as positive control (PC). Experiments are expressed as mean ± SD (%) from three independent experiments, *** $p < 0.001$.



443

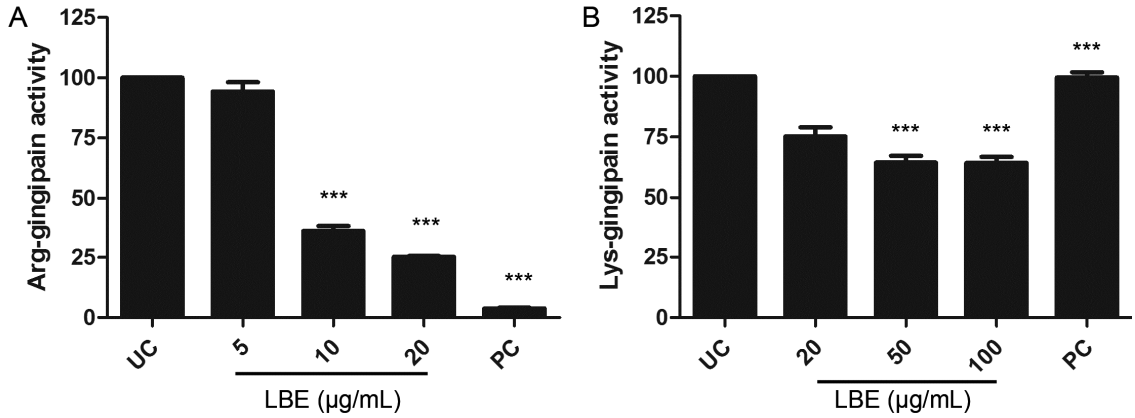
444 **Figure 2:** Influence of LBE on the relative adhesion of FITC-labelled *P. gingivalis* to

445 KB cells within co-incubation experiments (90 min) in a 96 well plate fluorimetric as-

446 say (**A**) and by flow cytometric evaluation (B). Results are related to the untreated con-

447 trol UC (= 100%). Results are expressed as mean ± SD (%) from n = 3 independent

448 experiments. * $p < 0.05$; *** $p < 0.001$.



449

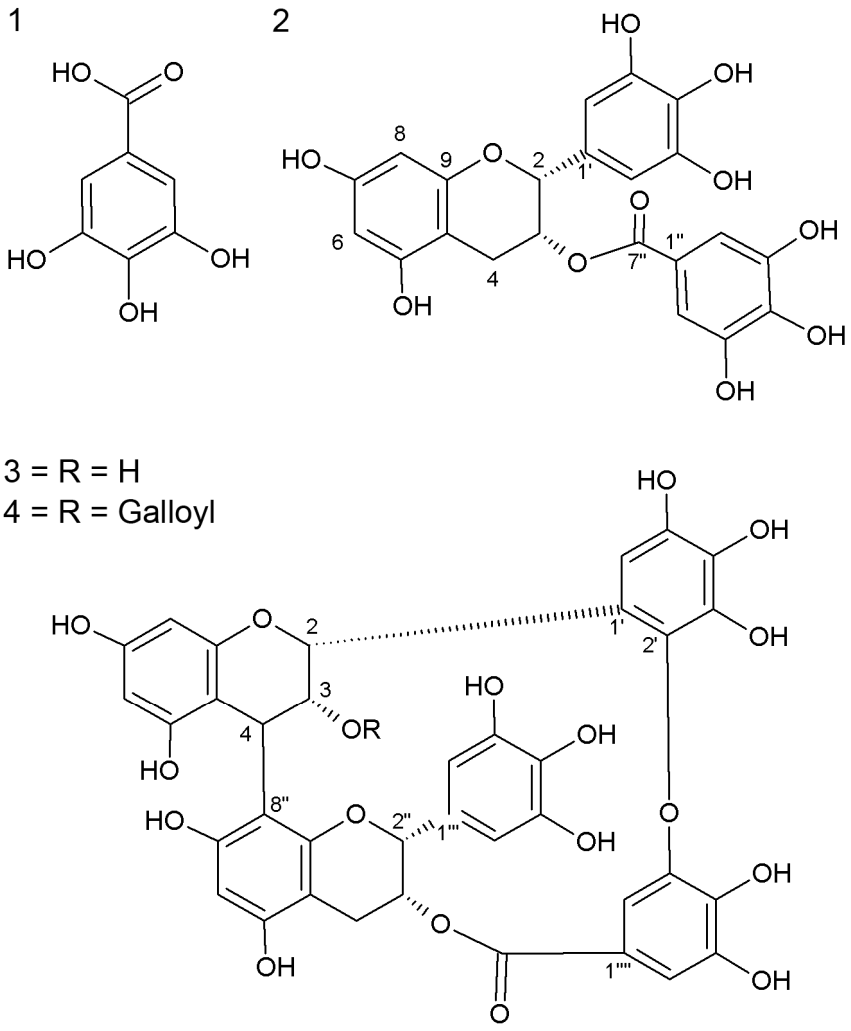
450 **Figure 3**:

451 Influence of LBE (5, 10 and 20 µg/mL) on Arg-gingipain (**A**) and Lys-gingipain (**B**)

452 protease activity in relation to untreated *P.gingivalis*. Leupeptin at 5 µM served as posi-

453 tive control (PC), untreated bacteria as negative control (UC). Data are mean ± SD (%)

454 from 3 independent experiments with n = 3 replicates . ***: $p < 0.001$.



455

456 **Figure 4:** Structural features of identified compounds from *L. brasiliense*. **1** gallic acid;

457 **2** epigallocatechin gallate; **3** samaragenin A **4** samarangenin B.

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CAPÍTULO 02

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Helicobacter pylori infection therapy: Promising new synthetic compounds

Caleare Ade O *et al Helicobacter pylori* therapy by synthetic compounds
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Abstract

Helicobacter pylori is a Gram-negative bacterium, an important pathogen that colonize the human stomach and survives in the adverse conditions of the gastric mucosa in approximately 50% of worldwide population. The infection is highly prevalent and causes gastrointestinal diseases as gastritis and gastric cancer that is one of the leading causes of cancer related death in the word. To date, there is no efficient strategy for the eradication of these microorganism and the current therapy strategies against *H. pylori* infection are becoming less efficient due the problem of the antibiotic resistance. Thus, by the enormous medical importance given the carcinogenic potential of *H. pylori*, new options for the treatment are needed to win the battle against these bacteria. In this context, we review the recent findings available in the literature about new synthetic compounds with antibacterial activity against *H. pylori*. The new studies for the synthesis of this type of molecules are related to the structural modification of moieties and substitutions in known synthetic substances. Thus, **r**ecently, the development of a series of *in vitro* studies have demonstrated that the class of quinoxaline, oligomers of acylated lysines, quinones, pyrazolopyrimidinediones, pyrazolines, coumarin-3-carboxamides and xanthones are promising compounds for the therapeutic eradication. However, further research is needed to use them in clinical treatment regimes.

Key-Words: *Helicobacter pylori*; Gastric cancer; Anti-bacterial agents; Gastrointestinal disease, Treatment, Antibiotic resistance

Caleare AO, Lancheros CAC, Nakamura TU, Mello JCP, Nakamura CV. Specific antibiotic therapy against *Helicobacter pylori* by new synthetic compounds.

Core tip: The infection caused by *Helicobacter pylori* is a recent discovery and the use of antibiotics to treat it and other infections, especially the broad spectrum, contribute to the complication of this condition. In the face of this, new alternatives are necessary and in a search on literature shows an immensity of

articles on natural products, mainly plants, that have activity against this bacterium, so we decided to consider the new options that the synthetic compounds can bring as an alternative to the antibiotics used.

Introduction

No one would be able to imagine that to let some blood agar plates inoculated with gastric biopsy specimens in the incubator for a few more days, in addition to the protocol, could take two researchers to win the Nobel Prize in Physiology or Medicine[1]. After the positive culture, Marshall and coworkers performed the characterization and identification of a Gram-negative bacterium that was oxidase, catalase and urease positive. It has different morphologies, when cultured *in vitro* is present as straight rods in comparison with gastric biopsies where it is S-shaped or curved[2]. This bacteria was primarily classified as *Campylobacter pyloridis*, but some different features and grammatical interpretations lead the researchers to transfers this microorganism to a new genus and species *Helicobacter pylori*[3].

Microbiological aspects

The most information about the physiology of *H. pylori* is about the primary culture after gastric biopsy. The microaerophilic characteristic is found in this culture. After successful grow, the established subcultures can grow in an incubator with 50 mL/L CO_2 and high umidity[4]. These bacteria are cultivated in an enriched medium, in agar plate, the tryptic soy agar plus 5% of sheep blood and for liquid culture, brain heart infusion (BHI) plus 10% of fetal bovine serum[5]. Exploring the antibiotic susceptibility of this bacterium is possible to formulate a selective medium. *H. pylori* is naturally resistant to vancomycin, trimethoprim, polymyxins, and some strains show susceptibility for nalidixic acid, cefsulodin, and antifungal compounds[6]. The 2,3,5-triphenyltetrazolium chloride (TTC) is a useful reagent and can be added at 40 μg/mL, in order to differentiate the *H. pylori* colonies[7,8]. Different selective media were proposed, but the most important factor is the use of a selective medium and later a non-selective medium, in order to achieve the successful isolation of *H. pylori*[9].

Epidemiology

It is well known that *H. pylori* infects nearly half of adult population in developed countries and can reach 90% in developing countries. If not treated, the infection can be persistent for the entire life[10,11]. *H. pylori* causes various gastrointestinal disorders such as peptic ulcers in 10% of infected individuals and gastric adenocarcinoma in around 1-2%[12].

The researchers quickly notice that this bacterium can be involved with cancer and in 1994 the World Health Organization classified it as a carcinogen class I in humans[13]. The mode of transmission is still unknown, because there is no definitive evidence about which is the route responsible for the infection, and there are no natural reservoir established[10].

Virulence factors

To achieve a successful colonization the bacteria must face a lot of challenge in host environment[14]. To live in the adversity of the stomach, *H. pylori* has mechanisms that protect it of the aggressions of this organ, leading to its establishment and the progression of the related diseases.

Urease

To circumvent the low pH of the stomach, this bacterium has the enzyme urease which breaks urea to ammonia and carbon dioxide, and the ammonia creates a coat with neutral pH encircling the bacteria (it is clear here that *H. pylori* is not an acidophilic microorganism[15]). The ureases are found primarily in the intracellular environment and secondly attached to the outer membrane, because of these, some bacterial cells are lysed[16].

In *H. pylori*, this enzyme is different from other bacterial ureases being consisted of two structural subunits (UreA and UreB), while for example, *Proteus mirabilis* has three subunits (UreA, UreB and UreC). Besides the two genes *ureA* and *ureB*, which code the structural region, the urease cluster has more seven genes (*ureC* and *ureD* in the regulatory region and *ureI*, *ureE*, *ureF*, *ureG*, *ureH* in the accessory region)[17]. Another important component of this enzyme system is the UreI, a proton-gated channel, where the urea is

translocated to the cytoplasm, to be cleaved by urease. Interestingly, it is closed at neutral pH and open in an acid environment, being essential for *H. pylori* infection[18,19].

Flagella

The flagella are needed to overcome the mucus layer overlying the stomach to reach the gastric epithelial cells and establish the infection. With polar flagella and a helical shape, the bacteria can easily penetrate and reaches the surface of the gastric cells by corkscrew movements[15,20].

Around 30 proteins compose the flagellar structure and between 40 to 100 are involved in produce a functional flagellum. The flagellin FlaA, the main filament protein and the FlaB the minor, are essential for motility and a study showed that mutants lacking this structure have a remarkably decrease in its ability to move throw the medium[21].

The flagellum can be divided into two substructures: the basal body and the extracellular filament. As *H. pylori* is a Gram-negative microorganism, the basal body starts in the cytoplasm and crosses the inner membrane, the peptidoglycan layers and the outer membrane. The hook is located outside the outer membrane and attached to basal body and the extracellular filament[20].

Adherence

The attachment of bacteria to the surface of host cells is the first step in the progression of an infection[22]. In *H. pylori*, various outer membrane proteins are able to bind to the gastric epithelium of the human stomach, which are classified as adhesins. The first adhesin described was BabA, that binds to the antigens of the blood group H type 1 and Lewis b expressed in gastric cells of secretor individuals[23]. After the attachment, *H. pylori* induces inflammation and enhances the antigen expression containing sialic acid in the gastric epithelium (sialyl-Lewis-X or sialyl Lewis A) thus, this bacterium passes to express another adhesin, which has an affinity for these antigens[24]. It was called sialic acid-binding adhesin (SabA) and severely contributes to the

chronicity of infection by this microorganism[25]. Several others adhesins have been identified in *H. pylori*, however its receptors remain unknown. In addition to these proteins, lipopolysaccharide (LPS) as well as integrins in the host play an important role in adhesion[26].

Exotoxins and other virulence factors

Different genes from *H. pylori* are related to the development of gastric cancer. The *cag* (cytotoxin-associated gene) island of pathogenicity, the most studied virulence factor of this bacterium, is a 40 kb segment of DNA and has 31 genes. Most of these genes encodes different proteins to form the secretion system type IV, which injects into the gastric epithelial cells, the CagA protein[12]. Within these cells, this protein can be phosphorylated by tyrosine kinases or follow a phosphorylation-independent pathway, inducing changes in the host cell signaling, which can cause a mitogenic response, leading to the development of neoplasms. The exact mechanism by which this protein promotes cancer is not yet clear[27]. It is not present in all strains, but in which it is present there is a higher risk to develop gastric cancer[28].

The vacuolating cytotoxin A (VacA), encoded by *vacA* gene, is also an important virulence factor in *H. pylori* for the development of gastric cancer. All strains contain this gene and its name is related to the ability to induce the formation of vacuoles in the mammalian cells[29,30]. Overall, the *vacA* genes display extensive polymorphism and have a three-domain structure, including the signal (which contains two distinct families of alleles, s1 and s2), intermediate (with i1, i2, i3 allelic families) and middle (with m1 and m2 allelic families). The combination of s1m1 allelic types results in enhanced virulence, leading to vacuolization and high risk to develop gastric cancer[29 31]. Among the structural and functional changes that it can cause, we can highlight the leaking of cytochrome c from mitochondria initiating apoptosis and membrane pore formation[32].

Other virulence factors play an important role in the progression to gastric cancer, among them we can emphasize the outer inflammatory protein (OipA), and the duodenal ulcer promoter gene (*DupA*)[33].

Not only the virulence factors of *H. pylori* can act in the development of gastric cancer, but also the host response, such as inflammation and reducing gastric secretion of acid and environmental factors as well[13]. In inflammation for example, macrophages are able to produce reactive oxygen species (ROS) that originates alterations to the DNA of gastric epithelial cells[34]. Environmental factors can increase the risk of developing gastric cancer, such as diet with high salt content, helminth infections and cigarette consumption[13].

Antibiotic treatment of *H. pylori* infections

The importance of *H. pylori* in emergence of severe gastroduodenal diseases as gastric cancer, peptic ulcer and other, have led to research in treatment strategies. Public health expenditures have greatly decreased with the discovery of *H. pylori* because antibiotics are cheaper than antacids, which the patient has to take it for a long period (about 10 times more expensive) and that the extreme procedure, vagotomy (removal of the vagus nerve). Usually the two-week regimen of antibiotic therapy is sufficient to eliminate the infection[35].

Among other options as probiotics and vaccines, the antibiotics are the first choice of treatment to eliminate the bacteria. However, due to the emergence of resistance, to date no therapy based on the use of a single antibiotic is useful.

Another challenge in the treatment of *H. pylori* is the gastric environment, firstly the low pH of stomach lead this bacteria to a non-replicate state, in that the antibiotics are usually not active[36]. Second, the pharmacokinetics in this organ is different from others. The drugs can face a lot of difficult to reach the bacteria, either by direct contact after oral ingestion, due to the permeability of the mucus layer or indirectly after intestinal absorption, due to the permeability of mucosa[37].

Another important point for a successful treatment is the determination of antimicrobial profile of *H. pylori* strains. The gold standard assay is based on the minimum inhibitory concentration (MIC)[38] which is performed in agar dilution test according to Clinical laboratory Standard Institute (CLSI)[39], due to the difficult of the bacteria to grow in the liquid medium. This method is laborious and does not allow a high throughput screening (HTS) for new molecules against this pathogen[9].

Specific therapy against single pathogens is emerging as a great opportunity to treat infections caused by bacteria. A successful example is the development of drugs for *Mycobacterium tuberculosis*, as isoniazid, pyrazinamide, ethionamide and ethambutol, that reaches the target differences between this and other bacteria, and clearly are not toxic to the host[40].

Three therapeutic lines that consist of combinations of antibiotics with other therapeutic agents are used for the treatment of these infectious diseases. Other treatment options have been described; however it is clear that the side effects are more pronounced, in addition to having a higher cost[36,41].

First line therapy

The first line treatment for eradication of *H. pylori* is called as triple theraphy, which generally uses two antibiotics, clarithromycin plus amoxicillin or amoxicillin plus metronidazole combined with inhibitors of the proton pump (PPI)[42]. The effectiveness of this treatment is 90% or more of healing, however, due to the prevalence of clarithromycin resistance by its wide use in gastrointestinal and respiratory infections, the success of this treatment has fallen to non-acceptable levels that is 80% or less in many countries. Thus, the first line therapy is recommended for regions with low clarithromycin resistance (< 20%), in regions with higher levels is suggested avoiding this antibiotic and use another therapy lines[43]. Among the alternatives are the sequential and concomitant therapies.

The sequential therapy emerges as an alternative in areas with high clarithromycin resistance. This regime has two stages, the first, 5 days of PPI

plus amoxicillin and the second, 5 days of PPI, clarithromycin and metronidazole treatment[42]. The concomitant therapy without bismuth salicylate, combines elements of the first and second line therapies consisting of PPI and three antibiotics administered concomitantly, amoxicillin, clarithromycin and metronidazole. This therapy also is effective in areas with clarithromycin and metronidazole resistance, with 90% of cure[41].

Second line therapy

This treatment line is an alternative to triple therapy and consists of metronidazole, tetracycline, proton pump inhibitor (PPI) and bismuth[41]. This is a quadruple treatment that displays good eradication rates when the first line therapy failed, especially in areas with high prevalence of clarithromycin resistance (> 20%). In the same way as the first line therapy, therapeutic problems have arisen, with the main difficulty related to side effects caused by the doses increase that result in an incomplete therapy. Another option of the second line, consists of levofloxacin, amoxicillin and PPI, showing satisfactory results in regions with high clarithromycin resistance[43].

Another alternative regime is the hybrid therapy is a combination of sequential and concomitant therapies proposed by Hsu *et al*[44]. This therapy has two treatment periods, each with seven days, the first with PPI and amoxicillin followed by a PPI, amoxicillin, clarithromycin and metronidazole.

Third line therapy

The lack of success with the first and second line therapies led to a last strategy where susceptibility testing is mandatory in the choice of the antimicrobial drug. A therapy showed good erradication rates is based on the rifabutin use, considered as anti-tuberculosis drug. On this case, side effects such as myelotoxicity, leucopenia, thrombocytopenia and the emergence of resistance are drawbacks related with less frequently use. The third line therapy is used after multiple treatment failures, and because, is known as salvage therapy[45].

Aim of this study

The goal of this work was the survey of new synthetic compounds against *H. pylori*. The surveys were carried out in the PUBMED database in the period from 2001 to the present year with the following terms: Helicobacter pylori; Synthetic Compounds; Treatments and anti-Helicobacter pylori activity.

New Perspectives in antibiotic therapy

Quinolones derivatives

Fluoroquinolones are synthetic quinolones that have biological activity against *H. pylori*, and this effect is related to the DNA gyrase activity inhibition. Minehart and Chalker (2001)[46] reported the activity of gemifloxacin, a new fluoroquinolone, against *H. pylori* clinical isolates from various different countries. By using the agar dilution method. MIC values of 0.06 mg/L, were reported for this compound, which was lower than ciprofloxacin, levofloxacin and moxifloxacin with (0.25 mg/L). In another way, Foroumadi *et al*.[47] investigated the structure–activity relationship (SAR) of a series of *N*-(phenethyl) piperazinyl quinolones derivated of ciprofloxacin, norfloxacin and enoxacin. The N-4 hydrogen of piperazinyl group of these substances was replaced with phenethyl moieties and the activity was evaluated against metronidazole-resistant and metronidazole-sensitive strains by the disc diffusion method (8, 16 and 32 µg/disc). This method relies on the dispersion of an antibiotic substance loaded in a paper disc (6 mm) through the solid agar medium on the plate. After the incubation period, the reading is made and the diameter of inhibition of the growth is measured. The classification in resistant, partially resistant and susceptible is standardized for the clinical antibiotics[48]. Among these substances, the ciprofloxacin derivatives showed significant activity, related to the cyclopropyl substituent at N-1 in the bicyclic ring. Against clinical isolates, the most active compound showed higher inhibition

zone diameter (22.2, 25.9 and 29.1 mm) than metronidazole (12, 16.3 and 17.6 mm) at 4, 8, and 16 µg/disc[47].

Bactericidal *in vitro* activity against *H. pylori*, similar to flouroquinolones was identified in another quinolone derivate, DX-619, a des-F(6)-quinolone. Takano *et al*.[49] evaluated the DX-619 by agar dilution method, against 293 *H. pylori* strains isolated from patients with gastrointestinal problems, in Japan and Russia. The results were compared with antimicrobial agents showing strong activity, MIC_{50} of 0.008 and MIC_{90} 0.06 mg/L, even against resistant strains to clarithromycin, amoxicillin, metronidazole and levofloxacin (The MIC_{50} and MIC_{90} mean the MIC values at which ≥50% or ≥90%, respectively of the isolates in a challenged population are inhibited)[50]. In levofloxacin resistant strains (32 mg/L) the DX-61 MIC values ranged from 0.06 to 0.5 mg/L. The authors conclude that these lower MIC values are related to a best interaction between the substance and the DNA gyrase[49].

Another group of quinolones, the fluoroquinolones, an antibacterial and anticancer agent was tested against *H. pylori* by Abu-Qatouseh *et al*[51]. In this study antimicrobial activity of five (3a-3e) fluoroquinolones with an 8-nitro substituent, the 8-nitrofluoroquinoline derivatives were tested in twelve clinical strains isolated from gastric biopsy samples and a metronidazole resistant strain. The disk diffusion method showed antimicrobial activity against *H. pylori* in all compounds (1 mg/mL), notably in compound 3c with maximum inhibition zones of 25 mm. In the same way, this compound showed the best activity by the two-fold agar dilution method with minimal inhibitory concentration (MIC) values ranged of 2-8 µg/mL. This compound also showed significant urease inhibition effect with IC50 value of 62.5 µg/mL. In another way, the compounds in combination with metronidazole were evaluated against resistant strains by the checkerboard titration method. The test showed synergistic activity of compounds 3a and 3d, that was related with alteration in the cell membrane permeability, which in turn, can favor the metronidazole entry.

1,3,4-thiadiazole Derivatives

The nitroheterocyclic compounds consist of 5-nitro group whose mode of action is related to formation of reactive oxygen species that cause DNA damage and results in cell death. Among these compounds, nitrofurans, nitroimidazoles and nitrothiophenes have been used by the pharmaceutical potential and nitroheterocyclic drugs as tinidazole and furazolidone have been studied as anti-*H. pylori* agents. In the same way, 1,3,4 thiadiazole derivates have shown interesting antibacterial activity, by the ability to bind to other molecules and thereby improve the pharmacological properties, related to the substituent and position of this attachment[52]. A broad series of molecules with these components were evaluated in similar studies. In this, the antibacterial activity of compounds and drugs as metronidazole and amoxicillin were tested by disk diffusion method against metronidazole-sensitive and resistant *H. pylori* strains and later, the antibacterial potential was evaluated against clinical isolates.

Foroumadi *et al*[53] evaluated 12 nitroheterocyclic compounds and the group of nitrothiophenes contain the ethylsulfonyl group showed remarkable antimicrobial activity against both strains (sensitive and resistant). In clinical isolates, the activity of this compound was higher than to metronidazole (8 µg/disk), nitrofuran and nitroimidazole derivatives (Table 1). Nevertheless, the compound showed greater cytotoxicity with IC_{50} values of ≤ 11.6 ± 2.2 mg/L against mouse fibroblasts (NIH/3T3). Other compounds showed less cytotoxicity, however less anti-*Helicobacter pylori* activity.

Similar results were obtained in other hybrid molecules with nitrofuran substituent at nitroaryl group[54]. This molecule had an additional group, the thiomorpholine ring that shown antimicrobial properties in drugs as nifurtimox and in linezolid analogs[55]. This compound shows potent activity (8 µg/disc) with inhibition zone diameter > 40 mm, against both (sensitive and resistant) strains. In clinical isolates, the most active compounds showed higher inhibition zone (36.1 mm) than by metronidazole (16.3 mm). Unlike the previous work, both nitrofuran and nitroimidazole showed better anti-*H. pylori* activity than

nitrothiophene derivatives. Promising results were also obtained in a nitrofuran with the substitution of the benzylthio moiety by the 2-chloro-6-fluoro group in the 1,3,4-thiadiazole nucleus. This compound showed anti-*H. pylor*i activity stronger than metronizadole, with inhibition zone at 8 µg/disk of 20 and 19 mm for sensitive and metronidazole resistant *H. pylor*i strains[56].

Nitrofuran synthetized compounds with another substitution containing 3-methoxybenzyl piperazine derivative at side chain 2-position, showed activity against metronidazole resistant strains. This moiety at C-2 position showed strong anti-*H. pylori* activity with inhibition zone diameters > 20 mm at concentrations from 25 to 100 µg/disk. When this moiety was combined with a nitrothiophen the activity was drastically reduced[57].

Compounds with 1-methyl-5-nitroimidazole moiety (5-position) and with the 3,5-dimethylpiperazinyl moiety (2-position) had strong anti-*H. pylori* activity with inhibition zone diameters of > 50 mm against both (sensitive and resistant) strains at 8, 16 and 32 µg/disk. In clinical isolates, the compounds showed similar activity that was dose dependent (0.5 - 16 µg/disk). The strong activity was confirmed in treatments of 0.5 and 1 µg/disk with inhibition zone > 20 mm while metronidazole showed inhibition zone < 10 mm[58].

Similar results were obtained in nitroimidazoles with a different moiety (4-methylphenyl) at 2-position. The activity in resistant strains was related to a possible interference of the compound in the resistance mechanism[59]. Nitroimidazoles with α-methylbenzylthio-pendant group at C-2 position were evaluated only against resistant strains (12.5 - 100 µg/disk) showed strong inhibitory activity with inhibition zone > 50 mm[60].

Studies with 1,3,4-thiadiazole derivatives concluded that the anti-*H. pylori* activity is altered by substituted-thio side chains at C2-position that's is also dependent of nitroaryl moiety (Table 1)[53,54, 56, 57, 58, 59, 60].

New compounds

SQ109 (Fig. 2.a) is an anti-tuberculosis drug that has a great accumulation at the stomach. Knowing this, Makobongo *et al*.[61] test this

compound against a set of strains and clinical isolates of *H. pylori* and found that it is active against all tested strains and isolates in a range of 6-10 μM for MIC and 50-60 μM for MBC. The mode of action against this bacteria is unclear, but the transmission electron microscopy analysis showed that the inner membrane and cytoplasmatic contend can be the target (Fig. 1).

Kamoda *et al*[62] evaluated the antibacterial activity of **TG44** (Fig. 2.b), an antimicrobial agent, against reference strains and clinical isolates of *H. pylori* and compared this activity with those of amoxicillin, clarithromycin and metronidazole. This agent showed excellent activity, even in resistant strains against these three antibiotics. By eletron microscopy the activity was related to detachment of outer membranes of *H. pylori* (Fig. 1). The activity against the type strain ATCC 43504 showed a MIC value of 0.5 mg/L. Against the clinical strains, it also showed good activity, where the maximum concentration was 1 mg/L. The pH of the stomach was not a problem for this compound, besides being highly stable in the pH range of 3 to 7, where at lower concentrations showed a bactericidal effect. The compound was found highly specific and showed no antibacterial activity against related bacteria.

In the same way, the molecule HPi1 (3-hydrazinoquinoxaline-2-thiol) (Fig. 2.c), was identified by high-throughput screen (HTS), and showed a MIC of 0.08-0.16 mg/L by broth microdilution with the ATCC 43504 strain. The compound had good selectivity for *H. pylori* when compared to microaerophilic and anaerobic gut bacteria. An *in vivo* experiment with mouse was also performed and this compound was also effective, where at the dose of 25 mg/kg body weight for 3 days, reduced the colony counts drastically. Both, *in vitro* and *in viv*o experiments, was not possible to isolate resistant mutants, another important feature of HPi1 as a promising agent in *H. pylori* therapeutics[63].

Makobongo *et al*.[38] tested the antimicrobial activity of synthetic peptides against five strains of *H. pylori*. Five peptide sequences of oligo-acyl-lysyl (OAK) group were synthesized, all of them showed antimicrobial activity and displayed stability and efficacy in temperatures ranging from 4 to 95°C since at

low pH. For the most effective peptide named $C_{12}K\text{-}2\beta_{12}$ (Fig. 2.d), an oligomer of acylated lysines, the MIC values ranged from 6.5 to 26 μM and the MBC values of 14.5 to 90 μM thus, with better activity than amoxicillin, in a molar-to-molar comparison. Besides, the activity regardless of bacterial growth was concluded that a possible mode of action of this substance is related to its interaction with phospholipids of cell membrane (Fig. 1). The following study with this compound was done in order to verify the *in vivo* activity in a model of infection using Mongolian gerbils and to solve the question about the mode of action. A treatment of 4 mg/kg body weight after 1 week of the initial infection with 3 doses reduced the bacterial load at the stomach and this compound has a mode of action, acting in the membrane and the biopolymers (DNA, RNA and proteins)[64].

Idebenone [6-(10-hydroxydecyl ubiquinone)] (Fig. 2.e), a quinone was effective against *H. pylori*. The ATCC 43504 strain was used and the MIC was 1.6-3.2 mg/L, this compound was approved for the use to treat the Friedreich's ataxia, so does not have toxicity for humans. As mode of action, it inhibits the respiration and reduces the ATP levels in this bacterium (Fig. 1)[65].

The series of pyrazolopyrimidinediones were found to contain great activity against an enzyme of peptidoglycan biosynthesis, glutamate racemase (Murl). The best substance was named *compound D* (Fig. 2.f) and it has a MIC of 1 mg/L against the strain SS1. This is a great results, but the authors stated that improvements are needed to ameliorates the solubility and the binding to proteins[66]. This enzyme was studied as target by the same author in 2015. The *compound A* (Fig. 2.g) showed a MIC of 0.25 mg/L against the strain SS1 but when tested *in vivo* failed to reduce the colonies counts[67].

Chimenti *et al*[68] used a series of N1-substituted 3,5-diphenyl pyrazolines to test against the ATCC 43504 strain and obtained that the compound named P8 (Fig. 2.h) had the better activity (1 mg/L).

Coumarin-3-carboxamides derivatives were also tested. In 2006, Chimenti *et al*[69] discovery that the compound C7 (Fig. 2.i), has a MIC of 0.25 mg/L by testing against the ATCC 43504 strain. Other series of synthetized

coumarins were evaluated and the compounds C10 e C11 (Fig. 2.j-k) showed a remarkably activity against the bacteria, the MIC against the strain ATCC 43504 was 0.031 and < 0.0039 mg/L respectively, and when tested against mammalian cells showed low toxicity by trypan blue method[70]. N-substituted-3-carboxamido coumarin derivatives were screened against this bacterium and for cytotoxicity for the selection of the best compound with selectivity index. The best MIC values of the compound 10 (Fig. 2.l) against the type strain were 4 µg/mL and for the cytotoxicity against the EAhy 926 cell line by the MTT method showed lower noxious effects at 50 mg/L[71].

Galano *et al*[72] demonstrated that flavodoxin inhibitors are good target for the therapy against *H. pylori*, this proteins is fundamental for the electron transfers in many bacterial metabolism. They synthetized compounds that not only have bounded to this protein, but those which had inhibited the growth of *H. pylori* and were not toxic to HeLa cells. The best compound (Fig. 2.m) has a selective index of 38, that means, the compound is 38 times more toxic to the bacteria than the mammalian cells.

Xanthones (Fig. 2.n-o) were also synthetized in order to evaluated its activity, and the compound 3 showed the best activity against the type strain (5 mg/L) and against the clarithromycin resistant strain, the compound 10 showed the best result (10 mg/L)[73].

Future perspectives

The specific therapy against *Helicobacter* pylori is a great opportunity for research. Once we have a compound with a good activity against this bacteria and good therapeutical index, not only with mammalian cells, but with other bacterial species, it can overcome the problem with the killing of our good microbiota, reduce the side effects of treatment and cure this infection.

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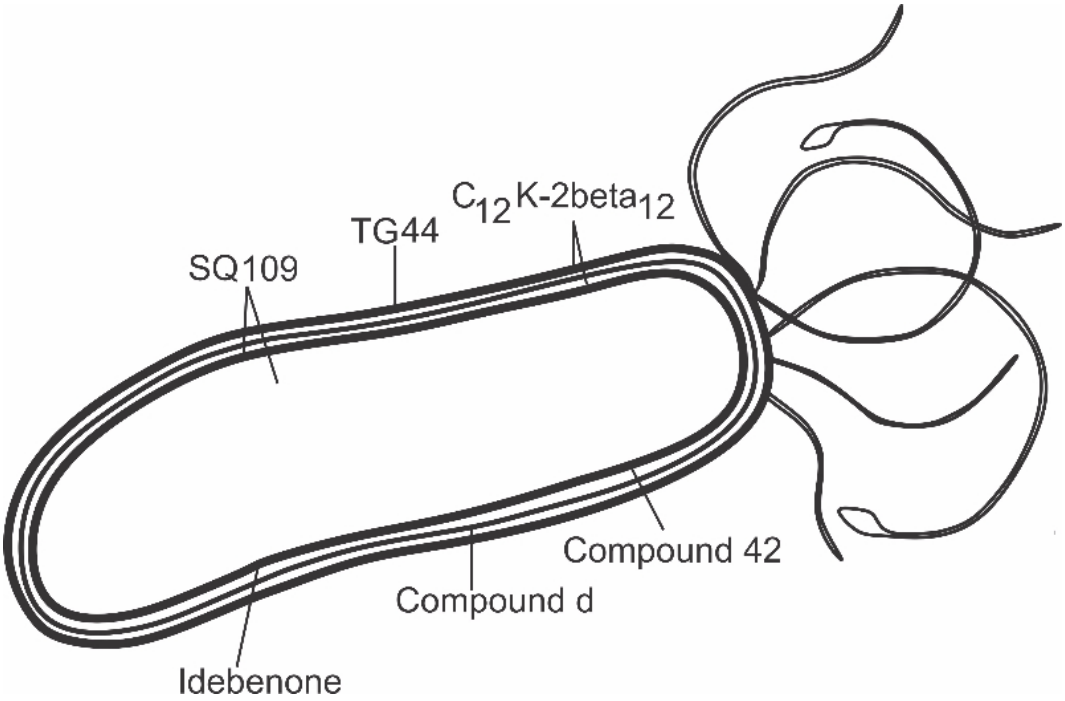


Figure 1 Mode of action of the compounds. SQ109 targets the inner membrane and the cytoplasmatic contend[61]. TG44 detached the outer membrane[62]. $C_{12}K$-$2\beta_{12}$ has as target the phospholipids of cell membranes[64]. Idebenone inhibits the respiration[65]. The *compound d* inhibits the peptidoglycan synthesis[66]. *Compound 42* meddles the flavodoxin electron transfer[72].

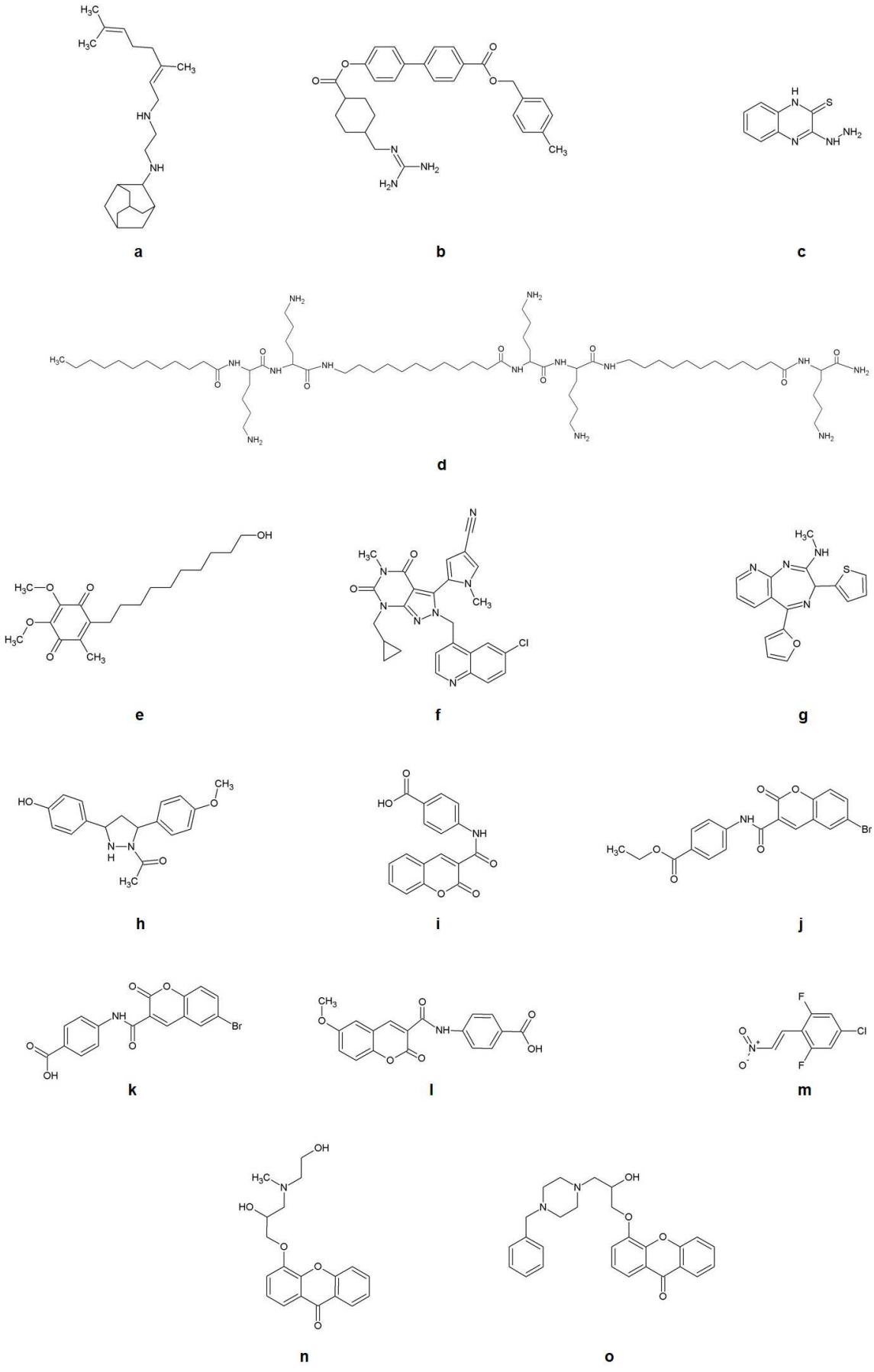


Figure 2. Chemical structure of anti-*H. pylori* agents

Table 1. Activity of some nitroaryl compounds with differents substituted-thiol side chain evaluated by disc diffusion against *H. pylori*. Measurements in millimeters (mm). *: Not evaluated. MNZ: Metronidazole

Nitroaryl moiety	**Substituted-thiol side chain**	**Metronidazole-sensitive strain (µg/disk)**			**Metronidazole-resistant strain (µg/disk)**			**References**
		8	**16**	**32**	**8**	**16**	**32**	
5-NO_2-thiophene	Ethylsulfonyl	> 50	> 50	> 50	> 50	> 50	> 50	[53]
	MNZ	18	23	27	11	12	14	
5-NO_2-furan	Thiomorpholine *S,S* dioxide	43	50	50	41	43	46	[54]
	MNZ	18	*	*	11	*	*	
5-NO_2-furan	2-chloro-6-fluoro	20	17	15	19	18	16	[56]
	MNZ	13	12	10	24	21	18	
1-Me-5-NO_2-imidazole	3,5-dimethylpiperazinyl	52	54	54	51	52	54	[58]
	MNZ	18	*	*	11	*	*	
1-Me-5-NO_2-imidazole	4-methylphenyl	23	32	42	21	29	38	[59]
	MNZ	18	*	*	11	*	*	

CAPÍTULO 3

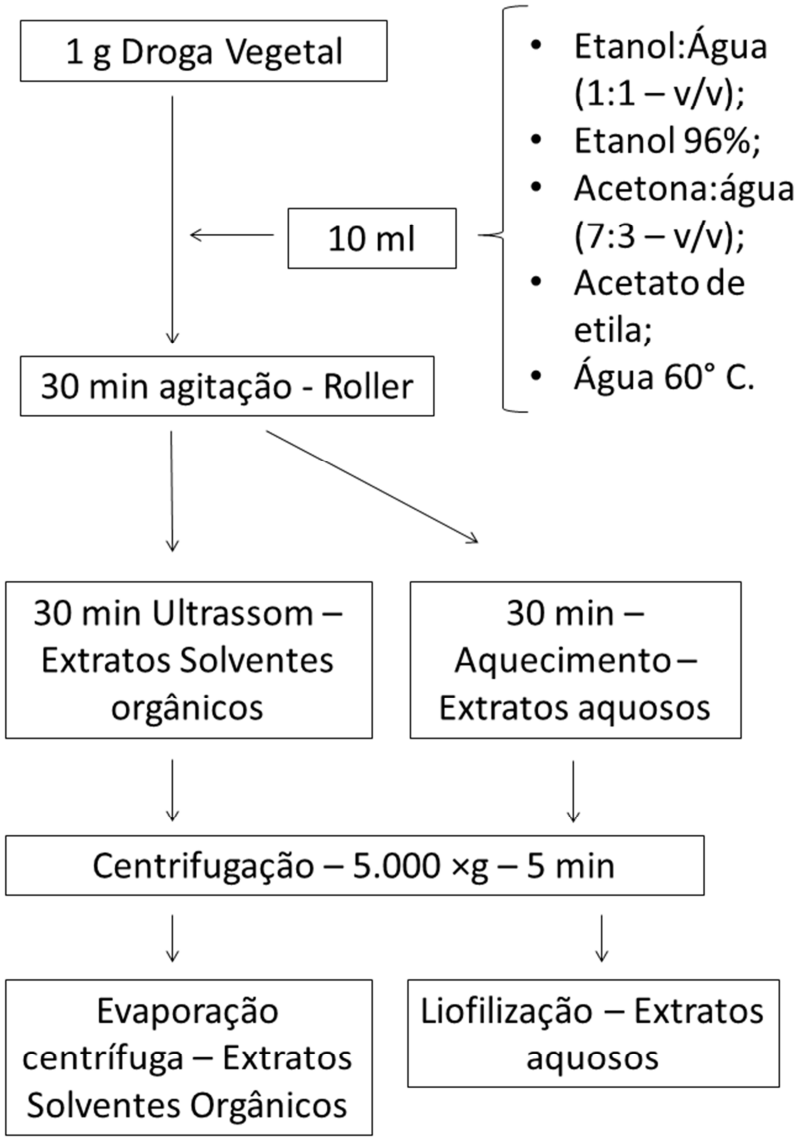
MATERIAIS E MÉTODOS

Material vegetal e extração

As sementes do guaraná (*Paullinia cupana*) foram obtidas no munícipio de Alta Floresta, Rodovia MT 325, Estrada Central, km 8, LT 376 A, Mato Grosso, Brasil. Os rizomas do baicuru foram os mesmos descritos no trabalho sobre a atividade anti-virulência contra *P. gingivalis*. O preparo dos extratos foi realizado no Instituto de Biologia Farmacêutica e Fitoquímica da Universidade de Münster, Alemanha.

Aproximadamente um grama do material vegetal foi submetido à extração usando diferentes solventes, estes foram, etanol:água na proporção 1:1 (v/v), etanol 96%, acetona:água 7:3 (v/v), acetato de etila e água a 60 °C (Fluxograma 01). Após a pesagem em tubo de centrífuga de 15 mL, 10 mL do respectivo solvente foram adicionados e submetidos à agitação em um agitador de rolos IKA® Roller 6 Digital (IKA, Staufen, Alemanha) por 30 minutos. Após esta etapa, os tubos foram submetidos à extração por ultrassom no banho Sonorex RK 100 (Bandelin, Berlim, Alemanha). Ambos os processos foram conduzidos à temperatura ambiente, exceto à extração por água quente, onde se utilizou o bloco de aquecimento com agitação por 30 min.

Após, as amostras foram centrifugadas por 5.000 ×g por 5 min (Centrífuga 5430 R, Eppendorf, Hamburgo, Alemanha), e o sobrenadante derivado da extração com solventes orgânicos, foi transferido para tubos de vidro e evaporado em um evaporador rotatório RVC 2-25 Plus (Martin Christ, Osterode am Harz, Alemanha) por 18 a 40 °C e a 1.000 rpm. O sobrenadante da extração com água quente foi transferido para um balão de fundo redondo e submetido à liofilização em um aparelho Alpha 1-4 LD Plus (Martin Christ, Osterode am Harz, Alemanha).



Fluxograma 01: Etapas no preparo dos extratos de *L. brasiliense* e *P. cupana*.

Cromatografia por camada delgada (CCD)

A CCD foi realizada utilizando-se placas de alumínio contendo sílica gel 60 F_{254} (0.2 mm, Merck, Darmstadt, Alemanha), como fases móveis e reagentes para visualização, utilizaram-se as seguintes combinações conforme a classe de composto de interesse, para flavonoides, utilizou o seguinte sistema solvente, acetato de etila/ácido fórmico/ácido acético glaci-

al/água (100:11:11:26 v/v/v/v) e como agente revelador o "*Natural products reagent A*" – difenilborato de aminoetanol solução metanólica a 1% (Carl Roth, Karlsruhe, Alemanha) que foi aplicado à placa utilizando-se um spray. Para detectar a presença de alcaloides, utilizou-se a seguinte fase móvel, acetato de etila/metanol/água (100:13.5:10 v/v/v) e como agente revelador o reagente de Dragendorff (Bladt, 1996). Para a detecção de proantocianidinas, utilizou-se o sistema solvente acetato de etila/ácido fórmico/água (90:5:5 v/v/v) e como agente revelador vanilina a 1% em solução etanólica seguida de ácido clorídrico concentrado (37%) (Spiegler *et al.*, 2015).

Teste de susceptibilidade por poço difusão

A partir de um cultivo de 72 h em placa, a bactéria foi coletada e transferida para o meio de cultura líquido e a densidade ótica a 660 nm ajustada para 0,1. Os extratos foram dissolvidos em PBS + 0,5% de DMSO. Foram feitos poços no ágar sangue (diâmetro de 0,6 cm e profundidade de 4 mm) e adicionaram-se a soluções dos extratos (80 µL) onde os poços teriam 400 ou 800 µg. A amoxicilina foi utilizada como controle a uma potência de 8 µg. A placa foi incubada por 5 dias à 37 °C em anaerobiose (Schmuch *et al.*, 2015).

Ensaio de citotoxicidade

As células KB foram cultivadas e tratadas para o teste de citotoxicidade conforme descrito no capítulo 1.

Ensaio de atividade anti-adesão.

O ensaio de atividade anti-adesão foi realizado conforme a metodologia de fluorimetria em microplaca que está descrita no trabalho referente à atividade anti-virulência do extrato de *L. brasiliense*.

UHPLC-ESI-QTOF/MS

Para a preparação da amostra para o cromatografia líquida acoplada à espectrometria de massas, 3 mg/mL do extrato foi dissolvido em água:acetonitrila (4:1 – v/v). A solução foi centrifugada por 5 minutos a 14.680 rpm e o sobrenadante foi transferido para outro tubo e submetido para análise.

A separação foi feita no aparelho Dionex Ultimate 3000 RS Liquid Chromatography System contendo a coluna Dionex Acclaim RSLC 120, C18 column (2,1 x 100 mm, 2,2 µm) com um Sistema binário de gradiente (A: água com 0,1% de ácido fórmico; B: acetonitrila com 0.1% de ácido fórmico a 0,4 mL/min. 0 a 5 min: isocrático a 5% B; 5 a 37 min : linear de 5 a 100% B; 37 a 47 min: isocrático a 100% B; 47 a 48 min: linear de 100 a 5% B; 48 a 55 min: isocrático a 5% B. O volume da injeção foi de 2 µL. Os compostos eluídos foram detectados usando o aparelho Dionex Ultimate DAD-3000 RS num intervalo de comprimento de onda de 200 a 400 nm e por espectrometria de massas usando-se o aparelho Bruker Daltonics micrOTOF-QII time-of-flight espectrômetro de massas equipado com fonte de ionização electrospray Apollo no modo positivo a 2 Hz num intervalo de massas de m/z 50-1500 usando os seguintes parâmetros: gás nebulizador nitrogênio, 4 bar; gás seco nitrogênio, 9 L/min, 200 °C; voltagem do capilar -4.500 V; end plate offset -500 V; tempo de transferência 100 µs; armazenamento pré-pulso 6 µs; configurações de energia de colisão e RF de colisão foram combinadas para cada único espectro de 2500 somatórios da seguinte forma: 1250 somatórios com energia de colisão de 80 eV e 130 Vpp + 625 somatórios com 16 energia de colisão eV e 130

Vpp + 625 somatórios com 16 energia de colisão eV e 130 Vpp . O conjunto de dados de calibração interna (modo HPC) foi realizado para cada análise utilizando o espectro de massa de uma solução 10 mM de formato de sódio em 50% de isopropanol, que foi infundido durante o reequilíbrio LC usando uma válvula de desvio equipados com um circuito de amostra de 20 uL (Spiegler *et al.*, 2015)

Cromatografia por coluna

Trezentos gramas do rizoma de *L. brasiliense* foram submetidos à extração por turbólise utilizando o dispersor VDI-25 a 9.500 rpm (VWR, Darmstadt, Alemanha) e como o solvente acetona:água na proporção de 7:3, v/v (3 L) em um ciclo de 5 min de agitação e 5 de maceração por 3 vezes (9 L de solvente). Após esta etapa o liquido extrativo foi filtrado em um Funil de Porcelana de Buchner contendo um filtro de papel qualitativo 413 (WVR, Darmstadt, Alemanha). O solvente orgânico foi removido por evaporador rotatório a 40 °C (Rotavapor® R-200, Büchi, Flawil, Suíça) e liofilizado.

Cinquenta gramas do extrato obtido conforme acima foi submetido à partição com água e acetato de etila (1:1 v/v, 1 L) por cinco vezes. Após a separação das duas fases, os solventes foram removidos por evaporação rotatória e os extratos liofilizados para obter a fração acetato de etila e a fração aquosa.

A fração acetato de etila (1,9 g) foi dissolvida em etanol e fracionada em uma coluna contendo Sephadex® LH-20 (690 × 28 mm) utilizando uma eluição por etapas com um aumento da polaridade do solvente (etanol, metanol e acetona:água 7:3 – v/v) nas seguintes proporções, 8.750 mL de etanol, 3.000 mL de metanol e 1.000 mL de acetona:água. Foram recolhidos 15 mL do eluente em fluxo de 0,75 mL/min, os tubos foram reunidos por semelhança do seu perfil cromatográfico em CCD e o solvente foi evaporado e as frações liofilizadas.

Cromatografia líquida de alta eficiência (CLAE)

As análises foram realizadas no Laboratório de Biologia Farmacêutica - Palafito (Prof. Dr. João Carlos Palazzo de Mello). Utilizou-se o aparelho da Marca Thermo Fisher Scientific equipado com bombas e degassificador interno (Finnigan Surveyor LC pump Plus), módulo de detecção espectrofotométrica PDA (Finnigan Surveyor PDA Plus detector, software (Chromquest) e injetador automático (Finnigan Surveyor Autosampler Plus) equipado com uma alça de 10 µL. As amostras foram preparadas em metanol:água (1:1, v/v) e filtradas por membrana (Millipore Milex – HC 0,45 µM) antes da injeção.

A corrida cromatográfica foi efetuada utilizando a coluna Agilent Zorbax SB-C18 250×4,6×5 mm e um sistema binário, onde a fase móvel A é composta por Água destilada:Ácido fosfórico (100:0,2 – v/v) e a fase B por Acetonitrila:ácido fosfórico (100:0,2 – v/v).

O seguinte gradiente foi empregado: 0 a 15 min, 90% A; 15 a 35 min, 85% A; 35 a 36 min, 81% A; 36 a 44 min, 20% A; 44 a 45 min, 20% A; 45 a 52 min, 90% A, 52 min, 90% A. Um fluxo de 0,4 mL/min foi utilizado, a temperatura do injetor a 10 °C e o comprimento de onda 280 nm (Blainski-Pinha, 2016).

Espectrometria de massas sequencial (ESI-MS/MS) e ressonância magnética nuclear de prótons (^{1}H-RMN)

A espectrometria de massas foi realizada no Complexo de Centrais de Apoio à Pesquisa (Comcap), da Universidade Estadual de Maringá e a ressonância magnética nuclear no Laboratório de Ecologia Microbiana (Prof. Dr. Admilton Gonçalves de Oliveira Junior) da

Universidade Estadual de Londrina, conforme as metodologias que estão descritas no trabalho referente à atividade anti-virulência do extrato de *L. brasiliense* frente a *P. gingivalis*.

RESULTADOS

Extratos

O rendimento dos extratos pode ser observado na tabela 01. Os extratos aquosos foram os que mostraram maior rendimento, sendo que o extrato hidroacetônico de *L. brasiliense* apresentou o melhor resultado.

Tabela 01: Rendimento dos diferentes extratos das espécies de plantas *Limonium brasiliense* e *Paullinia cupana*

Planta	**Extrato**	**Solvente**	**Rendimento (g)**	**Rendimento (%)**
Limonium brasiliense	**#01**	Etanol:H_20 (1:1) – v/v	0,2358	23,53
	#02	Etanol 96%	0,0429	4,250
	#03	Acetona:H_20 (7:3) – v/v	0,2639	26,29
	#04	Acetato de etila	0,0025	0,250
Paullinia cupana	**#05**	Etanol:H_20 (1:1) – v/v	0,1609	16,00
	#06	Etanol 96%	0,0282	2,810
	#07	Acetona:H_20 (7:3) – v/v	0,1575	15,69
	#08	Acetato de etila	0,0153	1,530
Limonium brasiliense	**#09**	Água quente 60 °C	0,2058	20,55
Paullinia cupana	**#10**	Água quente 60 °C	0,1450	14,47

Cromatografia por camada delgada dos extratos obtidos

Os diferentes extratos foram submetidos à CCD para verificar a presença de certas classes de compostos. Na fig. 01, foi aplicado o reagente "*Natural products reagent A*" que é específico para flavonoides e visualizados na luz ultravioleta de 366 nm. Podemos observar a presença de zonas fluorescentes azuis no baicuru, isto pode ser devido à presença de ácidos fenólicos e coumarinas, que são conhecidos por apresentar esta fluorescência azul quando utilizado este reagente(Bladt, 1996).

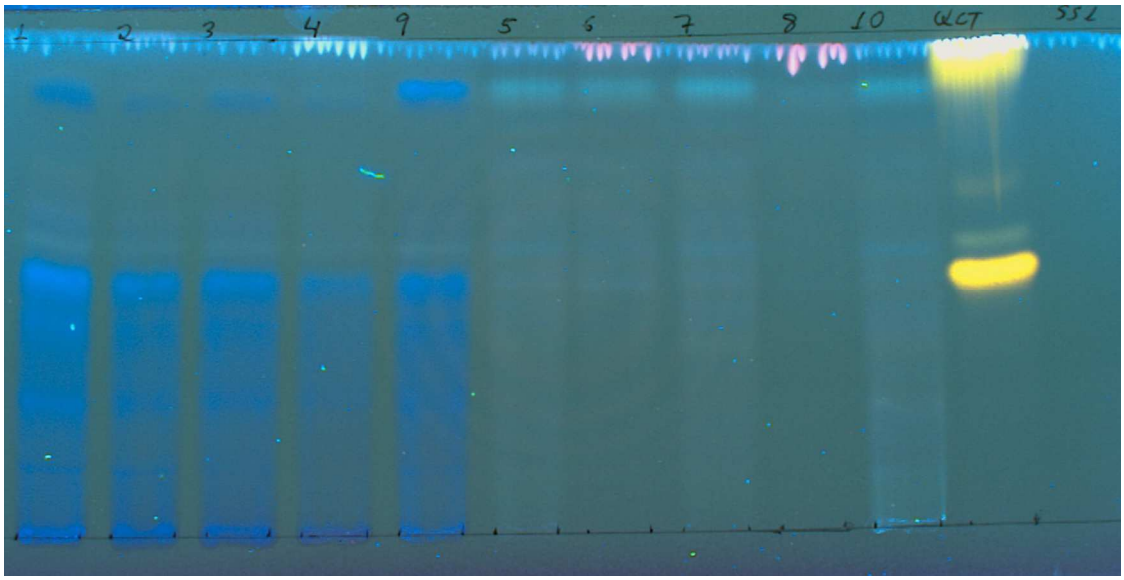


Fig. 01: Perfil por cromatografia de camada delgada dos extratos obtidos das plantas *L. brasiliense* e *P. cupana*. Os números na linha de chegada (LC) representam os extratos descritos na tabela 01. QCT: Quercetina (LC) e rutina.

Para verificar a presença de alcaloides, utilizou-se o reagente de Dragendorff (Fig. 02). Como o esperado, o guaraná, o qual apresenta altas quantidades de cafeína, apresentou zonas vermelhas, indicando a presença deste alcaloide, apesar deste reagentes não ser o mais adequado para alcaloides purínicos (Bladt, 1996). Não foi verificada a presença de alcaloides na espécie *L. brasiliense* por esta técnica.

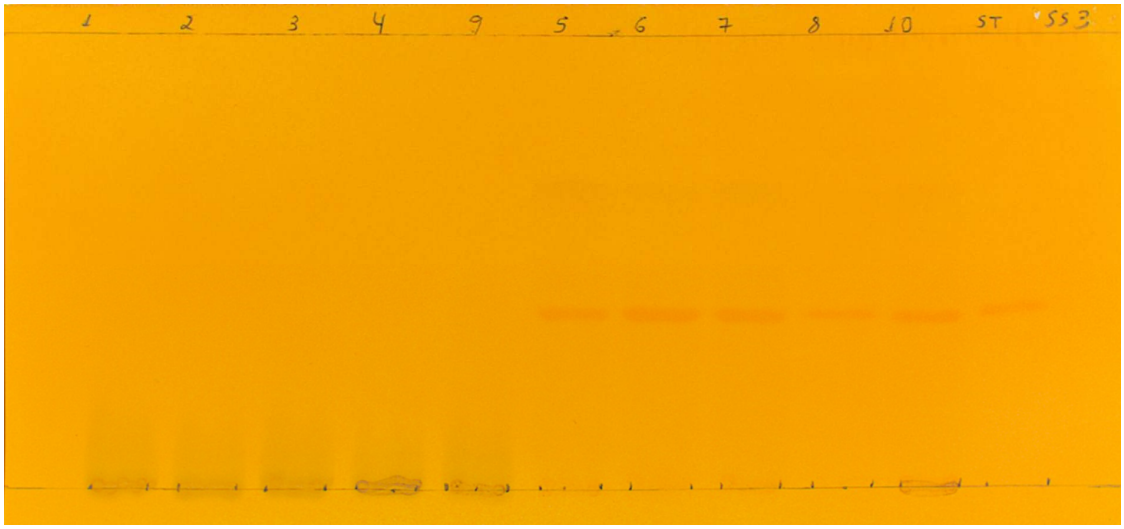


Fig. 02: Perfil por cromatografia de camada delgada dos extratos obtidos das plantas *L. brasiliense* e *P. cupana*. Os números na linha de chegada representam os extratos descritos na tabela 01. ST: Cafeína. SS3: sistema solvente número 3.

A presença de proantocianidinas foi verificada utilizando-se o reagente vanilina 1% em etanol seguido de HCl concentrado. No baicuru observou-se que os compostos ficaram próximos da linha de partida, o que pode ser indicativo de molécula grandes (protoantocianidinas poliméricas). Já no guaraná, notaram-se diferentes níveis de polimerização, onde as monoméricas ficaram na mesma linha da epicatequina (ECC).

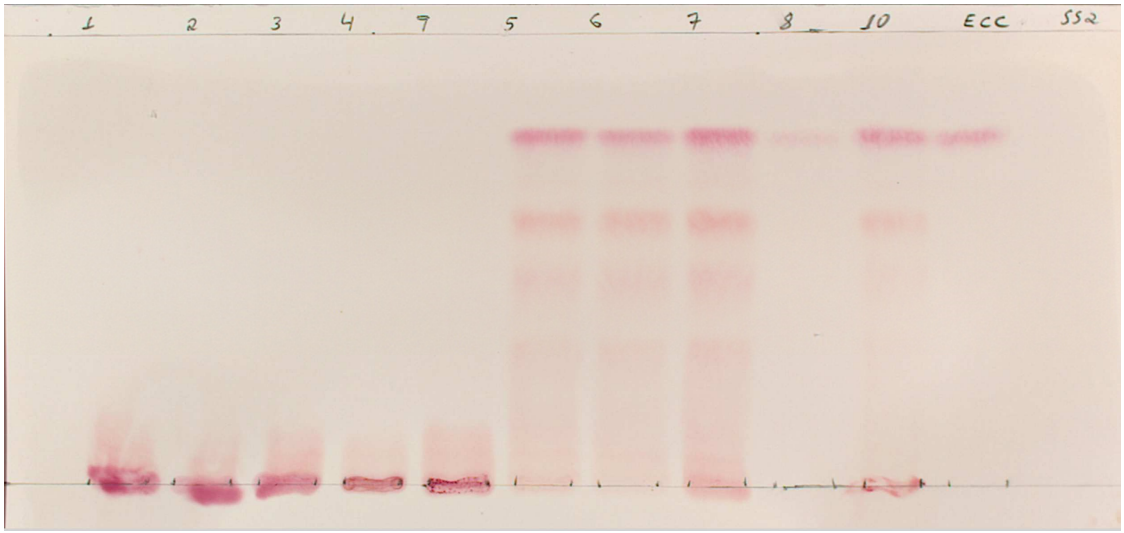


Fig. 03: Cromatograma por CCD dos extratos obtidos das plantas *L. brasiliense* e *P. cupana*. Os números na linha de chegada (LC) representam os extratos descritos na tabela 01. ECC: Epicatequina. SS2: Sistema solvente

número 2.

Citotoxicidade contra *P. gingivalis* e células de mamíferos

Nenhum dos extratos obtidos demonstrou halo de inibição quando testados via poço difusão contra *P. gingivalis*, somente o controle positivo, amoxicilina apresentou um halo de 50 milímetros (fig. 04).

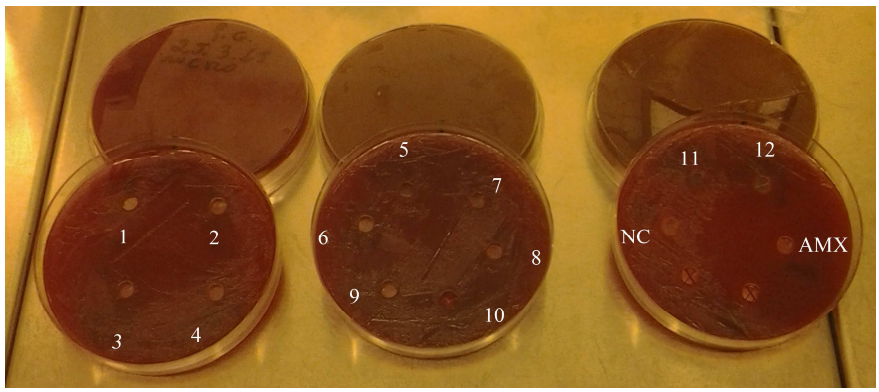


Fig. 04: Teste de susceptibilidade por disco difusão frente à bactéria *P. gingivalis*. **1**: LB - EtOH:H_20 1:1 - 800 µg e **2**: 400 µg. **3**: LB – Acetona 70% 800 µg e **4**: 400 µg. **5**: PC - EtOH:H_20 1:1 800 µg e **6**: 400 µg. **7**: PC – Acetona 70% 800 µg e **8**: 400 µg. **9**: LB – água quente 800 µg e **10**: 400 µg. **11**: PC – água quente 800 µg e **12**: 400 µg. **NC**: Controle negativo (Tampão salina fosfato (PBS) + dimetilsulfóxido (DMSO) e **AMX**: Amoxicilina. **LB**: *Limonium brasiliense*. **PC**: *Paullinia cupana*.

Quanto à viabilidade celular das células KB, praticamente todos os extratos testados, quer seja de *L. brasiliense* ou *P. cupana* (Figs. 5 e 6, tabelas 2 e 3) se mostraram pouco tóxicos. Os que tiveram um efeito mais deletério foram o extrato etanólico do baicuru que reduziu a viabilidade em cerca de 50% na concentração de 100 µg/mL e o hidroetanólico do guaraná (100 µg/mL) onde a viabilidade foi reduzida em cerca de 35%.

A escolha do extrato para o teste de atividade anti-adesão por fluorimetria em placa, baseou-se no rendimento dos extratos (água quente e acetona 70%) e por saber que a acetona a 70% favorece a extração de taninos (Santos e Mello, 2004).

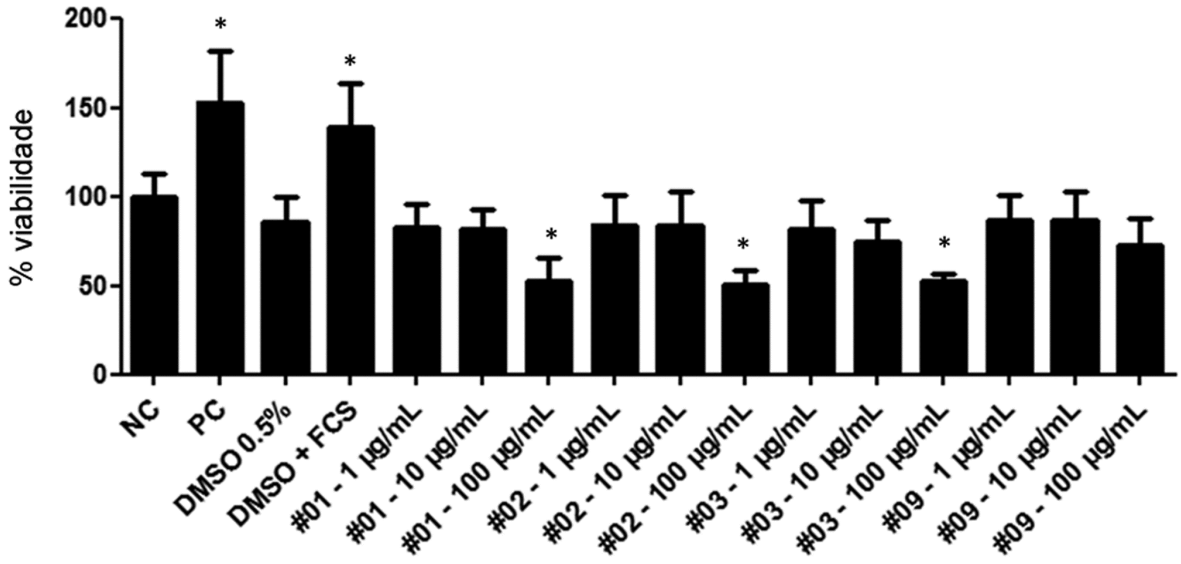


Fig. 05. Ensaio de viabilidade celular (24 h) das células KB desafiadas por vários tipos de extratos de *L. brasiliense* e diferentes concentrações pela técnica do MTT. NC: controle negativo, PC: controle positivo – 10% de soro fetal bovino (FCS), DMSO – Dimetilsulfóxido 0,5%: controle de solvente. Os números na legenda representam os mesmo extratos na tabela 01.

Atividade anti-adesão

Sabendo que os extratos não tem atividade anti-*Porphyromonas gingivalis* e também não se apresentaram tóxicos frente às células KB, eles foram testados para se verificar a atividade anti-adesão. Utilizou-se primeiro o teste em placa de 96 poços devido ao fato de que várias amostras podem ser analisadas num mesmo ensaio. O extrato "água quente" do *L. brasiliense* mostrou uma menor atividade anti-adesão, onde cerca de 40% da adesão foi reduzida (fig. 7, tabela 04). Já o extrato acetona:água do baicuru reduziu em 80% a adesão das células de *P. gingivalis* frente às células KB conforme descrito no capítulo 1.

Este resultados levaram a escolha do extrato acetona:água (7:3 – v/v) para os testes posteriores frentes aos principais mecanismos de virulência, que são as adesinas e as gingipaínas da bactéria *Porphyromonas gingivalis*, que deram origem ao primeiro artigo.

Tabela 02: Citotoxicidade dos extratos de *Limonium brasiliense* contra células KB por MTT

Amostras	**Concentração**	**Média (%)**	**DP (%)**	***n***
Controle negativo		100	12,83	12
Controle positivo FCS	10%	153,2	28,77	12
DMSO	0,5%	86,05	13,62	12
DMSO + FCS	0,5% & 10%	139,1	24,66	12
#01* EtOH:H_20 – 1:1	1 µg/mL	82,65	12,80	12
	10 µg/mL	81,78	10,67	12
	100 µg/mL	52,35	13,58	12
#02* EtOH 96%	1 µg/mL	84,03	16,18	12
	10 µg/mL	83,42	19,11	12
	100 µg/mL	50,54	7,647	12
#03* Acetona: H_20 7:3	1 µg/mL	81,88	15,77	9
	10 µg/mL	74,40	12,22	9
	100 µg/mL	52,64	4,455	9
#09* Água quente 60 °C	1 µg/mL	86,51	14,16	12
	10 µg/mL	86,20	16,80	12
	100 µg/mL	73,14	14,34	12

A média representa o percentual de células viáveis. DP: desvio-padrão. FCS: Soro fetal bovino. DMSO: Dimetilsulfóxido.

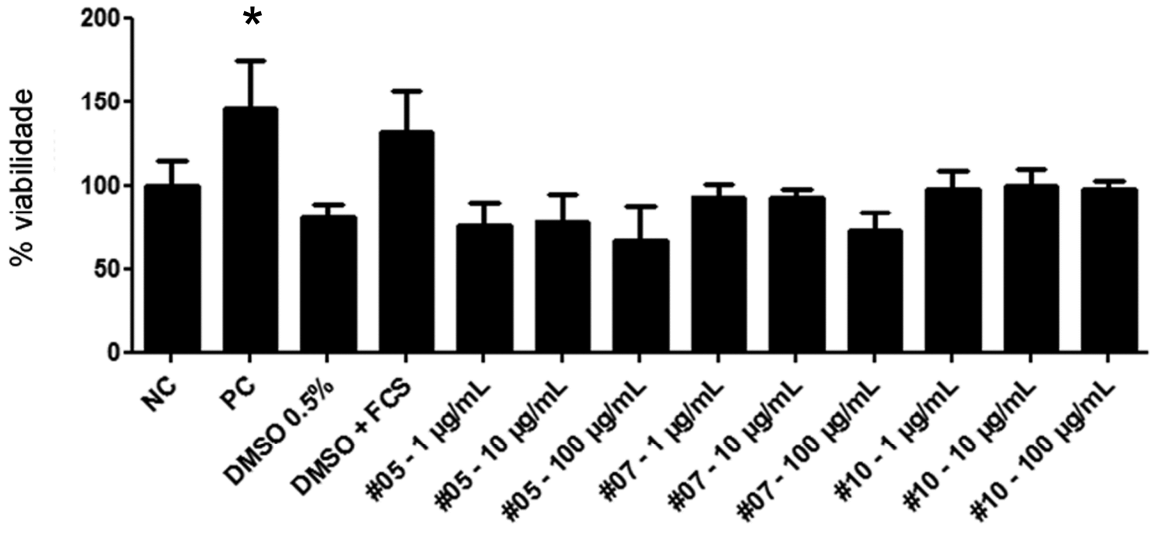


Fig. 06. Ensaio de viabilidade celular das células KB desafiadas por vários tipos de extratos de *P. cupana* e diferentes concentrações pela técnica do MTT. NC: controle negativo, PC: controle positivo – 10% de soro fetal bovino (FCS), DMSO – Dimetilsulfóxido 0,5%: controle de solvente. Os números na legenda representam os mesmo extratos na tabela 01.

Tabela 03: Citotoxicidade dos extratos de *Paullinia cupana* contra células KB por MTT

Amostras	**Concentração**	**Média (%)**	**DP (%)**	***n***
Controle negativo		100,0	14,43	9
Controle positivo - FCS	10%	145,8	29,34	9
DMSO	0,5%	81,00	8,006	9
DMSO + FCS	0.5% + 10%	131,9	24,43	9
#05	1 µg/mL	75,63	14,09	9
EtOH:H_20 – 1:1	10 µg/mL	78,12	16,98	9
	100 µg/mL	66,91	20,60	9
#07	1 µg/mL	93,10	7,383	9
Acetona: H_20 7:3	10 µg/mL	92,65	4,957	9
	100 µg/mL	72,82	11,21	9
#10	1 µg/mL	98,04	11,03	6
Água quente – 60 °C	10 µg/mL	99,80	9,893	6
	100 µg/mL	97,77	4,583	6

A média representa o percentual de células viáveis. DP: desvio-padrão. FCS: Soro fetal bovino. DMSO: Dimetilsulfóxido.

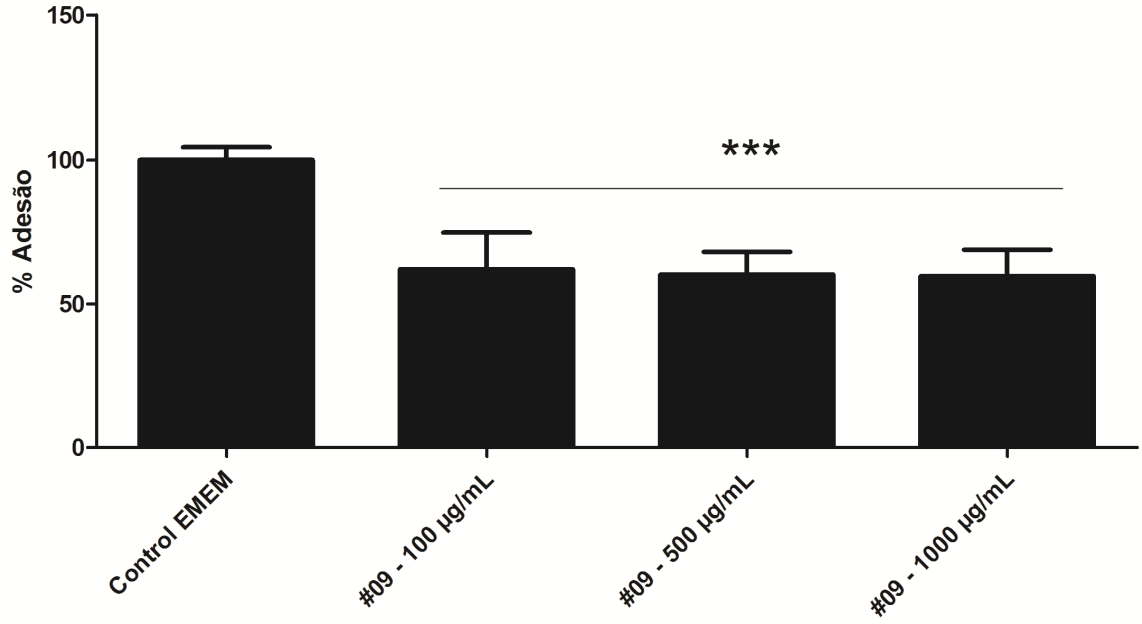


Fig. 07. Atividade anti-adesão do extrato "água quente" de *Limonium brasiliense* no experimento de co-incubação com células KB e *Porphyromonas gingivalis*. ***: p > 0,001 em relação ao controle.

Tabela 04. Atividade anti-adesão do extrato "água quente" de *Limonium brasiliense* no experimento de co-incubação com células KB e *Porphyromonas gingivalis*

Amostras	**Concentração**	**Média (%)**	**D.P (%)**	***n***
Controle negativo	-	100	4,4	15
#09 "Água quente" 60 °C	100 µg/mL	62,0	12,7	10
	500 µg/mL	60,1	7,9	10
	1000 µg/mL	59,6	9,1	10

UHPLC/+ESI-qTOF

O cromatograma do extrato acetona:água (7:3, v/v) do *L. brasiliense* (Fig. 8) mostrou três grandes picos e nestes picos foram encontrados os principais componentes descritos a seguir, isto foi realizado utilizando a função "dissect compounds" do programa Compass Data analysis 4.0 SP1. Após 8,11 min a primeira substância eluiu e o espectro de massas (Fig. 9) mostrou o íon molecular a uma relação massa/carga (*m/z*) de 761,1377 $[M+H]^+$ e 783,1193 $[M+Na]^+$. A segunda substância teve um tempo de retenção (tr) 10,20 min e um íon molecular de 459,0923 $[M+H]^+$ e 481,0749 $[M+Na]^+$ *m/z* (fig. 10). A terceira substância apresentou um tr de 10,80 min e o íon molecular de 913,1498 $[M+H]^+$ e 935,1328 $[M+Na]^+$ *m/z* (fig. 11).

A fim de fazer uma identificação tentativa dos principais compostos, o banco de dados METLIN (Smith *et al.*, 2005) e a literatura relacionada sobre a fitoquímica do gênero *Limonium* foram utilizados. O primeiro pico correspondeu as seguintes substâncias: teacitrina A, samarangenina A e prodelfinidina A2 3'-galato. O segundo pico correspondeu a algumas substâncias, mas quando comparamos os resultados com a fitoquímica de *L. brasiliense* descobrimos que epigalocatequina-3-*O*-galato é um componente abundante desta planta (Murray *et al.*, 2004). O terceiro pico, provavelmente o principal componente deste extrato, atingiu os compostos, teacitrina C e samarangenina B. A última é encontrado nas raízes de *Limonium sinense* (Lin *et al.*, 2000), sugerindo que o primeiro pico é o composto samarangenina A, apesar de que esta substância não foi descrita neste gênero, um estudo específico ainda não foi feito. Esta metodologia foi de suma importância para a confirmação da presença destes compostos no extrato de *L. brasiliense* via espectrometria de massa sequencial e ressonância magnética nuclear de prótons.

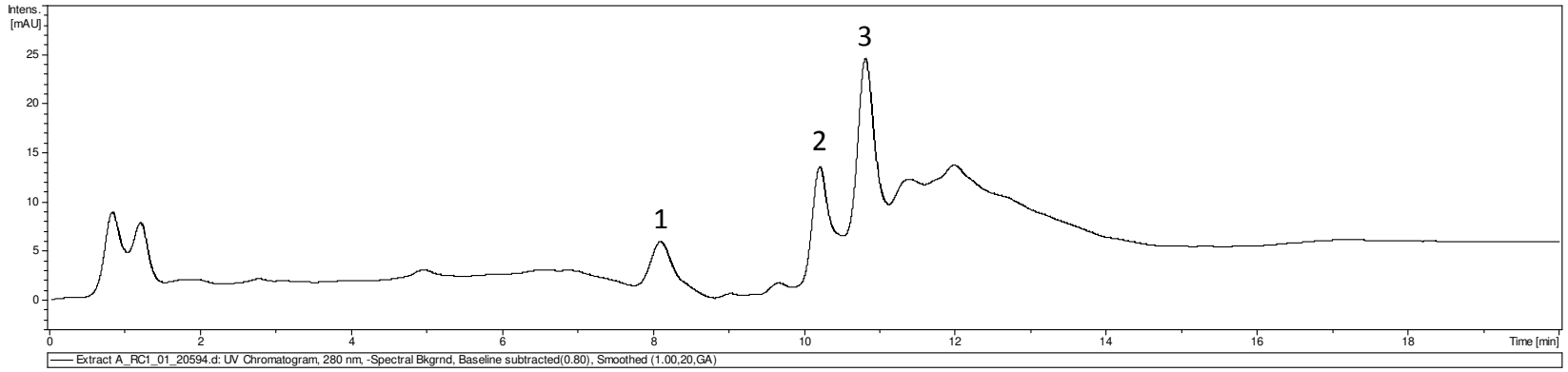


Fig. 08: Cromatograma derivado da cromatografia líquida de ultra eficiência (UHPLC) no comprimento de onda a 280 nm do extrato acetona:água (7:3) de *Limonium brasiliense* e compostos putativamente identificados presentes nos picos apresentados. 1) Samarangenina A. 2) Epigalocatequina galato e 3) Samarangenina B.

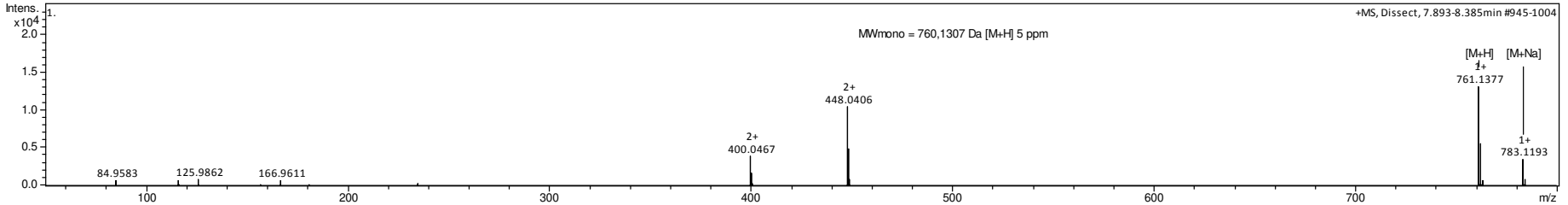


Figura 09. Espectro de massas +ESI-QTOF do extrato acetona 70% no intervalo de tempo de retenção apresentado, mostrando a presença do composto 1 correspondendo à substância samarangenina A.

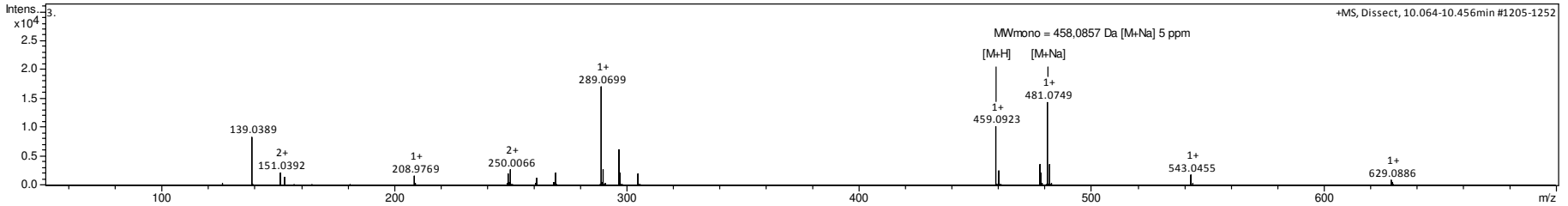


Figura 10. Espectro de massas +ESI-QTOF do extrato acetona 70% no intervalo de tempo de retenção apresentado, mostrando a presença do composto 2 correspondendo à substância epigalocatequina galato .

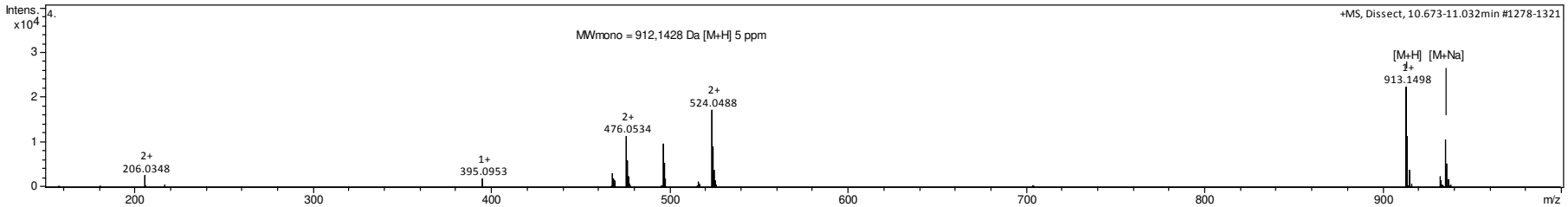


Figura 11. Espectro de massas +ESI-QTOF do extrato acetona 70% no intervalo de tempo de retenção apresentado, mostrando a presença do composto 3 correspondendo à substância samarangenina B.

Cromatografia em coluna

Verificada a excelente atividade anti-virulência do extrato do baicuru frente ao periodontopatógeno *P. gingivalis*, a separação dos compostos presentes no extrato se deu por cromatografia em coluna empregando como fase estacionária, a Sephadex LH-20. Após a partição do extrato acetona:água entre acetato de etila e água, e obtenção da fração acetato de etila (FAE), esta foi aplicada na coluna. Todo o processo rendeu 17 frações (tabela 05) onde, uma fração entre as de número XII e XIII (T.471-2), foi obtida por que o aparelho voltou à posição "zero".

Tabela 05. Rendimento do fracionamento em coluna empregando a Sephadex LH-20, onde se utilizou a fração acetato de etila (FAE) do *L. brasiliense*. Continua na próxima página.

Fração	**Miligramas**	**Tubos**	**Volume Solvente (mL)**	**Rendimento (%)**
I	49	1 – 22	165 – EtOH	2,58
II	98,6	23 – 28	45 – EtOH	5,19
III	18,2	29 – 34	45 – EtOH	0,96
IV	42,4	35 – 39	37,5 – EtOH	2,23
V	66,4	40 – 75	270 – EtOH	3,49
VI	62,03	76 – 96	157,5 – EtOH	3,26
VII	24,9	97 – 156	450 – EtOH	1,31
VIII	66,1	157 – 185	322,5 – EtOH	3,48
IX	29,6	186 – 239	810 – EtOH	1,56
X	242,6	240 – 277	570 – EtOH	12,77
XI	59,8	278 – 422	2.175 – EtOH	3,15
XII	75,6	423 – 470	720 – EtOH	3,98
T.471-2	52,8	*	400 – EtOH	2,78
XIII	206,7	471 – 521	765 – EtOH	10,9
XIV	163,3	522 – 662	1815 EtOH/300 MeOH	8,59
XV	390,9	663 – 785	2700 MeOH	20,57

XVI	207,4	**	1000 - Acetona:H20 (7:3)	10,92
Total	**1856,33**	-	-	**97,7**

Cromatografia em camada delgada das frações obtidas da coluna contendo Sephadex LH-20

Os cromatogramas em CCD mostraram-se com diferentes perfis, é interessante notar que as catequinas foram visualisadas a partir da fração VII, onde grandes quantidades foram observadas (fig. 12 à 17).

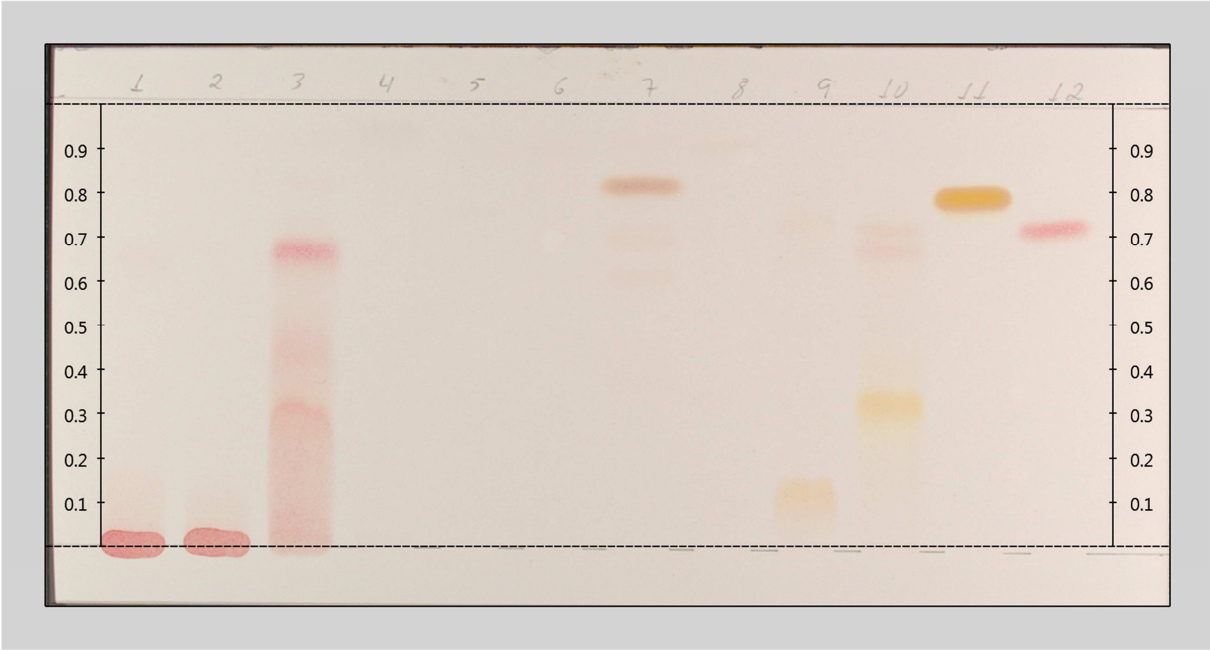


Fig. 12: Perfil por cromatografia de camada delgada dos extrato hidroacetônico (1), fração aquosa – FAq (2), fração acetato de etila – FAE (3), frações obtidas da cromatografia em coluna da FAE I (4), II (5), III (6), IV (7), V (8), VI (9), VII (10) obtidos da planta *Limonium brasiliense*, mostrando que não há proantocianidinas (não há reação com o reagente vanilina-HCl, formando os *pink spots*) nas frações obtidas da coluna. Os números em algarismos romanos correspondem aos da tabela 4. Controles: quercetina (11), epicatequina (12).

Foi utilizado para a revelação na CCD, além da vanilina-HCl, o reagente cloreto férrico ($FeCl_3$) que reage com polifenóis, gerando *spots* de cor azul (Santos e Mello, 2004). Este fato permitiu identificar o ácido gálico na fração VI, pois ele reage com o $FeCl_3$, mas não com o

tratamento com vanilina-HCl (Andreas Hensel, comunicação pessoal, 2016).

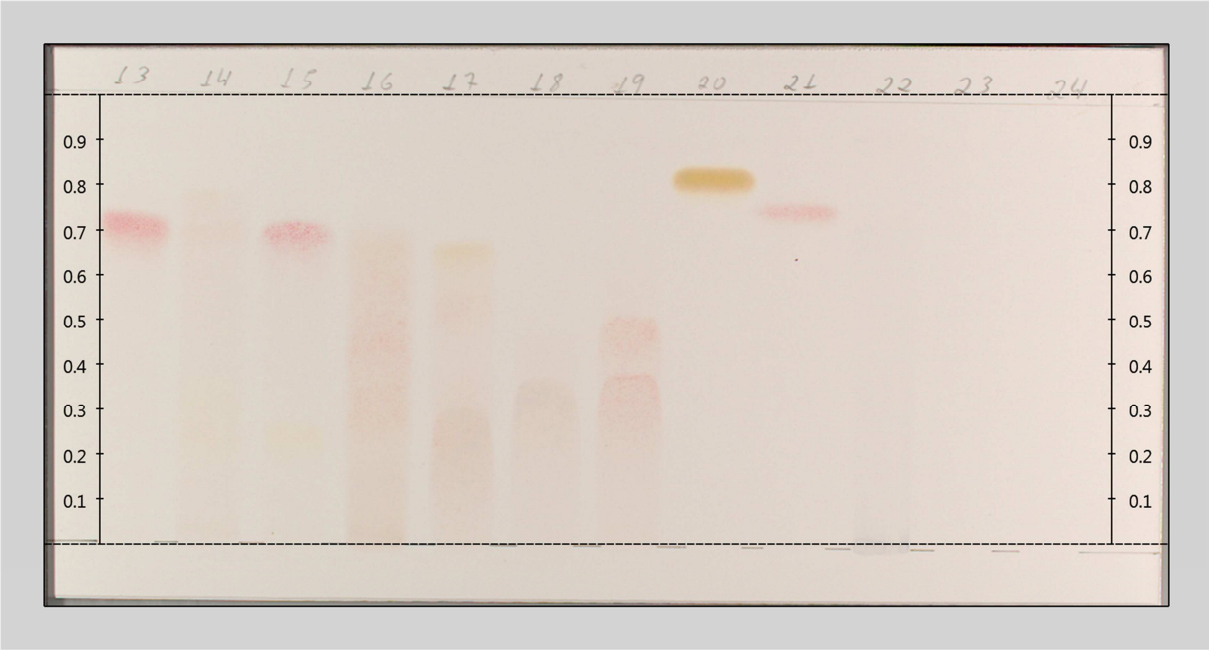


Fig. 13: Perfil por cromatografia de camada delgada das frações obtidas da cromatografia em coluna da FAE VII (13), IX (14), X (15), XI (16), XII (17), XIII (18), XIV (19) obtidos da planta *Limonium brasiliense*, mostrando que as proantocianidinas estão na fração 8 em diante. Os números em algarismos romanos correspondem aos da tabela 4. Controles: quercetina (20), epicatequina (21). Revelador: Vanilina-HCl.

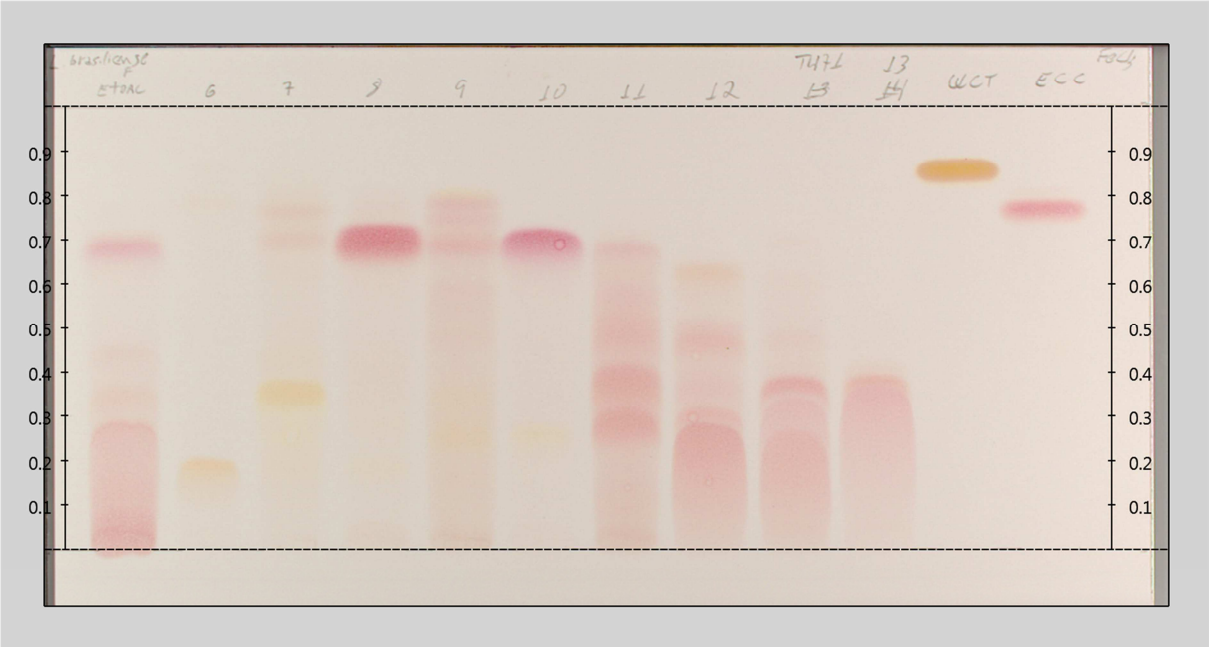


Fig. 14: Perfil por cromatografia de camada delgada das frações obtidas da cromatografia em coluna da FAE (EtOAc) obtidos da planta *Limonium brasiliense*, os números correspondem aos em algarismos romanos na tabela 4. Controles: quercetina (QCT), epicatequina (ECC). Revelador: Vanilina-HCl.

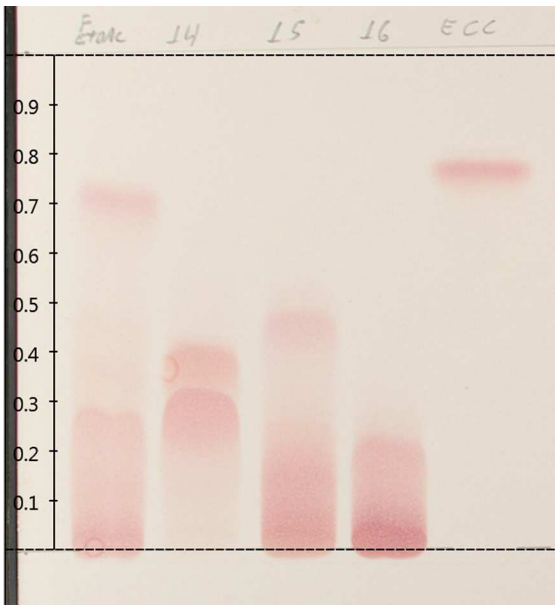


Fig. 15: Perfil por cromatografia de camada delgada das frações obtidas da cromatografia em coluna da FAE (EtOAc) obtidos da planta *Limonium brasiliense*, os números correspondem aos em algarismos romanos a tabela 4. Controles: quercetina (QCT), epicatequina (ECC). Revelador: Vanilina-HCl.

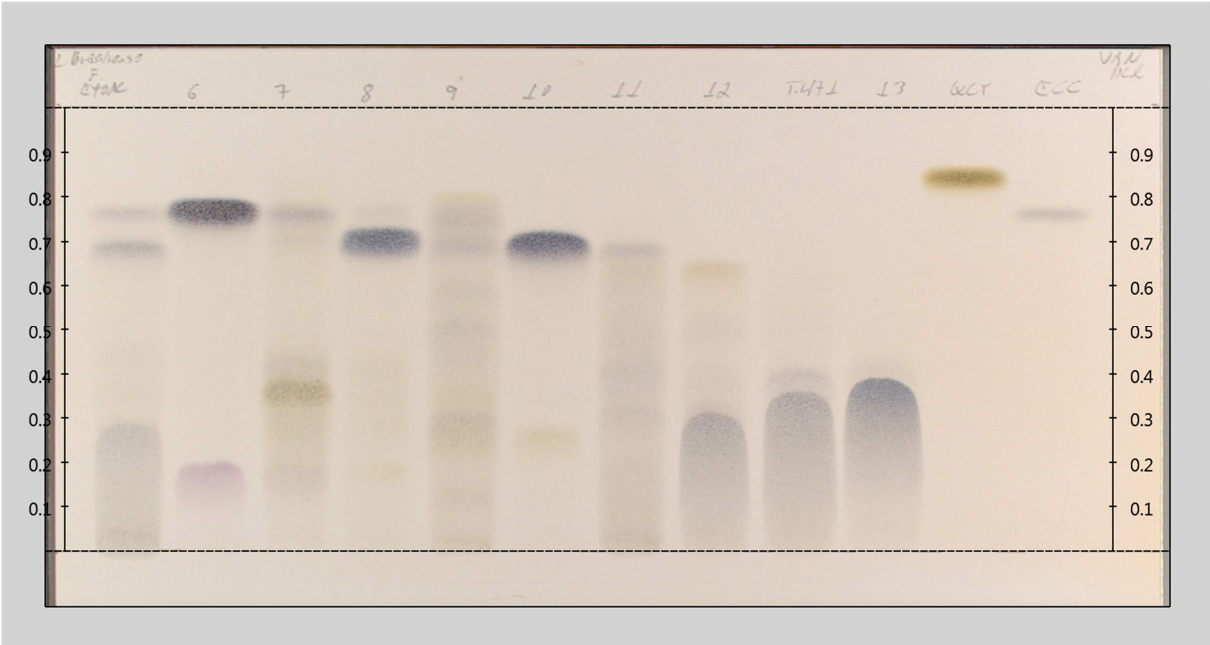


Fig. 16: Perfil por cromatografia de camada delgada da fração acetato de etila – FAE (EtOAc), frações obtidas da cromatografia em coluna da FAE obtidos da planta *Limonium brasiliense*, utilizou-se como reagente revelador cloreto férrico ($FeCl_3$) à 1% em etanol. Os números correspondem aos em algarismos romanos na tabela 4. Controles: quercetina (QCT), epicatequina (ECC).

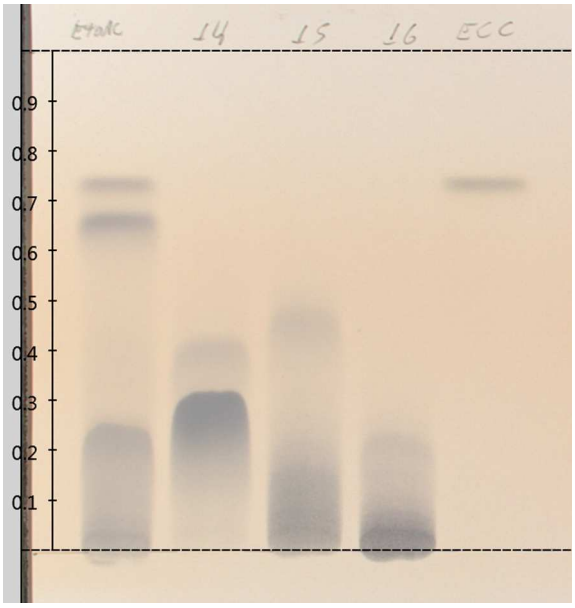


Fig. 17: Perfil por cromatografia de camada delgada das frações obtidas da cromatografia em coluna da FAE (EtOAc) obtidos da planta *Limonium brasiliense*. Os números correspondem a tabela 4. Controle: epicatequina (ECC). Utilizou-se como reagente revelador o cloreto férrico ($FeCl_3$) à 1% em etanol.

Cromatografia líquida de alta eficiência (CLAE)

A fim de verificar a presença de catequinas, o extrato, fração acetato de etila (FAE) e as frações mais puras, previamente avaliadas por CCD, foram submetidos à análise por CLAE (Fig. 18 à 23). As frações apresentaram somente um pico, no entanto pode-se observar a presença de um "ombro" na fração VI (fig. 20). Estes resultados levaram a escolha destas frações para a posterior análise por espectroscopia de massas e ressonância magnética nuclear (RMN).

Espectrometria de massas sequencial (ESI-MS/MS) e ressonância magnética nuclear de prótons (^{1}H-RMN)

Os compostos encontrados via ESI-MS/MS podem ser visualizados na tabela 5. O composto galocatequina-galato-dímero não é descrito na literatura para o gênero *Limonium*. Os outros foram encontrados na espécie do nosso estudo, *L. brasiliense* e a luteolina foi encontrada na espécie *L. bicolor* (Wei e Wang, 2006). Os espectros de ^{1}H-RMN confirmaram a presença das substâncias ácido gálico, epigalocatequina galato e samarangeninas A e B no baicuru (fig. 24 a 27).

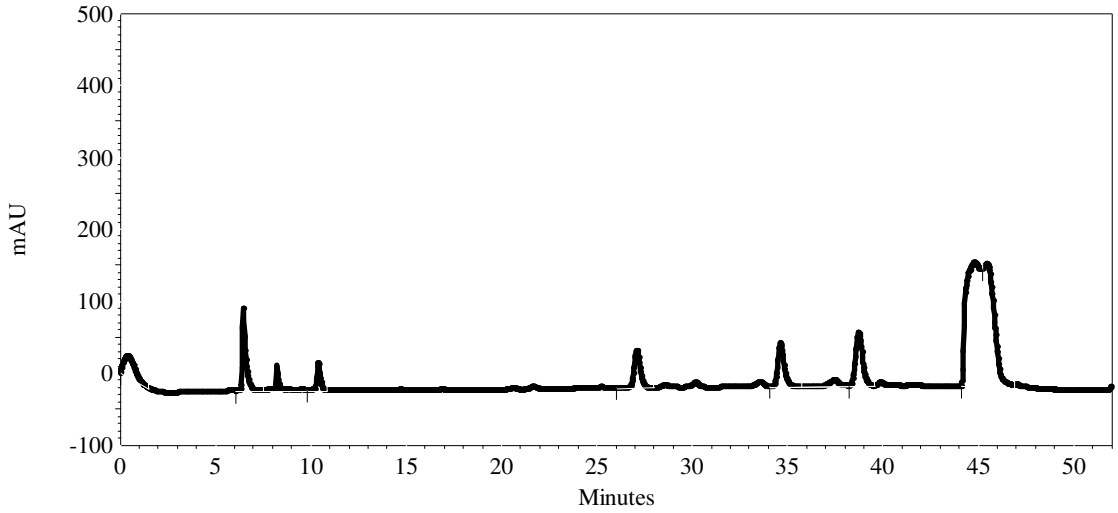


Fig. 18. Perfil cromatográfico à 280 nm do extrato bruto de *L. brasiliense*.

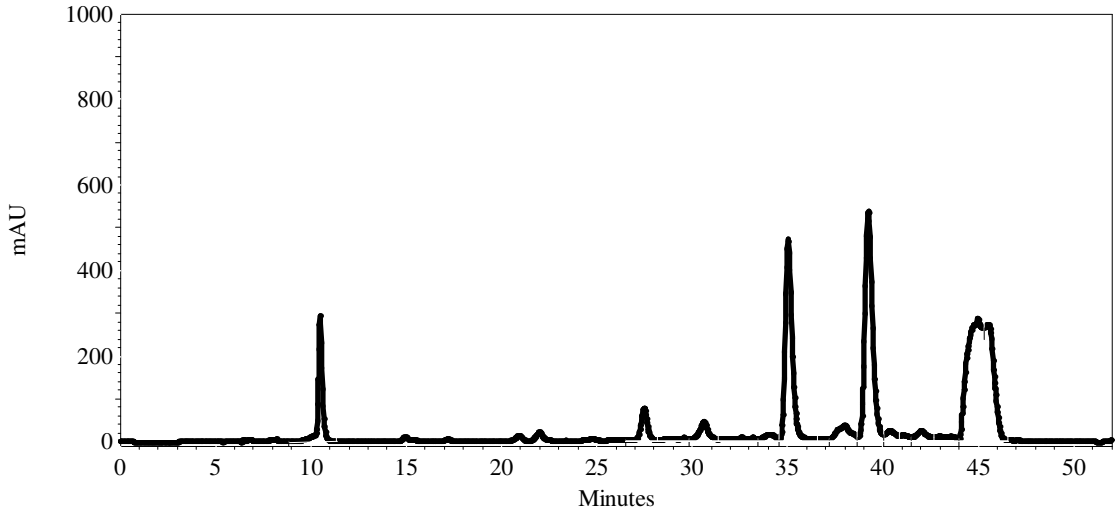


Fig. 19. Perfil cromatográfico à 280 nm da fração acetato de etila de *L. brasiliense*.

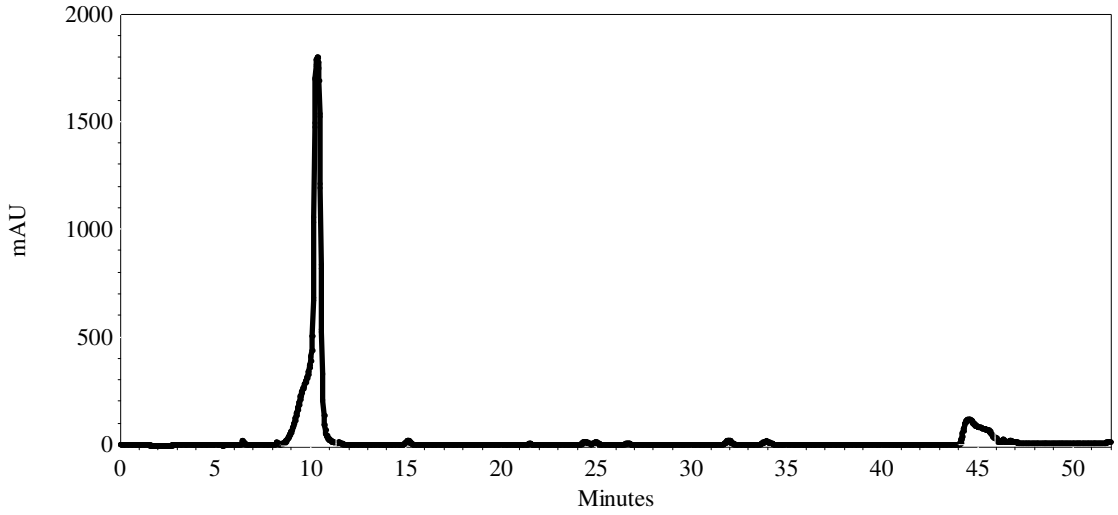


Fig. 20. Perfil cromatográfico à 280 nm da fração VI de *L. brasiliense*.

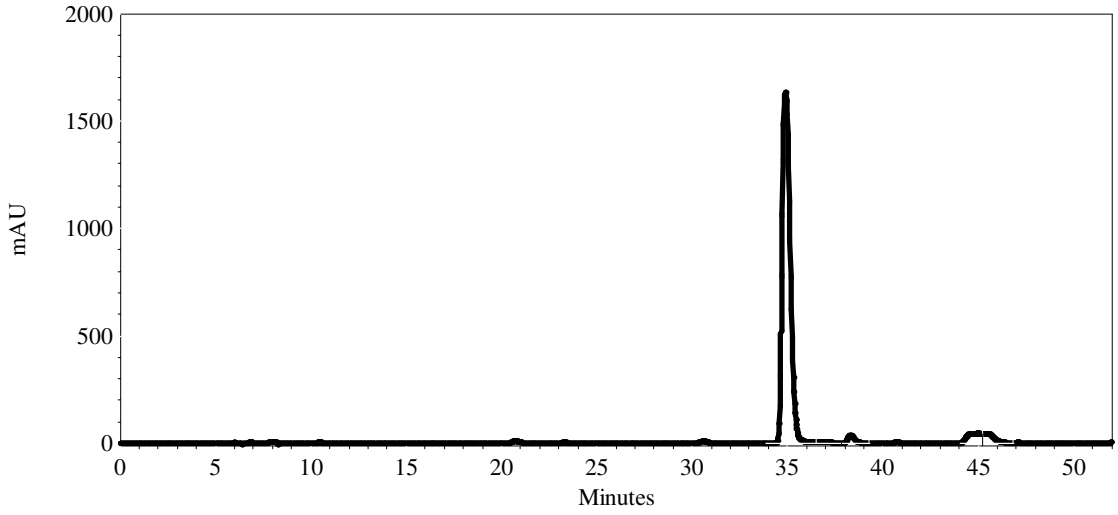


Fig. 21. Perfil cromatográfico à 280 nm da fração X de *L. brasiliense*.

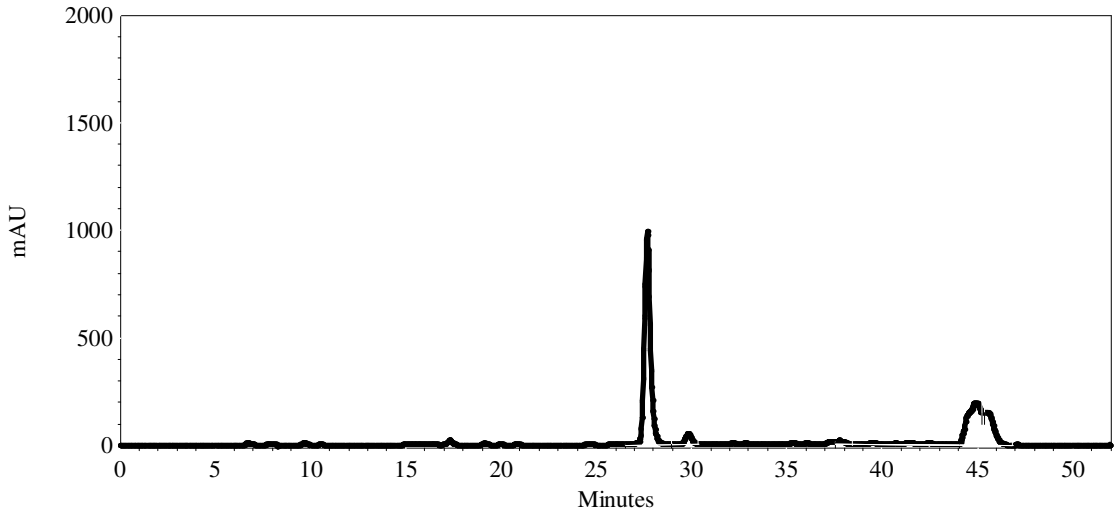


Fig. 22. Perfil cromatográfico à 280 nm da fração XII de *L. brasiliense*.

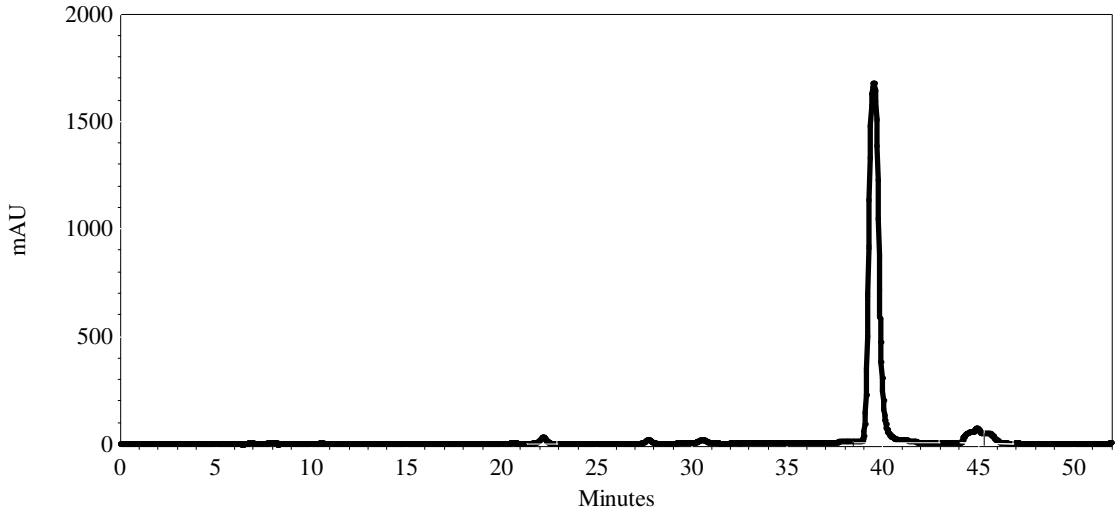


Fig. 23. Perfil cromatográfico à 280 nm da fração XIII de *L. brasiliense*.

Tabela 06. Compostos identificados por ESI-MS/MS nas respectivas frações.

Amostras Detectadas	**Composto**	**m/z [M- H]$^-$**	**MS/MS**	**Referência**
VI X XII XIII XIV	Acido gálico	169	125	(Fracassetti *et al.*, 2013)
X XII XIII XIV	Epigalocatequina galato	457	331, 305, 169	(Fracassetti *et al.*, 2013)
XII XIII	Samarangenina A	759	759→607, 589, 481, 463, 423	Andressa Blainski Pinha, comunicação pessoal, 2016.
XIII XIV	Samarangenina B	911	911→759, 741, 589, 571, 445, 423	Andressa Blainski Pinha, comunicação pessoal, 2016.
X XII	Galocatequina-galato-dímero	915	457, 169	(Fracassetti *et al.*, 2013)
Amostras Detectadas	**Composto**	**m/z [M+H]$^+$**	**MS/MS**	**Referência**
VI X XII XIII XIV	Luteolina	287	269, 241, 213, 175, 153	(Tsimogiannis *et al.*, 2007)

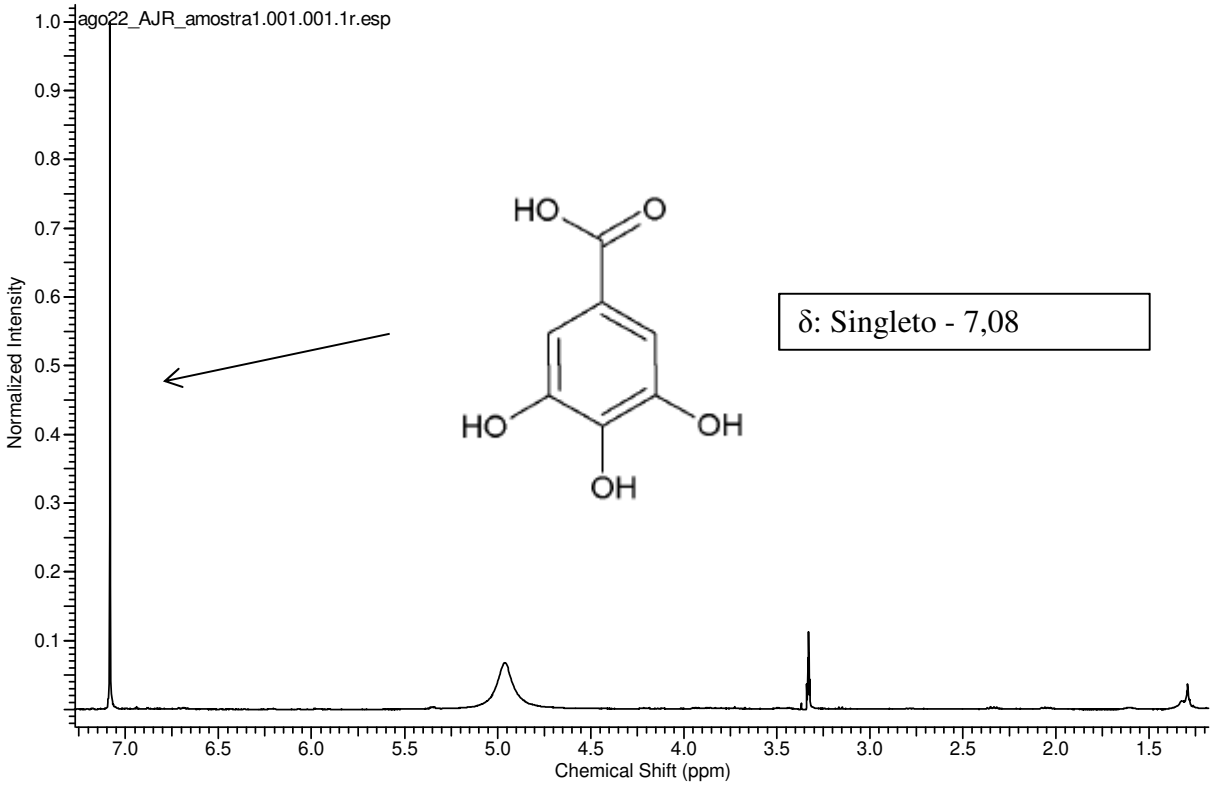


Fig. 24. Espectro de Ressonância magnética nuclear de hidrogênio (400 Hz – CD3OD) da amostra VI que contém a substância ácido gálico. Os deslocamentos a 3.33 e 4.96 ppm se referem ao solvente.

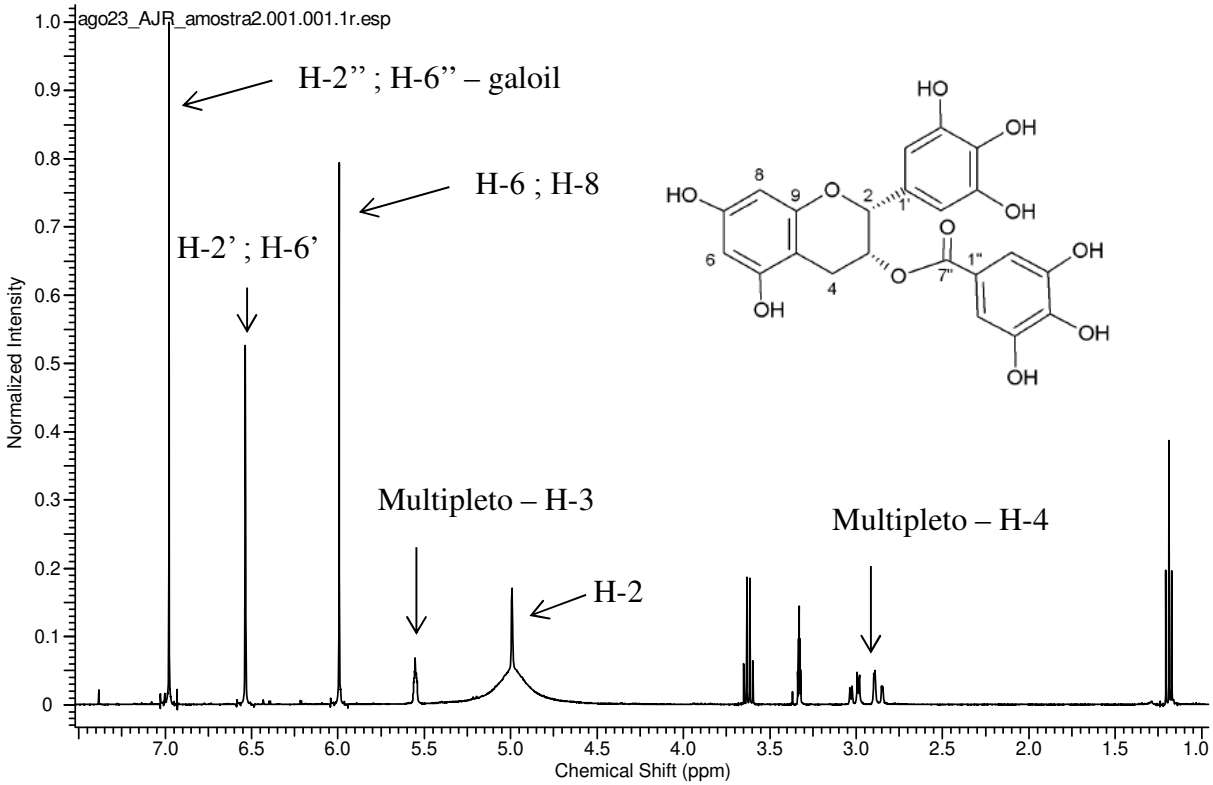


Fig. 25. Espectro de Ressonância magnética nuclear de hidrogênio da amostra X (400 Hz – CD3OD) que contém a substância epigalocatequina galato.

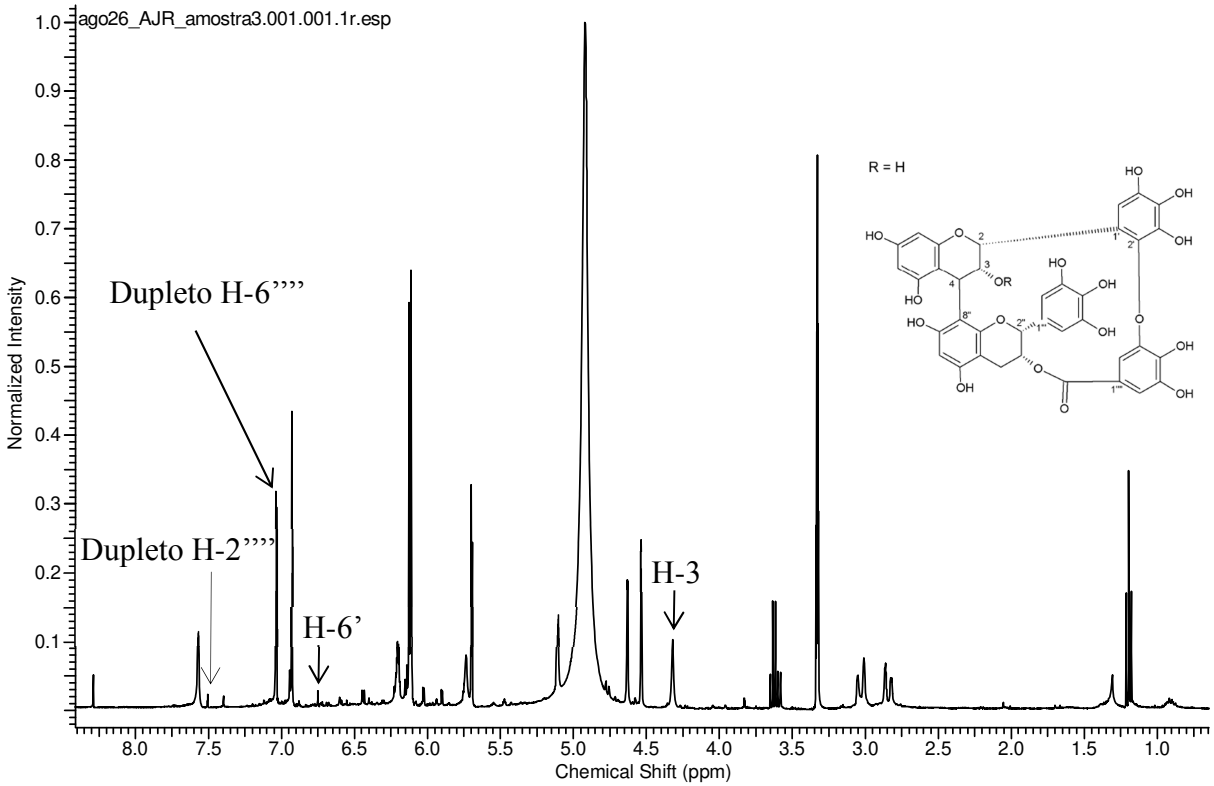


Fig. 26. Espectro de Ressonância magnética nuclear de hidrogênio (400 Hz – CD3OD) da amostra X que contém a substância samarangenina A. Os deslocamentos a 3.33 e 4.92 ppm se referem ao solvente.

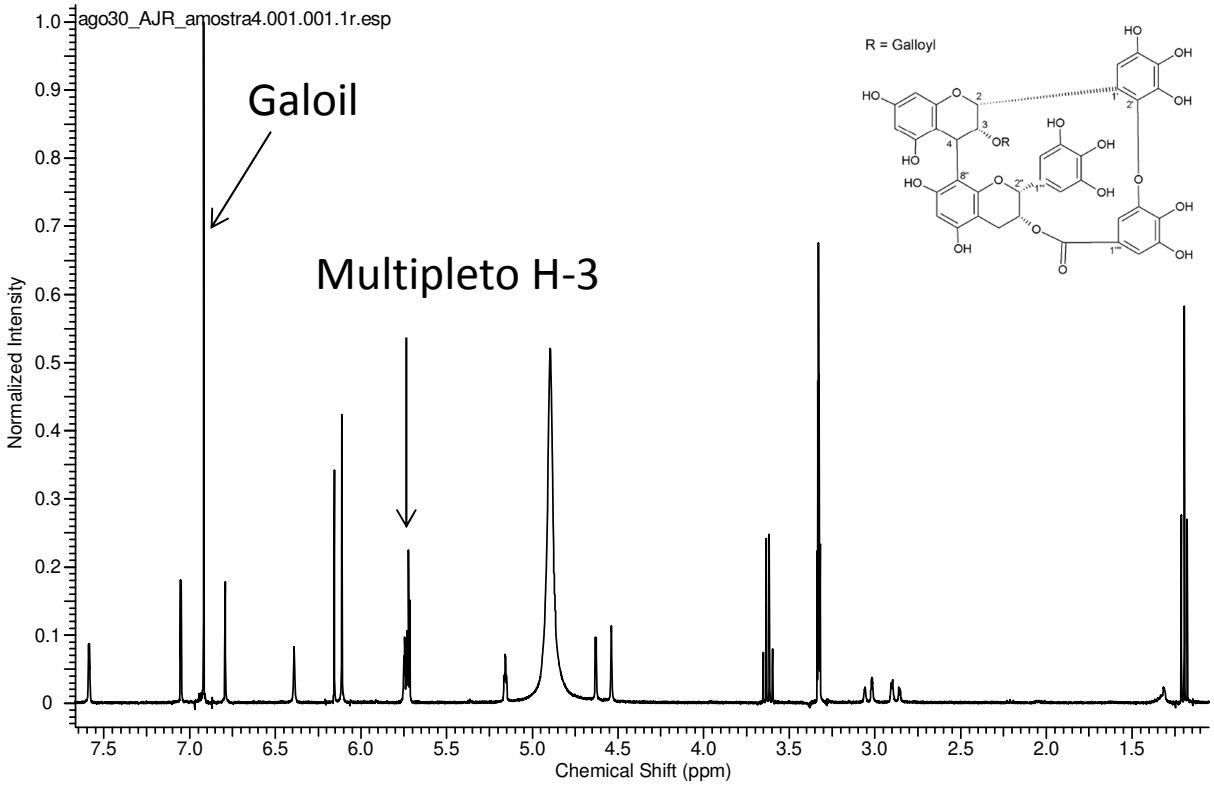


Fig. 27. Espectro de Ressonância magnética nuclear de hidrogênio (400 Hz – CD3OD) da amostra X que contém a substância samarangenina B. O deslocamento a 3.33 ppm se refere ao solvente.

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CONCLUSÃO

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O extrato hidroacetônico obtido da planta *L. brasiliense* é um agente promissor para combater a periodontite, pois inibiu os principais fatores de virulência de *P. gingivalis*, as gingipaínas, e também a adesão frente às células KB. Somado a esses dados, o extrato não mostrou citotoxicidade para a linhagem KB. Estudos com as proantocianidinas galoiladas isoladas são importante para determinar a substância por trás desta atividade. Estudos clínicos também são necessários para o desenvolvimento de um produto para uma terapia oral contra esta doença.

Novas moléculas de também são necessárias para o tratamento da infecção causada por *H. pylori* e classes de compostos com modos de ação diferentes dos tradicionais são essências para superar o problema da resistência em *H. pylori*. Na revisão de literatura as classes: quinoxalina, oligómeros de lisinas aciladas, quinonas, pirazolopirimidindionas, pirazolinas, cumarina-3-carboxamidas e xantonas são compostos promissores para a erradicação terapêutica. Testes *in vivo* e ensaios clínicos são de importância fundamental para o desenvolvimento de agentes efetivos frente a esta bactéria.