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ATIVIDADE ANTIMICROBIANA E ANTIOXIDANTE DE METABÓLITOS SECUNDÁRIOS E ÓLEO ESSENCIAL DE PLANTAS DA CAATINGA: *Myroxylon peruiferum* L.f E *Combretum leprosum*

FORTALEZA
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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia de Recursos Naturais da Universidade Federal do Ceará como requisito parcial para obtenção do título de mestre em Biotecnologia de Recursos Naturais.

Orientador: Prof. Dr. Edson Holanda Teixeira

Coorientador: Prof. Dr. Mayron Alves de Vasconcelos

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"Não existem fronteiras para quem quer atingir um objetivo. Seja um pássaro e voe alto, pois seus objetivos estão mais perto do que você imagina. Faça uma pergunta para você mesmo: Eu quero? Por que se você quer você pode! Portanto não pergunte: - Eu quero? Afirme: - Eu posso, eu consigo!"

Rogério Gozzi

RESUMO

O aumento de infecções microbianas juntamente com a resistência que estes agentes têm desenvolvido aos antimicrobianos convencionais tem levado a uma busca constante por alternativas terapêuticas eficazes, que possam oferecer melhores opções de tratamento aos doentes. Espécies do gênero *Combretum* e *Myroxylon* demonstram algumas atividades biológicas, como antibacteriana, antifúngica e antibiofilme. Desta forma, o triterpeno isolado de *Combretum leprosum* 3β-6β-16β-trihidroxilup-20(29)-eno (CLF1) e a isoflavona 7-hidroxi-4´,6-dimetoxi-isoflavona juntamente com o óleo essencial *M. peruiferum* foram utilizados neste estudo, com o objetivo de verificar a ação antimicrobiana e antibiofilme. A Concentração Inibitória Mínima (CIM), Concentração Bactericida Mínima (CBM) e a Concentração Fungicida Mínima (CFM) foram determinadas pelo método de microdiluição em caldo. O sinergismo foi avaliado pelo método de *checkerboard* com a combinação do triterpeno com as drogas ampicilina e tetraciclina. Já a formação do biofilme foi avaliada através da quantificação de biomassa pelo método de coloração com cristal de violeta (CV) e enumeração de células viáveis, enquanto que a atividade metabólica foi analisada pelo método XTT. Para a atividade antioxidante, foram utilizados os ensaios de 2,2-difenil-1-picril-hidrazil (DPPH), quelante de íons ferrosos (FIC), redução do ferro (FRAP), inibição da oxidação do β-caroteno (BCB). As espécies de bactérias e fungos tiveram o seu crescimento planctônico completamente inibido pelo triterpeno e isoflavona nas concentrações que variaram entre 2000 a 1,9 µg/mL, enquanto que para o óleo essencial variou entre 5 a 1,25%. O CLF1 apresentou efeito sinérgico com ampicilina para *S. aureus* ATCC 25923, *S. aureus* ATCC 700698 e *S. epidermidis* ATCC 35984 e *S. epidermidis* ATCC 12228 e com tetracilina para *S. aureus* ATCC 25923, *S. aureus* ATCC 700698 e *S. epidermidis* ATCC 35984, já para *S. epidermidis* ATCC 12228 apresentou efeito antagônico. Em relação aos ensaios antibiofilmes, CLF1 induziu uma redução na biomassa e número unidade formadora de colônias (UFC's), para todas as bactérias Gram-positivas. Já para o método XTT, a estirpe de *S. aureus* ATCC 25923 e *S. aureus* ATCC 700698 apresentou maior redução metabólica do que *S. epidermidis* ATCC 12228 e *S. epidermidis* ATCC 35984. Com relação às atividades antioxidantes, os tratamentos com óleo essencial e isoflavona resultaram em efeitos antioxidantes em todos os ensaios e concentrações realizadas, principalmente no BCB com uma inibição de 100%, mostrando-se mais ativa do que o controle positivo. Portanto, os resultados demonstraram que os compostos secundários e o óleo essencial utilizados nesse estudo, podem ser considerados potenciais agentes antimicrobianos e antibiofilmes, sugerindo assim o uso dessas moléculas no tratamento de infecções associadas a diferentes microorganismos.

Palavras-chaves: *Combretum leprosum. Myroxylon peruiferum* L.f. Metabólitos secundários. Atividade antimicrobiana. Atividade antioxidante.

ABSTRACT

The increase in microbial infections plus the resistance that these agents have developed to conventional antimicrobials has led to a search for effective therapeutic alternatives that offer better solutions for treatment. Species of the genus *Combretum* and *Myroxylon* demonstrate some biological activities such as antibacterial, antifungal and antibiofilm. Thus, the triterpene of *C. leprosum* 3β-6β-16β-trihydroxylup-20(29)-en (CLF1) and isoflavone 7-hydroxy-4',6-dimethoxy-isoflavone with the essential oil of *M. peruiferum* were used in the study with the objective of verifying the antimicrobial activity and antibiofilm. The Minimal Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (CFM) were determined by the broth microdilution method. Synergism was evaluated by the *checkerboard* method with the combination of triterpene with ampicillin and tetracycline. Biofilm formation was evaluated through the quantification of biomass by the crystal violet (CV) staining method and enumeration of viable cells, whereas the metabolic activity was analyzed by the XTT method. For the antioxidant activity, were realized the tests of 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrous ion chelating (FIC), iron reduction (FRAP), inhibition of β-carotene oxidation (BCB). Bacteria and fungi species had their planktonic growth completely inhibited by triterpene and isoflavone at concentrations ranging from 2000 to 1.9μg/mL; for essential oil ranged from 5 to 1.25%. CLF1 showed synergistic effect with ampicillin for *S. aureus* ATCC 25923, *S. aureus* ATCC 700698, *S. epidermidis* ATCC 35984, *S. epidermidis* ATCC 12228 and with tetracylin for *S. aureus* ATCC 25923, *S. aureus* ATCC 700698 and *S. epidermidis* ATCC 35984, whereas for *S. epidermidis* ATCC 12228 presented antagonistic effect. In relation to antibiotic tests, CLF1 induced a reduction in biomass and viable cells (CFU) for all Gram-positive bacteria. For the XTT method, *S. aureus* strain ATCC 25923 and *S. aureus* ATCC 700698 had a greater metabolic reduction than *S. epidermidis* ATCC 12228 and *S. epidermidis* ATCC 35984. About antioxidant activities, treatments with essential oil and isoflavone resulted in antioxidant effects in all assays and concentrations performed, mainly in BCB with a 100% inhibition, proving to be more active than the positive control. Therefore, the results demonstrated that the secondary compounds and the essential oil used in this study can be considered as potential antimicrobial agents and antibiotics, suggesting the use of these molecules in the treatment of infections associated with different microorganisms.

Keywords: *Combretum leprosum*. *Myroxylon peruiferum* L.f. Metabolites secondary. Antimicrobial activity. Antioxidant activity.

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LISTA DE ABREVIATURAS E SIGLAS

CBM	Concentração Bactericida Mínima
CIM	Concentração Inibitória Mínima
CLF1	Fração um de *Combretum leprosum*
CV	Cristal Violeta
DO	Densidade Ótica
DPPH	2,2-difenil-1-picril-hidrazil
FIC	Quelante do Ion Ferro
FRAP	Poder Antioxidante Redutor do ferro
LPS	Lipopolissacarídeos
MEV	Microscopia Eletrônica de Varredura
mL	Mililitros
MVA	Via do Mevalonato
PAL	Fenilalanina-amónia-líase
QS	*Quorum Sensing*
Acetil CoA	Acetilcoenzima A
SPE	Substâncias Poliméricas Extracelulares
UFCs	Unidades Formadoras de Colônias
µg	Micrograma
µL	Microlitros
IPP	Isopentenil-difosfato
OPP	Oxigênio-pirofosfato
GPP	Geranil difosfato
MEP	Metileritritol-fosfato
ERO	Espécieis reativas de oxigênio
AMPs	Peptídeos antimicrobianos de hospedeiro
PBPs	Proteínas de ligação a penicilinas
KI	Iodeto de potássio

SUMÁRIO

1 INTRODUÇÃO

O aumento de infecções microbianas juntamente com a resistência que estes agentes têm desenvolvido aos antimicrobianos convencionais tem levado a uma busca constante por alternativas terapêuticas eficazes, que possam oferecer melhores opções de tratamento aos pacientes (PIERCE *et al*., 2013).

As doenças infecciosas são a segunda maior causa de mortalidade mundial, e é considerada esta problemática, atrelada às altas taxas de resistência dos microrganismos, especialmente em ambientes hospitalares, justifica a urgência no desenvolvimento de novos agentes antimicrobianos (GUIMARAES *et al*., 2010). As doenças infecciosas são consideradas como grave problema de saúde coletiva, em virtude do impacto que geram na sociedade. São provocadas por microrganismos patogênicos que invadem o organismo do hospedeiro, evitam suas defesas e provocam danos aos tecidos (GUIDO *et al*., 2010).

O aparecimento global de microrganismos resistentes a antimicrobianos tem sido diretamente associado ao mau uso da terapia antimicrobiana em hospitais e pela própria população através da automedicação, além do aumento da utilização de antibióticos na indústria de alimentos e da falta de drogas específicas que sirvam para o tratamento de pacientes de risco, os quais geralmente são acometidos por múltiplas co-morbidades (BUFFET-BATAILLON *et al*., 2012).

Nas últimas décadas, os estudos sobre as comunidades microbianas têm chamado atenção para a análise das complexas interações que surgem entre microrganismos da mesma espécie e espécies distintas durante a formação de comunidades microbianas, denominadas biofilmes (PARROT *et al*., 2016).

No processo de desenvolvimento de novos compostos farmacologicamente ativos, substâncias isoladas de vegetais representam fontes importantes para a prospecção de novas drogas (TOLMACHEVA; ROGOZHIN; DERYABIN, 2014). De fato, o conhecimento popular acerca das propriedades curativas de plantas tem sido aplicado por muitos povos desde períodos remotos (SEMWAL *et al*., 2014).

Muitas plantas podem ser consideradas como fontes de moléculas úteis, as quais atuam como agentes antimicrobianos na tentativa de superar a resistência aos antibióticos utilizados no tratamento clínico (SIMOES; SIMOES; VIEIRA, 2010). Por outro lado, os vegetais são fontes de compostos secundários com atividade antioxidante, sendo úteis na terapêutica de doenças relacionadas ao estresse oxidativo celular (AHMAD *et al*., 2015; MARINO *et al*., 2015).

O estresse oxidativo resulta em um desequilíbrio na produção de espécies reativas de oxigênio (ERO) e a capacidade antioxidante das células. Os EROs, tais como o superóxido (O_2^-), o peróxido de hidrogénio (H_2O_2) e os radicais hidroxilo (OH) são constantemente produzidos em células aeróbicas, por redução incompleta de O_2 molecular a H_2O durante a fosforilação oxidativa mitocondrial. (BIRBEN *et al*., 2012).

As plantas medicinais são fontes de substâncias bioativas empregadas na medicina popular por muitos grupos populacionais rurais, quilombolas, indígenas e ribeirinhos (SILVA *et al*., 2012). *M. peruiferum* e *C. leprosum* são espécies com atividade antimicrobiana e são encontradas nas famílias Fabaceae e Combretaceae.

M. peruiferum é conhecida popularmente como cabriúva, pau-de-bálsamo e bálsamo. É uma planta decídua, heliófita, possuindo cerca de 10 a 20 metros de altura. Suas folhas são de ocorrência em quase toda a extensão do país, e a madeira é adequada para mobiliário, revestimentos, construção civil, dentre outras atribuições, por causa da sua alta resistência ao apodrecimento (LORENZI, 1998). Substâncias como 3',4',7-trimetil isoflavonas**,** 6-Hidróxi-4',7-metoxi-isoflavona e germacreno D foram isoladas a partir das folhas de *M. Peruiferum* e apresentaram atividades frente a *Mycobacterium tuberculosis*, *Mycobacterium avium* e *Mycobacterium kansasii* (CARVALHO *et al*., 2008). A isoflavona 7-hidroxi-4´,6-dimetoxi-isoflavona isolada do extrato acetato de etila, sendo o composto mais importante da espécie, apresentou atividade antibiótica comprovada frente à *Helicobacter pylori*. (OHSAKI *et al*., 1999).

C. leprosum é um membro da família Combretaceae, planta arbustiva nativa da região Nordeste do Brasil, conhecida popularmente de “mufumbo” ou “cipoaba” (LIRA *et al*., 2002), e apresenta em sua constituição vários compostos resultantes do seu metabolismo secundários, como o triterpeno pentacíclico 3β,6β,16β-trihidroxilup-20(29)-eno, composto capaz de desempenhar atividade antibacteriana e antibiofilme sobre bactérias Gram-positivas. (FACUNDO *et al*., 1993; EVARISTO *et al*., 2014, 2017).

Desta forma, este trabalho teve como objetivo de avaliar o potencial do triterperno isolado de *C. leprosum* e da isoflavona e óleo essencial de *M. peruiferum* frente a microrganismos patogênicos.

2 REFERÊNCIAL TEÓRICO

Plantas medicinais

Considerações iniciais

Os vegetais são utilizados como fontes de alimentos, fibras, medicamentos e muitos outros produtos úteis pela humanidade (GURIB-FAKIM, 2006). O uso de plantas medicinais no tratamento de enfermidades humanas e animais são bem conhecidos, mas a sua utilização no controle de fitopatógenos é mais recente (TAPWAL *et al*., 2011).

O primeiro registro arqueológico do uso de vegetais importantes é datado em 60.000 anos, tendo sido encontrado em um túmulo humano localizado no Iraque. Várias descobertas arqueológicas apontam o uso de espécies psicoativas no Timor (Indonésia), datado em cerca de 11.000 anos a.*C* (ALLEN, 2012). Parte do conhecimento médico dos antigos egípcios está registrada nos papiros denominados "Edwin Smith", "Kahun" e "Ebers". O papiro de Ebers (1550 a.C.), inicia-se com a afirmação "aqui começa o livro relativo à preparação dos remédios para todas as partes do corpo humano" (SAAD, 2013). De acordo com Aboelsoud (2010), o arquivo é considerado como o primeiro tratado egípcio sobre o uso de plantas medicinais, descrevendo as aplicações médicas de *P. somniferum* L. (ópio), *Cannabis sativa* L (maconha), *Commiphora myrrha* (mirra), *Boswellia serrata* Roxb. ex Colebr. (incenso), *Sena alexandrina* Mill (sena), *Lawsonia inermis* L. (hena) e *Aloe vera* (L.) Burm. f. (babosa).

No Brasil, a utilização de plantas medicinais é anterior ao Período Colonial, integrando as práticas tradicionais das diversas nações indígenas. Os relatos da flora brasileira iniciaram-se após a descoberta, por exemplo, na carta de Caminha, são relatadas várias espécies vegetais e seus usos, dentre estas o *Bixa orelana* L. (urucum). Pedro Álvares Cabral observou entre os povos indígenas daquela época o uso de produtos de origem vegetal para alimentação, tratamento de doenças e finalidades cosméticas. Posteriormente, Gabriel Soares de Souza em seu "Tratado Descritivo do Brasil" de 1587, denomina os produtos empregados na medicina indígena como "árvores e ervas da virtude" (WALKER, 2013).

Numerosos relatórios científicos mostraram que as plantas têm um alto potencial para sintetizar diferentes substâncias antimicrobianas (DAGLIA, 2011), que atuam como mecanismos de defesa da planta e protegem contra estresses abióticos (radiação UV, seca, temperaturas altas ou baixas, salinidade excessiva do solo) e estresses bióticos (por exemplo, microrganismos, insetos, e herbívoros). Os agentes antimicrobianos derivados de plantas podem ser classificados em fenólicos e polifenóis, terpenoides, alcaloides, entre outros componentes. (SIMOES; BENNETT; ROSA, 2009).

O Brasil é rico em espécies vegetais com metabólitos secundários de importância terapêutica, que também podem ser utilizados em doenças de plantas. Os produtos naturais, como compostos puros e extratos de plantas padronizados, proporcionam oportunidades para novos fármacos devido à disponibilidade da diversidade química (JAMUNA; RAI, 2011).

Plantas da Caatinga

A Caatinga é o único bioma exclusivamente brasileiro, ocupando cerca de 800.000 km². O nome “caatinga” tem origem Tupi-Guarani, na qual significa "mata branca", e refere-se à paisagem esbranquiçada da vegetação, adaptada à seca e aos ciclos climáticos (FILIZOLA; SAMPAIO, 2015). Este bioma compreende os estados de Alagoas, Bahia, Ceará, Pernambuco, Paraíba, Rio Grande do Norte, Piauí e Sergipe.

No Brasil, o estudo de plantas medicinais em resposta a tendência mundial de preservação da biodiversidade está crescendo de forma vertiginosa. Esse acontecimento deve-se a grande diversidade química e potencialidade farmacológica das espécies. Mesmo que os metabólitos secundários isolados não venham a se tornar fármacos, podem ser utilizadas como protótipos e originar compostos com utilidade clínica. Muitos dos vegetais com tais substâncias bioativas são encontadas no bioma Caatinga. (MONTANARI; BOLZANI, 2001; SILVA *et al*., 2012; SOUZA; MENDONÇA; SILVA, 2013; ALVES *et al*., 2014; MALAQUIAS *et al*., 2014; SILVA *et al*., 2014).

As plantas com atividades farmacológicas são encontradas em diferentes famílias botânicas, destacando-se entre as angiospermas as famílias Fabaceae e Combretaceae, dentre estas famílias, encontra-se as espécies de *M. peruiferum* e *C. leprosum.*

Myroxylon peruiferum

M. peruiferum é normalmente reconhecida pela combinação de pontos e listras translúcidas nos folíolos e fruto do tipo sâmara. É uma árvore na qual cresce até 15 ou 25 metros de altura, de copa arredondada e pouco densa, tronco cilíndrico de 60 a 80 cm de diâmetro (Figura 1). É encontrada no México, Honduras, Colômbia, Peru, Bolívia, Argentina, Brasil e Equador, em áreas de savana estépica, savana, floresta ombrófila densa, amazônica e atlântica (LIMA; MANSANO, 2011).

Conhecida popularmente como “bálsamo, cabreúva, bálsamo-do-peru e óleo-cabreúva”, *M. peruiferum* é uma planta nativa do bioma Caatinga, que também possui aplicações medicinais para as comunidades do semiárido. A casca do caule é utilizada para lavar o local afetado, como um antisséptico (CARTAXO *et al*., 2010). Além disso, esta

espécie produz um bálsamo (substância aromática que contém óleos essenciais e resinas em sua composição), que tem sido usado há séculos pelos povos indígenas da América Central e América do Sul para tratar a asma, reumatismo, feridas externas, bronquite, resfriado, tuberculose, dores de cabeça e abscesso (CUSTÓDIO; VEIGA-JUNIOR, 2012).

Figura 1: Imagem da espécie de *M. peruiferum.* A (caule), B (folhas jovens), C (hábito) e D (Folha composta, folíolos elípticos, alternos).

Fonte: Sartori (2000 e 2015)

Combretum leprosum

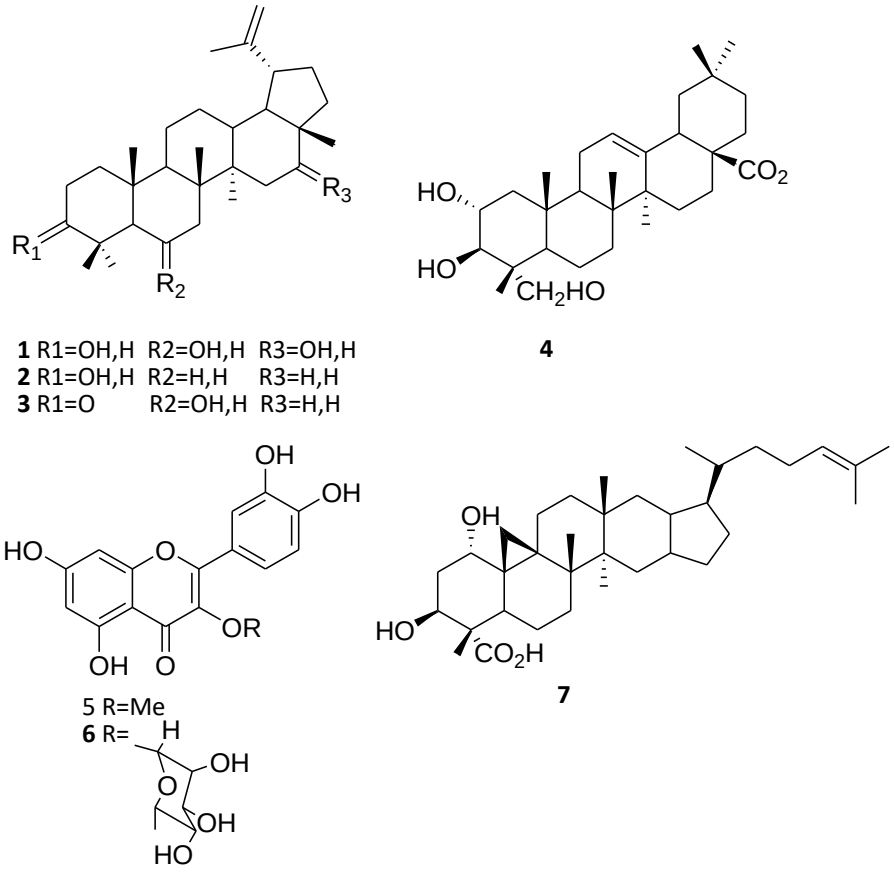
Na região brasileira, *C. leprosum* é uma planta que vem sendo bastante estudada (Figura 2). Estas espécies são conhecidas popularmente como “mofumbo, mufumbo, pente-de-macaco, carne-de-vaca ou cipoaba”, e além de ser uma espécie melífera, está distribuída nos nas regiões do Norte e Nordeste e nos estados de Mato Grosso e Mato Grosso do Sul (CHAVES *et al*., 2007). *C. leprosum* é utilizada na medicina popular, principalmente pela preparação de infusões de suas partes aéreas que proporcionam o tratamento de feridas e irritações cutâneas, ação expectorante, anti-hemorrágico, sedativo e analgésico (AGRA *et al*., 2007; FACUNDO *et al*., 2005).

Facundo *et al*. (1993) analisaram quimicamente as folhas e raízes de *C. lemprosum* e isolaram os triterpenos 3β,6β,16β-trihidroxilup-20(29)-eno e seus derivados (1-3), 2α, 3β, 23-trihidroxiolean-12-en-28-óico (4), ácido mólico (7) e flavonoides, 3-O-metilquercetina (5-6) e 3-O-α-L-ramnopiranosilquercitrina (7), assim como mostrados na figura 3.

Figura 2. Imagem da espécie de *Combretum leprosum*. A (folhas), B (folhas e inflorescência).

Fonte: Google imagem

Figura 3. Triterpenos e flavonoides isolados de *C. leprosum*.



Adaptado de Facundo *et al.*, *(*1993)

Estudos farmacológicos realizados com extrato etanólico de *C. leprosum* e com o triterpeno 3β,6β,16β-trihidroxilup-20(29)-eno, obtidos em diferentes partes da planta, demonstraram atividades biológicas incluindo propriedades antimicrobianas, antinociceptivas, anti-inflamatórias, anticolinérase e antiulcerogênicas. (PIETROVSKI *et al*., 2006; LONGHI-

BALBINOT *et al*., 2009, 2012; NUNES *et al*., 2009; HORINOUCHI *et al*., 2013; EVARISTO *et al*., 2014, 2017).

Metabólitos secundários de plantas

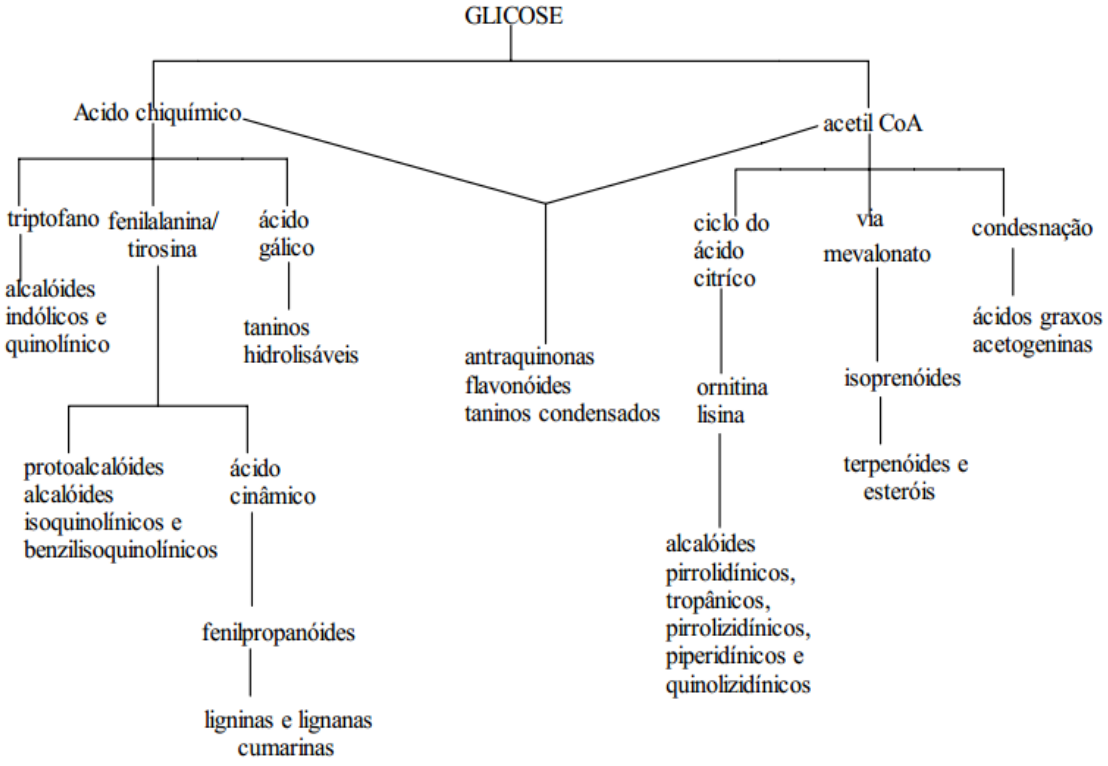
De acordo com Nelson e Cox (2014), o metabolismo refere-se ao conjunto de todas as transformações das moléculas orgânicas, envolvendo a participação das enzimas como biocatalisadoras. Ocorrem nas células vivas, renovando suas moléculas e permitindo a continuidade do estado organizado por meio do suprimento de energia.

As substâncias extraídas dos vegetais estão divididas em dois grupos: metabólitos primários e metabólitos secundários. Os metabólitos primários compreendem as classes de biomoléculas indispensáveis à vida, sendo os lipídios, carboidratos, proteínas e os ácidos nucléicos. Já os metabólitos secundários conferem propriedades para o desenvolvimento dos seres vivos, que são os terpenos, alcaloides, glicosídicos, flavonoides, compostos fenólicos, carotenoides, entre outros (ZHENG; WANG, 2001; CAI; SUN; CORKE, 2003; MILIAUSKAS; VENSKUTONIS; VAN BEEK, 2004; MATOS, 2009; KAMILOGLU *et al*., 2014; KARADENIZ *et al*., 2015; OZKAN *et al*., 2016).

Os metabólitos secundários apresentam diversidade química estrutural, alta variação intraespecífica e são produzidos conforme as condições ambientais, nutricionais e físicas do habitat (HARTMANN, 1996, 2007; BOURGAUD *et al.*, 2001; WAHID *et al.*, 2007). A origem de todas essas moléculas pode ser resumida a partir de vias metabólicas fundamentais para os organismos, como a fotossíntese, glicose e ciclo de Krebs (Figura 4) (DIAS; URBAN; ROESSNER, 2012).

Os metabólitos secundários são distinguidos em dois grupos diferentes, os compostos nitrogenados como os alcaloides, aminoácidos não protéicos, aminas, alcamides, glicosídeos cianogênicos e glucosinolatos e compostos não nitrogenados como os flavonoides, terpenos, saponinas, esteroides e cumarinas (PAIVA *et al.*, 2010).

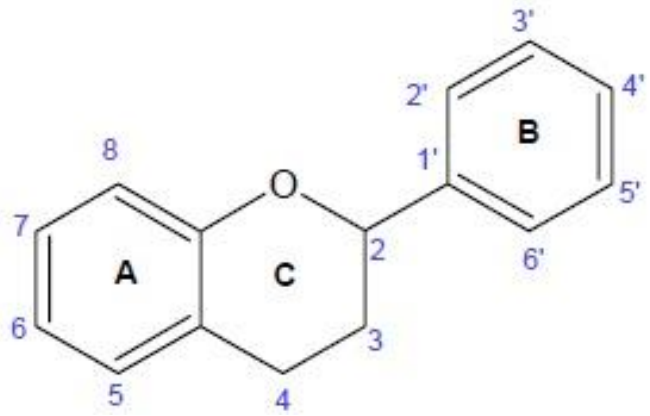
Figura 4 - Rota metabólica dos metabólitos secundários. Fonte: Santos (2004).



Flavonoide

Os flavonoides são considerados um dos maiores grupos de metabólitos secundários das plantas e distribuídas amplamente em frutas, folhas, chás e vinhos. São pigmentos naturais importantes e nas plantas tem como principal função proteger estes organismos contra agentes oxidantes (LOPES *et al*., 2010).

Figura 5 – Estrutura química básica dos flavonoides.

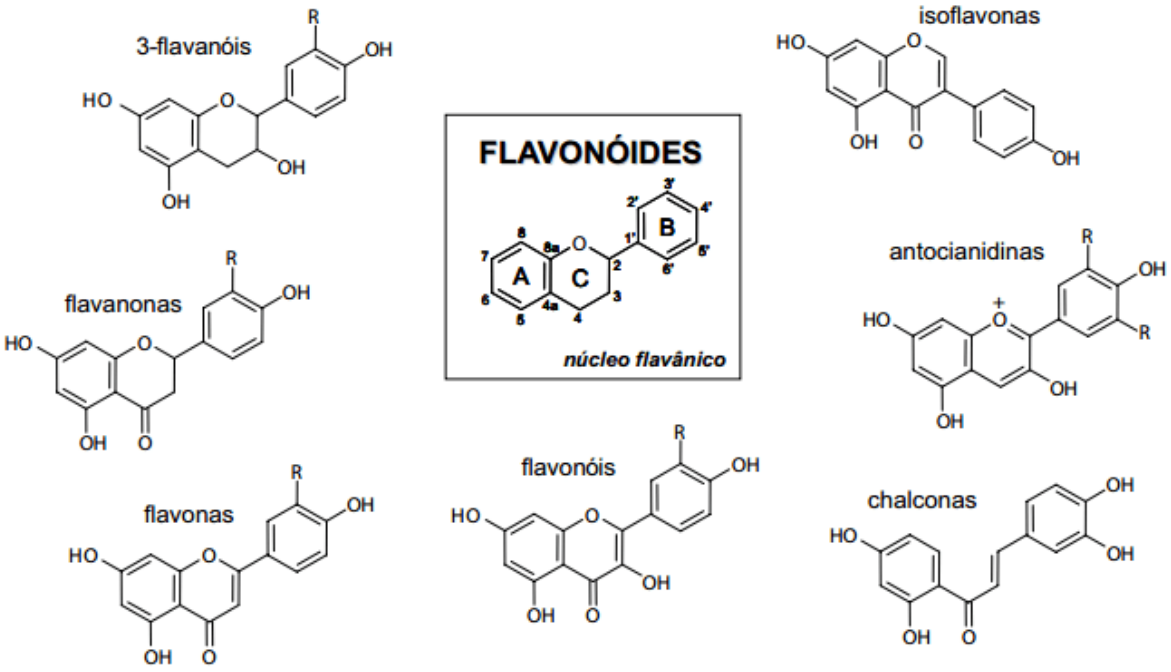


Fonte: Simões *et al.*, (2010).

A estrutura básica dos flavonoides consiste de 15 carbonos distribuídos em dois anéis aromáticos, A e B (Figura 5) e conectados via carbono heterocíclico do pirano. De acordo

com o estado de oxidação da cadeia heterocíclica do pirano, têm-se distintas classes de flavonoides (Figura 6) como as flavonas, flavonois, chalconas, auronas, flavanonas, flavanas, antocianidinas, leucoantocianidinas, proantocianidinas, isoflavonas e neoflavonoides (BRAVO, 1998; CHEYNIER, 2005; NOLVACHAI; MARRIOTT, 2013).

Figura 6 – Esqueletos básicos de flavonoides.

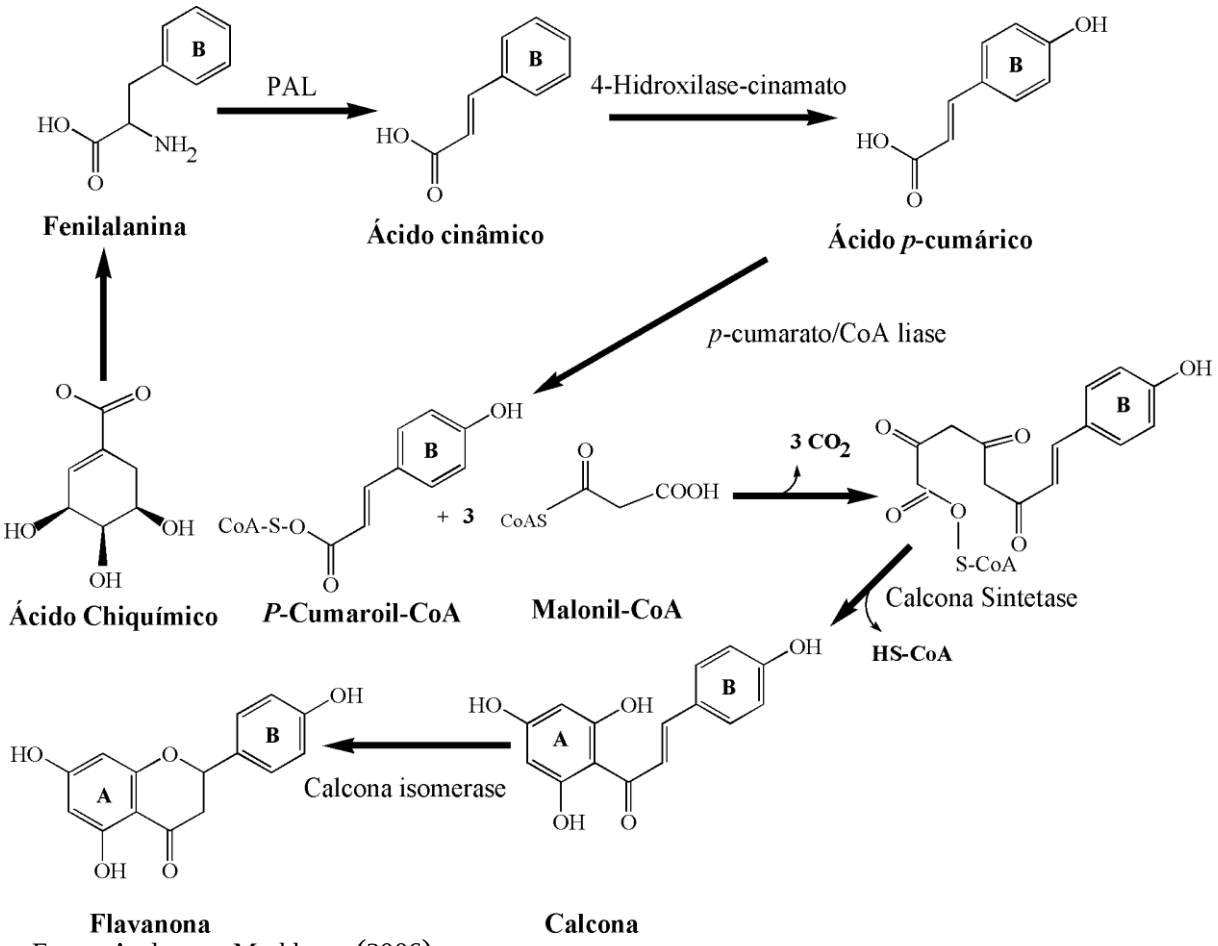


Fonte Google imagem

A estrutura comum dos flavonoides é biossintetizada a partir dos metabólitos que são derivados do ácido chiquímico e acetil-coA (Figura 7). O ácido chiquímico produz a fanilalanina que é o precursor inicial da síntese dos flavonoides. O aminoácido fenilalanina é desaminado pela enzima fenilalanina-amónia-líase (PAL) e produz o ácido cinâmico. Este é convertido em ácido *p*-cumárico por ação da enzima 4-hidroxilase cinamato. Por conseguinte, ocorre a adição da CoA, catalisada pela enzima *p*-cumarato/CoA liase originando a *p*-cumaroil-CoA. A *p*-cumaroil-CoA, ao reagir com três moléculas de malonil-CoA forma a calcona. Esta reação é catalisada pela enzima calcona sintetase. Finalmente, ocorre a ciclização do anel da calcona pela ação da enzima calcona isomerase, originando a flavanona (naringenina), com o núcleo básico de todos os flavonóides. Assim, nos flavonoides o anel A é formado via acetil CoA, o anel B via ácido chiquímico e o anel C deriva do fosfoenolpiruvato (FORMICA; REGELSON, 1995; DEWICK, 2002; HAVSTEEN, 2002;

IBRAHIM, 2001; MARTENS; MITHÖFER, 2005; ANDERSEN; MARKHAM, 2006; WINKEL, 2006; HURBER *et al*., 2008; DIXON; PASINETTI, 2010).

Figura 7- Biossíntese de flavonoides.



Fonte: Andersen; Markham, (2006).

Isoflavonas e suas atividades biológicas

As isoflavonas pertencem a uma classe do grupo dos flavonoides e são caracterizados por sua estrutura polifenólica. Diferente dos flavonoides, as isoflavonas são encontradas em raras famílias botânicas, concentrando-se principalmente na família Fabaceae. Esta distribuição reduzida das isoflavonas no reino vegetal pode ser devido às enzimas presentes na família das leguminosas. Estas enzimas atuam na rota biossintética dos flavonoides, as quais catalisam a transformação das flavanonas em isoflavonas (AGUIAR *et al*., 2007; COWARD *et al*., 1993; TSAO; YANG; YOUNG, 2003).

As isoflavonas têm chamado atenção devido à capacidade de reduzirem os riscos de doenças cardiovasculares e de promover a inibição do crescimento de células cancerígenas. Além disso, as isoflavonas possuem um importante papel na prevenção de várias doenças,

como a osteoporose e nos sintomas da menopausa. São também inibidores da proteína tirosina quinase, o que leva a inibição do ciclo celular e indução de apoptose em células tumorais. Os efeitos destas isoflavonas são fortemente influenciados pela estrutura química (FRITSCHE; STEINHART, 1999; VILLARES *et al*., 2011).

Os isoflavonoides que exibem grupos pernil apresentam atividade antibacteriana sobre bactérias Gram-positivos tais como, *Staphylococcus aureus* e *Bacillus subtilis*. Esta atividade aumenta quando os grupos prenil localizam-se nas posições C-6 ou C-8 no anel A e em C-3´ou C-5´ no anel B (BOJASE *et al*., 2002; MUKNE *et al*., 2011).

A maioria dos antibacterianos exibe atividade através da inibição da enzima DNA topoisomerase e desta forma, interfere na replicação de DNA, expressão e recombinação de genes. Através desta teoria, conseguiram provar que a isoflavona extraída da soja, é capaz de inibir significativamente a atividade da DNA topoisomerase através da formação de complexos que interferem com as ligações a esta enzima (WANG *et al*., 2010).

Os isoflavonoides, bem como outros flavonoides, podem bloquear o ciclo celular e induzir a apoptose em células cancerígenas que se encontram em divisão (SANDHAR *et al*., 2011; HAVSTEEN, 2002).

Terpenos

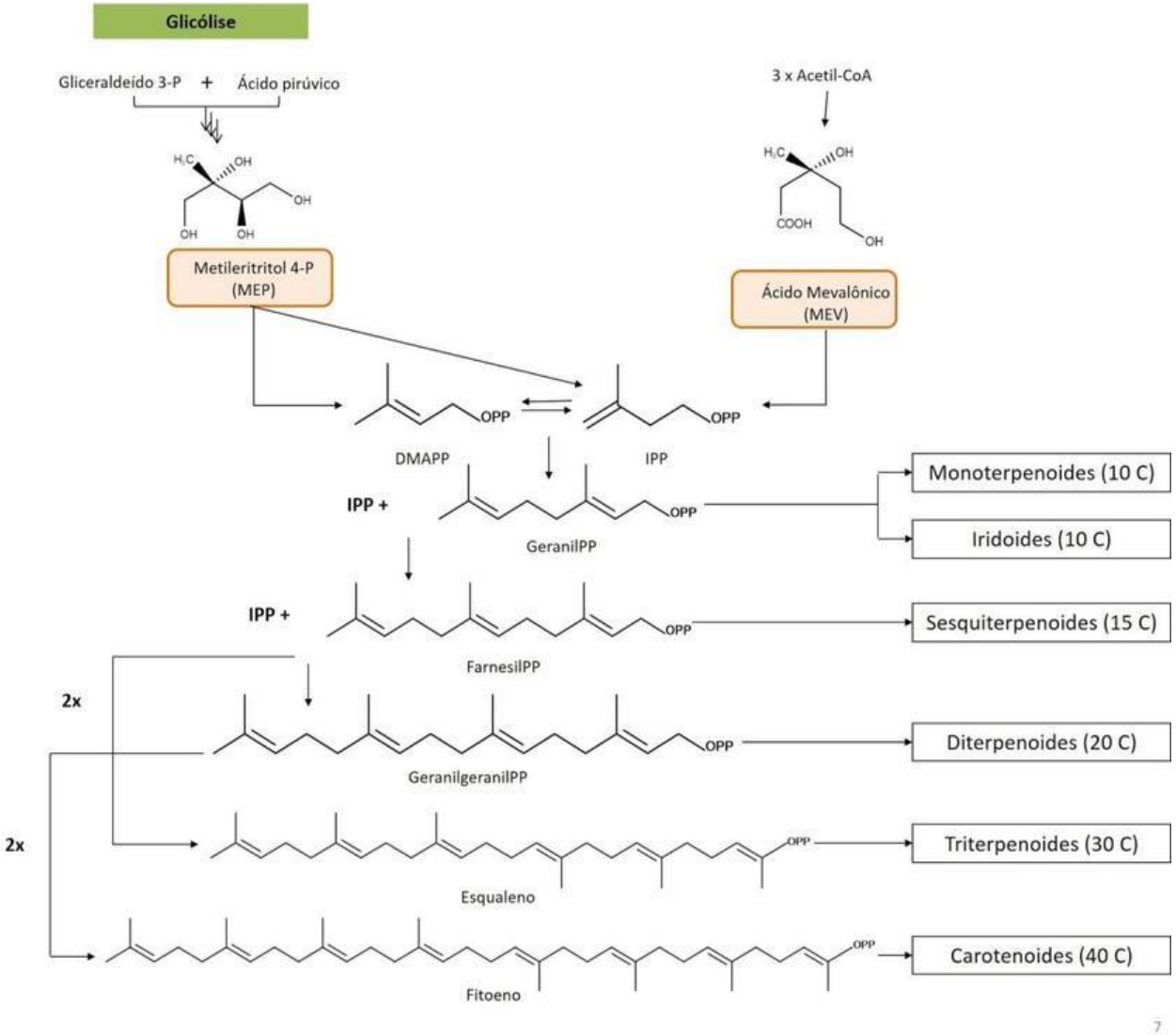
Terpenos são moléculas de hidrocarbonetos, enquanto que terpenoides são terpenos que tiveram suas moléculas modificadas em, pelo menos, uma adição de um átomo de oxigênio (ZWENGER; BASU, 2008). Os terpenoides representam o maior grupo de moléculas naturais presentes na natureza, exercendo diferentes funções em vários organismos como nas bactérias, leveduras, fungos filamentosos, plantas, animais e em alguns organismos marinhos (FAUSTINO, 2015).

Os terpenos ou isoprenoides são distribuídos no reino vegetal e apresentam grande diversidade estrutural e geralmente são insolúveis em água, formados pela fusão de unidades isoprênicas de 5 carbonos (C5), sendo subdividido e classificado em monoterpenos (2 unidades C), sesquiterpenos (3 unidades C5), diterpenos (4 unidades C5), triterpenos (6 unidades C5); tetraterpenos (8 unidades C5) e politerpenóides (mais de 8 unidades C5) (TAIZ, ZEIGER, 2009; BORGES, 2013).

As rotas principais na biossíntese de terpenos ocorrem em organelas diferentes da célula vegetal. Na rota do ácido mevalônico, que ocorre no citoplasma da mesma (Figura 8), três moléculas de acetil-CoA reagem para formar o ácido mevalônico e este, após sofrer reações de piro-fosforilação, descarboxilação e desidratação, resulta no isopentenil-difosfato

(IPP) (TAIZ; ZEIGER, 2004). Este composto ativado de fósforo se converte em seu isômero dimetilalil-difosfato (DMAPP), o qual é biossintetizado pela rota do metileritritol-fosfato (MEP) que ocorre nos cloroplastos, possui um grupo de saída, o oxigênio-pirofosfato (OPP). Após a protonação do seu oxigênio e, consequentemente, a formação do carbocátion alílico, ocorre à dimerização, com formação do geranil difosfato (GPP). Na rota do MEP, ocorre a reação de condensação entre uma molécula de gliceraldeído-3-fosfato e piruvato, que origina o DMAPP que se converte em seu isômero IPP (DEWICK, 2009).

Figura 8. Esquema da síntese de terpenos pelas vias MEV e MEP.



Fonte: Rezende *et al*., (2017)

Os monoterpenos são constituintes que fazem parte da composição dos óleos essenciais, que por sua vez são empregados na indústria, na produção de perfumes e cosméticos (EDRIS, 2007), além de apresentarem diversos efeitos farmacológicos, entre eles:

antihipertensivo, anti-inflamatório, antimicrobiano (ZORE *et al*., 2011) e antioxidante (QUINTANS-JÚNIOR *et al*., 2011).

Triterpenos e suas atividades biológicas

Nos últimos anos, os triterpenos têm sidos apresentados como uma nova classe de agentes terapêuticos antimicrobianos e antibiofilmes (KATERERE *et al*., 2003; FONTANAY *et al*., 2008; NASCIMENTO *et al*., 2014; GOSSAN *et al*., 2016). Tal interesse é amplamente justificado pelo rápido aumento da incidência de microrganismos patogênicos que representa um importante problema de saúde pública mundial (SOTO, 2013).

Em relação à atividade biológica dos terpenos, Evaristo *et al*. (2014; 2017), relataram que o triterpeno 3β,6β,16β-trihidroxilup-20(29)-eno, denominado como CLF1, apresentou atividade bactericida e bacteriostática sobre o crescimento planctônico e em forma de biofilmes das bactérias *Streptococcus mutans* e *Streptococcus parasanguinis* caracterizando-se como potencial agente antibacteriano.

Nascimento-Neto *et al*. (2015) verificaram que este mesmo triterpeno apresenta atividade pró-cicatrizante em feridas cutâneas de camundongos, através de uma progressão moderada de vasos no tecido de granulação da derme, rápida deposição de matriz extracelular e organização de miofibroblastos para tração da derme.

Gossan *et al*., (2016) demonstraram o pontencial antibacteriano dos triterpenos 28-*O*-β-D-glucopiranosil-2α,3β,21β,23-tetrahidroxiolean-18-en-28-oato e arjungenina frente as cepas de *Escherichia coli* (DHSα) *e Enterococcus faecalis* (ATCC 1054), *Staphylococcus aureus* (CIP53154 e 8325-4)**,** respectivamente.

Óleos essenciais

Óleos essenciais (OEs) são produzidos por plantas aromáticas e são compostos voláteis, produtos do metabolismo secundário, com composição complexa de substâncias lipofílicas, geralmente odoríferas e líquidas (LINDE *et al.*, 2016).

São misturas altamente complexas de compostos químicos presentes em diversas espécies vegetais. Estes óleos podem ser extraídos de distintas regiões do vegetal, como em folhas, galhos, raízes, frutos, sementes e flores. Determinadas estruturas vegetais são anatomicamente especializadas para acumular e secretar componentes dos óleos essenciais, como idioblastos secretores, ductos e tricomas glandulares. No organismo vegetal, os OEs exercem várias funções ecológicas, agindo como mensageiros internos, substância de defesa contra predadores e como atrativo de agentes polinizadores (FRANZ; NOVAK, 2010).

Constituintes químicos dos OEs são compostos voláteis, como hidrocarbonetos, álcoois, ácidos, aldeídos, cetonas, lactonas, fenóis, óxidos e ésteres, os quais podem ser classificados em terpenos e fenilpropanoides (DJILANI; DICKO, 2012).

Geralmente, as propriedades biológicas dos óleos essenciais são determinadas por seus componentes principais, incluindo dois grupos de origem bio-sintética distintas. (BOWLES, 2003; PICHERSKY; NOEL; DUDAREVA, 2006). Terpenos e terpenoides compreendem os grupos principais, enquanto que os constituintes aromáticos e alifáticos compreendem o outro grupo, todos caracterizados por baixo peso molecular. (CHOUHAN; SHARMA; GULERIA, 2017).

A atividade antimicrobiana dos EOs tem sido atribuída aos seus compostos fenólicos e sua interação com membranas celulares dos micorganismos que causam a liberação de íons e seu conteúdo citoplasmático e, portanto, podendo levar à morte celular (BURT, 2004; SUNTRES, COCCIMIGLIO; ALIPOUR, 2015).

Os OEs obtidos da flora nativa apresentam amplo potencial no controle de fitopatógenos, devido à sua ação antifúngica direta, inibindo o desenvolvimento micelial e a germinação de esporos ou pela indução de fitoalexinas (OOTANI, 2010). Além de possuir propriedades antifúngicas, ainda apresenta ações antibacterianas e inseticidas com baixo risco de toxicidade ao homem e ao meio ambiente (TOMAZONI, 2013).

Raut e Karuppayil (2014) relatam que diversos óleos essenciais podem apresentar atividade antibacteriana, antifúngica, antiviral e antiprotozoária, possuem, ainda, propriedades capazes de prevenir o desenvolvimento de câncer e mutações genéticas, apresentam potencial antidiabético e atividade anti-inflamatória.

Geralmente os OEs exibem maiores propriedades bacteriostáticas e bactericidas contra bactérias Gram-positivas do que Gram-negativas, devido à camada de lipopolissacarídeos na sua membrana externa (TECHATHUVANAN *et al*., 2014; TEHRANI; SADEGHI, 2015).

A parede celular das bactérias Gram-positivas consiste em peptidoglicano (90-95%), juntamente com ácido teicoico e proteínas ligadas a ele. Como os principais constituintes dos EOs são hidrofóbicos, eles interagem com a membrana celular e passam facilmente através do citoplasma. Enquanto que a estrutura da parede celular em bactérias Gram-negativas é mais complexa, pois é composta por uma monocamada de peptidoglicano rodeada por uma membrana externa constituída por proteínas e lipopolisacarídeos. (KHORSHIDIAN *et al*., 2018). Esta camada externa tem natureza hidrofílica, no entanto, os compostos hidrofóbicos

podem ultrapassar por essa barreira (VAARA, 1992; NIKAIDO, 1994; NAZZARO *et al*., 2013).

Existe a necessidade de encontrar estratégias alternativas para lidar com infecções resultantes de bactérias resistentes a medicamentos, devido ao aumento de bactérias resistentes aos antibióticos e à falta de novos antibióticos trazidos para o mercado. (CHOUHAN; SHARMA; GULERIA, 2017). O desenvolvimento de alternativas aos antibióticos e a descoberta ou desenvolvimento de adjuvantes estão entre as estratégias potenciais propostas (BUSH *et al*., 2011). Combinação de antibióticos com outras drogas que não são antibióticas é uma dessas possibilidades.

Biofilmes microbianos

Conceito e aspectos gerais

As pesquisas científicas vêm dando maior ênfase a estudar os biofilmes, sendo esta a sua forma natural em que a maioria dos microrganismos é encontrada. O primeiro relato científico sobre biofilme foi descrito em 1638 por Antonie Van Leeuwenhoek em seu artigo para a Sociedade Real de Londres. Ele relata sua observação sobre placa dentária: “O número de animalículos na superfície de um dente são tantos que eu acredito excederem o número de homens em um reino” (GULATI; NOBILE, 2016).

Os estudos sobre as comunidades microbianas têm chamado atenção para a análise das complexas interações que surgem entre microrganismos da mesma espécie e espécies distintas durante a formação de comunidades microbianas, denominadas biofilmes (PARROT *et al*., 2016).

Biofilmes são comunidades microbianas caracterizadas por células aderidas a um substrato abiótico ou biótico e incrustadas numa matriz de substâncias poliméricas extracelulares (SPE), produzida pelos próprios microrganismos (HURLOW *et al*., 2015).

Além de auxiliar os microrganismos em sua adesão, a matriz é capaz de conferir-lhes maior resistência, provendo-lhes mecanismos adicionais de adaptação a fatores de estresse (presença de antibióticos, desenvolvimento da resposta imunológica do hospedeiro e a dessecação da comunidade microbiana) (PAUL; JEFFREY, 1985; STOODLEY *et al*., 2002; BEECH; SUNNER; HIRAOKA, 2005; FLEMMING; WINGENDER, 2010).

Além da barreira física produzida pela matriz, pesquisadores acreditam que os microrganismos em biofilmes têm seu metabolismo e taxa de crescimentos reduzidos, conferindo aos microrganismos uma maior tolerância aos agentes antimicrobianos (DUNNE, 2002; DAVIES, 2003; DOUGLAS, 2003; AN; DONG; ZHANG, 2009). Além do mais, genes

expressos em algumas bactérias codificam enzimas capazes de inibir a ação dos antibióticos (ANDERL *et al*., 2000).

Etapas de formação de biofilme

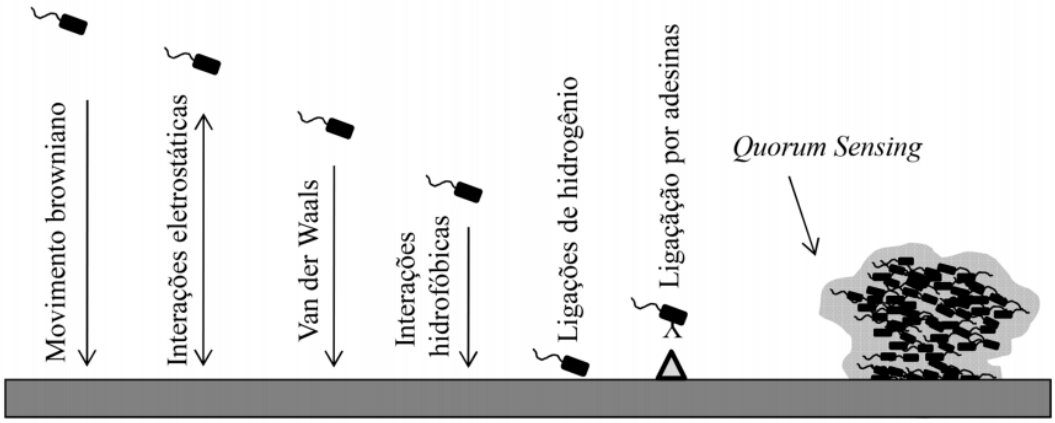
A formação de biofilme começa com a adesão, que é seguida por crescimento e expansão do biofilme (KOKARE *et al*., 2009). Principalmente a adesão das células irá ocorrer em superfícies mais ásperas, mais hidrofóbicas e revestidas por uma superfície de condicionamento (DONLAN; COSTERTON, 2002; KOKARE *et al*., 2009).

A adesão primária compreende o primeiro passo para formar um biofilme maduro e envolve a ligação reversível de microrganismos planctônicos a uma superfície. (Figura 9) (HOIBY *et al*., 2011).

A adesão das superfícies abióticas é mediada por interações físico-químicas inespecíficas, como as forças hidrodinâmicas, interações eletrostáticas, forças de Van der Waals e interações hidrofóbicas, sendo que as células planctônicas aderem a uma superfície aleatoriamente com movimento browniano e força gravitacional ou de maneira direcionada via quimiotaxia, mobilidade dos flagelos e pili (PAVITHRA; DOBLE, 2008; TRENTIN *et al*., 2013).

Enquanto a adesão a superfícies bióticas é realizada por interações moleculares mediadas por lectinas ou adesinas (DUNNE, 2002). É importante ressaltar que as bactérias anaeróbicas facultativas têm a característica de serem colonizadores iniciais, aderindo às superfícies e produzindo o EPS, criando então um ambiente favorável para os microrganismos anaeróbicos nos estágios posteriores (MARANGONI *et al*., 2013).

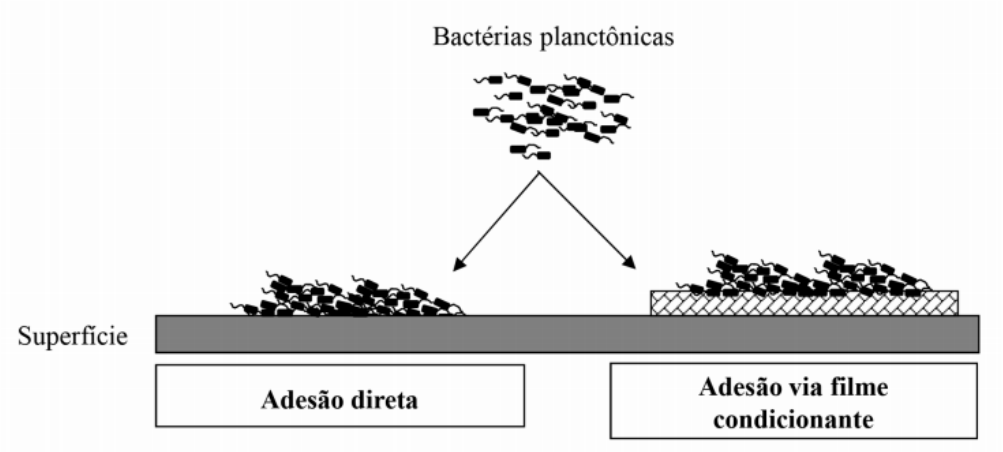
Figura 9: Etapa de adesão dos microrganismos.



Fonte: adaptado de Trentin; Giordani; Macedo (2013).

Ao analisar que a superfície do material em questão pode ser um dispositivo relacionado ao médico, como por exemplo, cateteres urinários, cateteres venosos centrais, tubos endotraqueais, próteses e válvulas cardíacas e que esse dispositivo será inserido em um hospedeiro, a adesão reversível entre a bactéria e a superfície pode acontecer de modo direto ou através de um filme condicionante (Figira 10). Este filme é um filme orgânico na qual possui composição variável, de acordo com o sítio de inserção, mas é constituído, principalmente, por proteínas, como albumina, imunoglobulina, fibrinogênio e fibronectina (HERRMANN *et al*., 1988; GOTTENBOS *et al*., 2002; ROCHFORD; RICHARDS; MORIARTY, 2012).

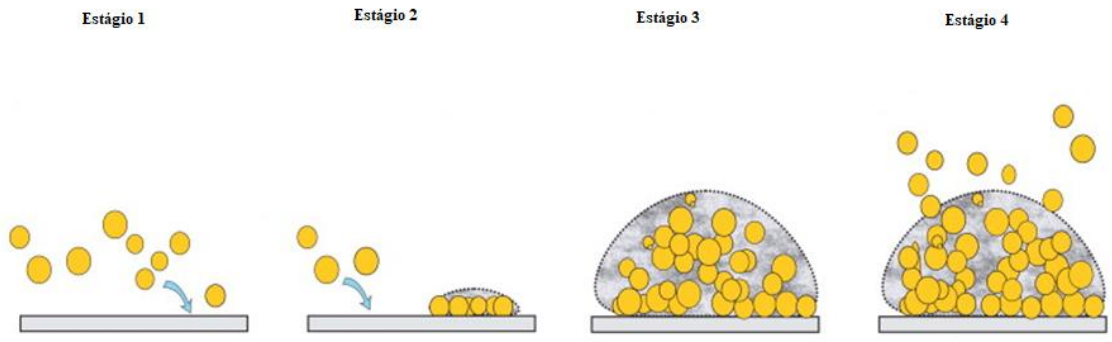
Figura 10. Adesão bacteriana em superfícies abióticas.



Fonte: Trentin; Giordani; Macedo (2013).

A formação de um biofilme é um processo complexo, que requer uma interação célula-célula coordenado por fatores físicos, químicos e biológicos (TA; ARNASON, 2015). Zhang *et al*. (2017) definiram a formação de formação de biofilme em quarto estágios, sendo que no estágio 1, os microrganismos são aderido a uma superfície e formam-se uma camada confluente (figura 11). No estágio 2, os microrganismos se multiplicam e depois se juntam para formar uma microcolônia. A terceira etapa deste processo envolve a formação de macrocolônias das microcolônias reunidas. Finalmente, no estágio 4, uma vez que o biofilme maduro é formado, os microrganismos saem e reentram na estrutura do biofilme.

Figura 11. Estágios do desenvolvimento dos biofilmes.



Fonte: Adaptado Tang *et al.* (2016)

Biofilme bacteriano de infecções causadas por *Staphylococcus aureus*

Biofilme microbiano é considerado um desafio mundial, devido à resistência antibiótica inerente conferida pelo estilo de vida. Ao viver em comunidade em uma situação clínica, os organismos microbianos são responsáveis por casos graves e perigosos de infecção. O combate a esta organização de células geralmente requerem altas doses de antibiótico por tempo prolongado, e essas abordagens muitas vezes falham, contribuindo para a persistência da infecção (RIBEIRO *et al*., 2016).

Biofilme de *S. aureus* tornou-se notório por causar infecções crônicas, devido à sua capacidade de resistir ao tratamento terapêutico formando biofilmes em dispositivos médicos domésticos, incluindo válvulas cardíacas artificiais implantadas, cateteres e próteses articulares. (RIBEIRO *et al*., 2012; MCCONOUGHEY *et al*., 2014).

De fato, as infecções relacionadas aos biofilmes, estão associadas ao aumento da morbidade e mortalidade, com dispositivos médicos infectados que, muitas das vezes, requerem remoção cirúrgica e aumento da duração da hospitalização. Como resultado, a prevalência dessas e outras doenças levaram a um aumento significativo nas despesas associadas às infecções por *S. aureus* na última década, com custos anuais estimados de US $ 450 milhões (PARVIZI *et al*., 2010; SONG *et al*., 2010).

S. aureus é capaz de se desenvolver em vários locais dentro do corpo, devido à sua impressionante variedade de fatores de virulência, incluindo adesinas, toxinas e proteínas de evasão imune (FOSTER *et al*., 2014; THAMMAVONGSA *et al*., 2015)

Mais recentemente, a relevância fisiológica da análise de biofilme *in vitro* foi aprimorada medindo a formação de biofilmes em condições de fluxo para recriar forças de cisalhamento em ambientes naturais, por exemplo, vasos sanguíneos e válvulas cardíacas. O

uso de ensaios de biofilme que combinam sistemas de células de fluxo microfluídico e microscopia de lapso de tempo gerou um modelo de desenvolvimento de biofilme de estafilocóco caracterizado por cinco fases distintas: (i) anexo, (ii) multiplicação, (iii) êxodo, (iv) maturação e (v) dispersão (MOORMEIER; BAYLES, 2017).

Essas experiências revelaram um novo nível de sofisticação, em que subpopulações metabolicamente heterogêneas são susceptíveis de melhorar significativamente a capacidade de resposta ambiental e a adaptabilidade da comunidade de biofilmes como um todo (MOORMEIER *et al*., 2013; 2014; MOORMEIER; BAYLES, 2017).

Na verdade, vários estudos de biofilme sugerem que os biofilmes de *S. aureus* se desenvolvem em espessas camadas de células em que os mecanismos de desprendimento são desencadeados e as subpopulações do biofilme são dispersas, criando microcolônias na biomassa que permanece (YARWOOD *et al*., 2004; BOLES; HORSWILL, 2008; PERIASAMY *et al*., 2012).

No entanto, nem todas as infecções por *S. aureus* envolvem o desenvolvimento do biofilme e há evidências crescentes de que a formação de agregação ou "microcolônia" é mais relevante em muitos casos. Muitas das vezes, esses agregados estão incorporados em material do hospedeiro, como proteínas de matriz extracelular, fibrinogênio, fibronectina e colágeno. Essas microcolônias não são necessariamente ligadas à superfície, são geralmente menores do que os biofilmes e não formam as torres de cogumelos que são observadas com o crescimento do biofilme *in vitro*. (BJARNSHOLT *et al*., 2013).

Há vários estudos na literatura mostrando que a conversão de fibrinogênio em fibrina pela enzima coagulase de *S. aureus* forma um tipo de escudo protetor, no qual as bactérias são incorporadas durante a patogênese de infecções por este microrganismo (CHENG *et al*., 2010; MCADOW *et al*., 2011; GUGGENBERGER *et al*., 2012; THOMER *et al*., 2016), e foi recentemente ampliado para demonstrar que a acumulação de biofilme mediada por fibrina e dependente de coagulase em dispositivos médicos implantados, revestidos com plasma sanguíneo rico em fibrinogênio é um mais novo mecanismo de biofilme de *S. aureus* que é independente de adesinas, tais como PNAG, eDNA e parede celular (proteínas ancoradas que são necessárias para a formação de biofilmes *in vitro*) (ZAPOTOCZNA *et al*., 2015; KWIECINSKI *et al*., 2016).

O fenótipo do biofilme não é apenas um processo acumulativo, com a expressão de proteases, modulinas fenossolúveis e nucleases que são usadas para degradar as adesinas de biofilme e promover a dispersão (LISTER; HORSWILL, 2014). De fato, a dispersão do biofilme afeta significativamente a progressão da doença, promovendo a disseminação do

patógeno da infecção para locais secundários através do sistema circulatório (O'GARA, 2017).

Biofilme bacteriano de infecções causadas por *Staphylococcus epidermidis*

Outra espécie bacteriana do gênero *Staphylococcus* é *S. epidermidis*, um membro permanente da microbiota humana normal, comumente encontrada na pele e mucosas. Ao aderir às porções de superfície do tecido do hospedeiro através de adesinas específicas, *S. epidermidis* é capaz de estabelecer uma relação comensal ao longo da vida com seres humanos na qual começa no início da vida. Embora os isolados de *S. epidermidis* comensais exibam altas taxas de resistência a antibióticos de relevância clínica (MORGENSTERN *et al*., 2016), seu status padrão como bactéria comensal torna esse fenômeno bastante irrelevante para o hospedeiro humano saudável (BRESCÓ *et al*., 2017).

Para persistir na pele humana, *S. epidermidis* desenvolveu diversos mecanismos para detectar e superar as características físicas e químicas da defesa antimicrobiana do hospedeiro. Tais mecanismos incluem adesinas superficiais que permitem a ligação ao hospedeiro (COATES *et al*., 2014), sistemas para detectar peptídeos antimicrobianos de hospedeiro (AMPs), moléculas de comunicação (por exemplo, hormônios) (LI *et al*., 2007; N'DIAYE *et al*., 2016) e mecanismos contra AMPs (JOO; OTTO, 2015).

A capacidade de aderir a uma superfície representa o primeiro passo na formação de biofilmes, comumente considerado o fator de virulência mais importante possuído por *S. epidermidis* (BRESCÓ *et al*., 2017).

A presença de matriz exopolissacarídea e a capacidade de formação de biofilme apresentadas por *S. epidermidis* são importantes fatores que favorecem a resistência deste microrganismo, visto que reduzem a capacidade de penetração de antibióticos, dificultando a ação dos mesmos (NANVAR *et al*., 2014).

Biofilme bacteriano de infecções causadas por *Pseudomonas aeruginosa*

Pseudomonas aeruginosa é uma bactéria Gram-negativa e aeróbica, sendo encontrada em diversos habitats bióticos e abióticos, incluindo solo, água, insetos, plantas e animais (GELLATLY; HANCOCK, 2013).

P. aeruginosa é um antigo formador de biofilme que está implicado em infecções crônicas e agudas. Pode causar infecções crônicas particularmente devastadoras ou permitir infecções nosocomiais que ameaçam a vida em curta duração (BILLINGS *et al*., 2013)

Esta bactéria pode ser encontrada no ambiente em duas principais formas de crescimento, como bactérias planctônicas e biofilmes (MULCAHY; ISABELLA; LEWIS, 2014). As bactérias planctônicas possuem vários apêndices de superfície que medeiam motilidade (flagelo, pilus) e virulência (sistemas de secreção) (HUBER *et al*., 2016).

P. aeruginosa, residente em biofilmes, torna-se metabolicamente menos ativo e mais resistente aos ataques do sistema imunológico ou a qualquer tratamento médico (TOLKER-NIELSEN, 2014; RYBTKE *et al*., 2015; RASAMIRAVAKA *et al*., 2015). O biofilme é um dos fatores de virulência mais importantes que surgem na superfície das bactérias que estão incorporadas na matriz extracelular (COULON *et al*., 2010; WEI; MA, 2013).

Além da regulação típica do desenvolvimento do biofilme, a formação de biofilmes também envolve outros tipos de regulação, como a sinalização mediada por ácidos graxos, que pode desempenhar um papel na regulação da dispersão de biofilme de *P. aeruginosa*. O sinal ácido cis-2-decenoico, parece estar envolvido na dispersão de biofilmes maduros (AMARI; MARQUES; DAVIES, 2013).

Existem várias razões para a tolerância ao biofilme, incluindo o crescimento lento e a presença de uma matriz extracelular constituída por vários biopolímeros. A formação e proliferação de um biofilme com sua matriz circundante são influenciadas por uma multiplicidade de fatores, incluindo vários sistemas regulatórios complexos que se mostraram envolvidos em diferentes estágios do ciclo de vida do biofilme. Para o patógeno oportunista *P. aeruginosa*, vários foram identificados, incluindo o sistema de comunicação célula-célula, *quorum sensing*, o mensageiro secundário bis-(3´-5´)-ciclico-dimériconguanosina monofosfato e, mais recentemente, a cascata Gac / Rsm. (JAKOBSEN; TOLKER-NIELSEN; GIVSKOV, 2017).

Muitos genes em *P. aeruginosa* são regulados e expressos pelo sistema *quorum sensing* incluindo genes de patogênese, como aqueles para protease alcalina, piocianina, pioverdina, cianeto, lipase, movimento de espasmos, alginato, azurina, quitinase, catalase, superóxido dismutase, *lasA*, *lasB*, máquina de transporte XCP, etc. (MOGHADDAM; KHODI; MIRHOSSEINI, 2014).

Devido ao papel do *quorum sensing* na regulação, controle e formação de biofilme e muitos fatores de virulência, a inibição de *quorum sensing* tem sido sugerida como alvo potencial para novas estratégias preventivas e/ou terapêuticas de infecções por *P. aeruginosa*. (FAVRE-BONTÉ *et al*., 2007; MOGHADDAM; KHODI; MIRHOSSEINI, 2014). De fato, o conhecimento sobre formação de biofilmes e detecção de *quorum sensing*, resulta na

identificação de novos alvos para terapêutica contra infecção por *P. aeruginosa* (SHARMA *et al*., 2014).

Os exopolissacarídeos Psl, Pel e alginato são constituintes principais da matriz de biofilme de *P. aeruginosa* envolvidos na adesão superficial e, juntamente com o eDNA, determinam a arquitetura do biofilme. Esses SPE desempenham um papel importante na resistência a respostas imunes e tratamentos antibióticos (GHAFOOR *et al.*,2011; GELLATLY; HANCOCK, 2013; STREMPEL *et al*., 2013).

Semelhante a Psl, Pel é importante para iniciar e manter a interação célula-célula em biofilmes (COLVIN *et al*., 2011). Pel e/ou Psl são os polissacarídeos estruturais da matriz primária em cepas de *P. aeruginosa* como um fenótipo ambiental predominante. (MORADALI; GHODS; REHM, 2017).

Alguns estudos de biofilme *in vitro* mostraram que a composição dos alginatos pode influenciar muito as características do biofilme, como propriedades viscoelásticas, biovolume, densidade celular e arquitetura, além da interação célula-célula, agregação celular e agentes de superfície (TIELEN *et al*., 2005; MORADALI *et al*., 2015).

Mecanismo de Resistência dos Biofilmes

Antibióticos

Os antibióticos têm mecanismos de ação diferentes que são capazes de modificar a tradução, a replicação de DNA e síntese da parede celular (MORAR; WRIGHT, 2010). Além do mais, nas últimas décadas têm sido relatados vários casos de resistência à maioria de classes de antibióticos utilizados na prática clínica. Tal fato tornou-se um dos maiores problemas de saúde pública, principalmente nos países em desenvolvimento porque estas infecções causam alta taxa de mortalidade e morbidade na qual culminam na perda de recursos humanos e econômicos (ASKARIAN *et al.*, 2013).

A “era de ouro” dos antibióticos não durou muito tempo e um novo impedimento surgiu, sendo este a resistência bacteriana. Esse acontecimento, causado pelo uso maciço e inadequado dos antibióticos, culminou na seleção de isolados resistentes a múltiplas drogas, deixando os doentes sem opções de tratamento e vulneráveis a doenças perigosas (NISNEVITCH, 2013).

Nos dias atuais, tem sido relatado que várias bactérias como as *Pneumococcus* sp. *Staphylococcus* sp. e *Streptococcus* sp., desenvolveram resistência aos antibióticos e ainda, mais de que 70 % dessas espécies, causam infecção. Vale lembrar que, apesar dos graves quadros de infecção proeminentes dessas bactérias Gram-positivas, o maior alerta de saúde

pública está em relação aos integrantes da família Enterobacteriaceae, pois estes apresentam resistência a ampicilina (β-lactâmicos), trimetropin, sulfametoxazol, tetraciclina, cloranfenicol vancomicina e ácido nalidíxico (MOSQUITO *et al.*, 2011).

As bactérias do grupo Gram-positivas podem desenvolver resistência através do efluxo de antibióticos, utilizando uma coleção de proteínas que estão associadas à membrana, na qual funcionam como bomba de efluxo ou ainda, através da síntese de enzimas como, por exemplo, as β-lactamases, que rompem o anel lactâmico e causam mutações em PBPs (proteínas de ligação a penicilinas) conferindo-lhes, assim, resistência aos β-lactâmicos (WRIGHT, 2005; MOSQUITO *et al.*, 2011). Além do mais, em Gram-positivas também ocorrem às alterações no sítio de ligação que atribuem resistência aos macrolídeos ou então, na reprogramação da via de biossíntese da parede celular, como na resistência aos antibióticos glicopeptídicos (AKTAS *et al.*, 2007; CETIN *et al.*, 2010).

Em relação às bactérias Gram-negativas, são descritos que as β-lactamases e PBPs, na qual conferem resistência aos β-lactâmicos e apresentam uma ação seletiva de porinas, que dificultam a penetração do fármaco e a presença de bombas de efluxo, que bombeiam a antibiótico para fora da célula bacteriana (LLARRULL *et al.*, 2010). O fenótipo de resistência pode ser perceptível em função da presença de um ou mais mecanismos moleculares de resistência antibiótica na bactéria. Entre os diversos tipos de mecanismos moleculares de resistência, destacam-se, por sua relevância, a inativação enzimática, as alterações no sítio ativo e as alterações na permeabilidade (MOSQUITO *et al.*, 2011).

Fármacos anteriormente abandonados por apresentarem espectro de ação parecido aos usuais, porém com alta toxicidade, têm sido reintroduzidos (MAALEJ *et al.*, 2011). Ainda assim, no tratamento clínico adotou-se como estratégia uma melhor utilização dos antibióticos licenciados, sendo combinado junto com os agentes terapêuticos a fim de obter uma melhora no tratamento (ELOUENNASS *et al.*, 2012). Contudo, em alguns casos a combinação pode resultar em alterações dos parâmetros farmacocinéticos e consequentemente, em uma maior toxicidade (PETROSILLO *et al.*, 2008).

Para que os antibióticos tenham um efeito eficaz, é de extrema importante que a sua concentração no local da infecção seja suficiente. Os antibióticos podem apresentar duas funções diferentes: a inibição do crescimento bacteriano através da ação bacteriostática e a destruição de uma população bacteriana por uma ação bactericida. A primeira impede o crescimento das bactérias, mantendo o mesmo na fase estacionária. (PANKEY; SABATH, 2013), enquanto a bactericida atua em processos vitais para a célula levando à morte celular. (GOODMAN; GILMAN'S, 2008; KATZUNG, 2007; LAGO, 2011).

Diferentes tipos de antibióticos afetam as paredes celulares, como os glicopeptídeos, fosfomicina e β-lactâmicos, enquanto outros exercem a sua atividade inibido a síntese de proteínas através da interação com subunidades ribossomais incluindo, o cloranfenicol, a tetraciclina, aminoglicosídeos e macrolídeos. Outro tipo de antibiótico é a fluoroquinolonas e rifampicina onde interfere a síntese de ácido nucleico, enquanto outros exercem os seus efeitos por interferirem nas vias metabólicas como, por exemplo, as sulfonamidas e análogos de ácido fólico. Existem, ainda, os antibióticos que causam ruptura da estrutura da membrana bacteriana, como lipopeptídeos e polipeptídeos (LIMA *et al.*, 2013). Os diferentes mecanismos de ação destas moléculas podem ser visualizados na Figura 12.

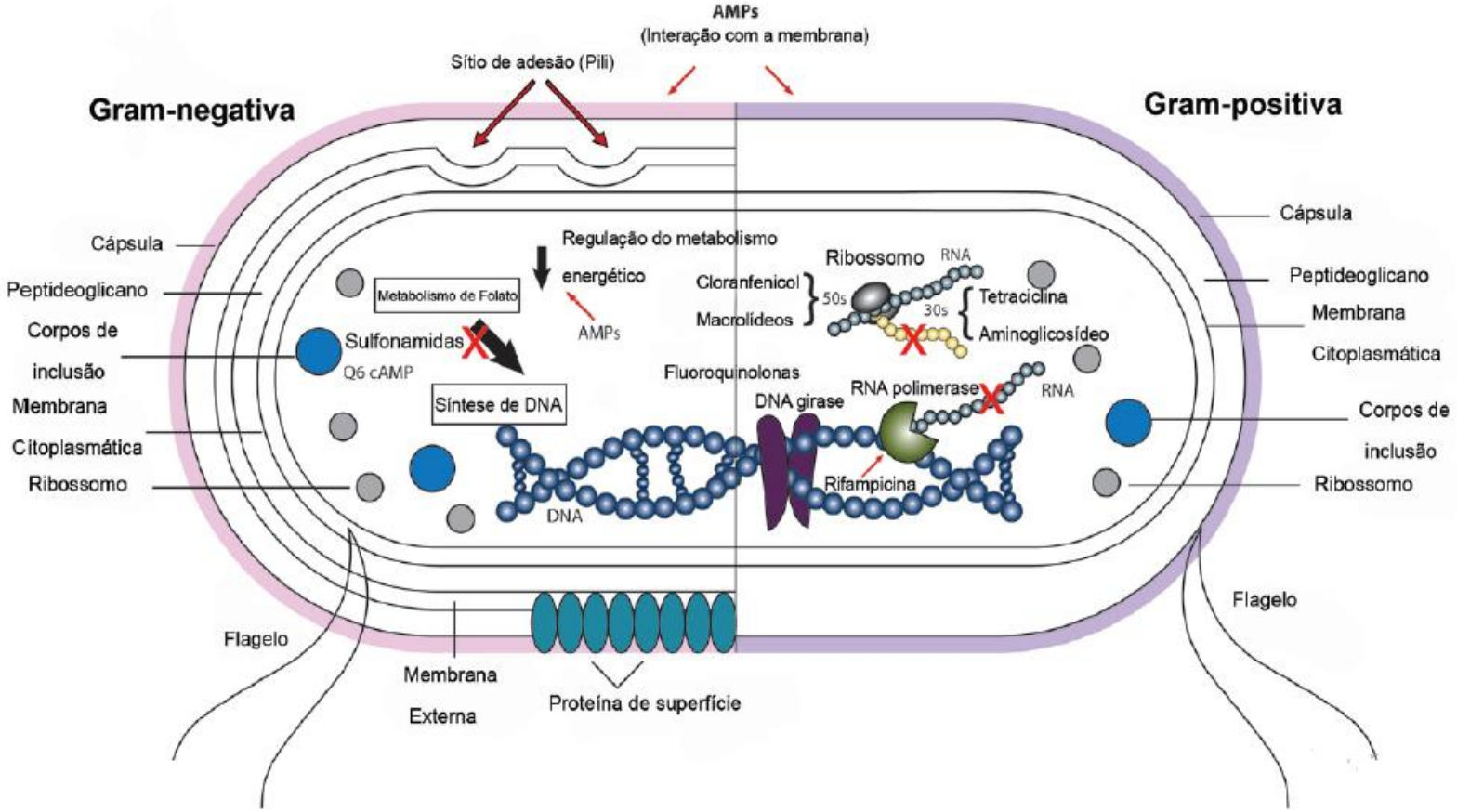
Antifúngicos

Os antifúngicos surgiram logo após as drogas antibacterianas com o uso tópico de soluções compostas de ácido benzoico, ácido salicílico e iodo (MEZZARI; FUENTEFRIA, 2012). No início do século XX, começou a ser utilizado o iodeto de potássio (KI), fármaco pouco efetivo e não específico usado no manejo do tratamento de dermatoses inflamatórias, como eritema polimorfo, granuloma anular e eritema nodoso, dermatoses neutrofílicas, como síndrome de Sweet e pioderma gangrenoso e dermatoses infecciosas. Sob o pretexto de que sua dose terapêutica era muito similar da dose tóxica, o KI foi substituído por outros novos fármacos, entretanto ele desempenha um grande papel na micologia médica sendo ainda hoje uma droga de primeira escolha para tratamento de esporotricose e segunda escolha para zigomicose (COSTA *et al*., 2013; MACEDO *et al*., 2015).

É bastante limitado o arsenal antifúngico disponível comparado com o antibacteriano. Isto pode estar relacionado à dificuldade de descobrir um fármaco, que seja eficaz apenas em célula fúngica eucariótica sem possuir toxicidade para as células do hospedeiro (SILVA *et al*., 2012).

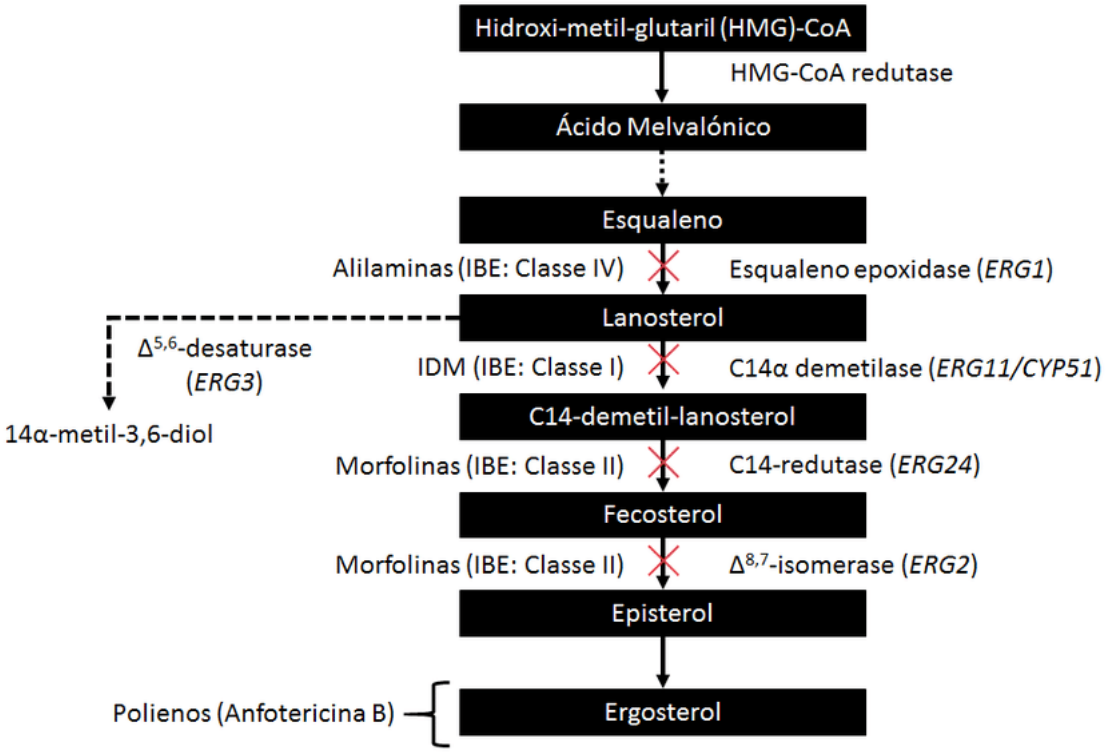
Os antifúngicos azólicos possuem amplo espectro de ação e são divididos em duas classes: imidazólicos e triazólicos. O primeiro imidazólico de ação tópica, o clotrimazol, foi lançado no ano de 1960 e ainda é usado para candidíase superficial. Os triazólicos são divididos em primeira geração (itraconazol e fluconazol) e de segunda geração (voriconazol e posaconazol). Os azólicos agem inibindo a enzima denominada de lanosterol-14-α-desmetilase, uma enzima chave no processo de produção do ergosterol. Sem essa enzima, o ergosterol não é formado e a célula fúngica tem sua membrana enfraquecida (Figura 13) (SILVA *et al*., 2012; COLOMBO *et al*., 2013).

Figura 12: Representação dos principais alvos de antibióticos. Os antibióticos que são capazes de inibir o metabolismo do folato, que é um componente necessário para a síntese de ADN (sulfonamidas). Além disso, os antibióticos que inibem a biossíntese de proteínas podem agir sobre os ribossomos utilizando a subunidade 30S (tetraciclina e aminoglicosídeos) ou 50S (cloranfenicol e macrolídeos). Antimicrobianos envolvidos na replicação de DNA e RNA podem causar uma inibição da transcrição por ligação a RNA polimerase, prevenindo assim a sua ligação ao DNA (rifampicina). Finalmente antibióticos que se ligam à DNA girase (quinolonas).



Fonte: Lima *et al.*, (2013)

Figura 13. Mecanismo de ação de compostos antifúngicos afetando a rota Biosintética do ergosterol. Enzimas alvo à direita das setas com genes codificadores entre parênteses. Compostos antifúngicos à esquerda das setas. Setas indicam passos para a biossíntese do ergosterol.



Adaptado de Lupetti *et al*., (2002).

Estresse oxidativo e atividade antioxidante de óleos e metabólitos secundários

O estresse oxidativo resulta em um desequilíbrio na produção de espécies reativas de oxigênio (ERO) e a capacidade antioxidante das células. Os EROs, tais como o superóxido (O_2^-), o peróxido de hidrogénio (H_2O_2) e os radicais hidroxilo (OH) são constantemente produzidos em células aeróbicas, por redução incompleta de O_2 molecular a H_2O durante a fosforilação oxidativa mitocondrial. (BIRBEN *et al*., 2012).

Além disso, os EROs são gerados durante uma série de processos, como inflamação, infecção, tensões mecânicas e químicas, exposição a radiação UV e radiação ionizante. Os níveis basais de EROs atuam como moléculas de sinalização para ativar a proliferação celular, a sobrevivência, a apoptose, a diferenciação, as respostas imunes, a motilidade e as vias sensíveis ao estresse. (RHEE, 2006; SENA; CHANDEL, 2012; CHEN *et al*., 2016)

No entanto, a produção excessiva de EROs resulta em danos irreversível ao DNA, tais como modificações nas ligações de base, levando à morte celular. (GORRINI; HARRIS;

MAK, 2013) Portanto, a regulação celular de EROs é fundamental para a manutenção da homeostase celular. (CHIKARA *et al*, 2018)

EROs são átomos ou moléculas que proporcionam uma redução no número central de seus elétrons, muito instáveis, tornando-se altamente reativo e com grande habilidade para combinar-se com distantes moléculas celulares. Nos organismos, seu excesso cumpre efeitos danosos sobre enzimas, proteínas teciduais e comprometimento da integridade das membranas celulares (BARREIROS; JORGE, 2006; AZEVEDO *et al*., 2014)

Devido à produção excessiva de radicais ou à velocidade diminuída de sua remoção, e conduz à oxidação de biomoléculas com desequilíbrio homeostático ou perda de seus desempenhos biológicos, acarretando danos potenciais em células e tecidos. Os lipídios, que são componentes essenciais das membranas celulares, são alvos principais do ataque por radicais livres de oxigênio. A recorrência do processo de oxidação lipídica implica risco aumentado para doenças crônicas não transmissíveis (GIL-CHÁVEZ *et al*., 2013; YIN; XU; PORTER, 2011).

Desta forma, os EROs oxigenados são produzidos constantemente *in vivo* para fins fisiológicos e atuam como mediadores para a transferência de elétrons nas diversas reações bioquímicas, sendo úteis na proteção frente a microrganismos patógenicos, possibilitando a geração de ATP (energia) por meio da cadeia transportadora de elétrons, fertilização do óvulo, ativação de genes, etc. O excesso pode resultar em estresse oxidativo (BARBOSA *et al*., 2010; ALVES *et al*., 2010).

Com base nisso, os antioxidantes são substâncias com a habilidade de retardar ou inibir a oxidação de substratos orgânicos, protegendo as células contra os efeitos danosos dos radicais livres e espécies reativas de oxigênio (ERO), sendo estas substâncias envolvidas em um amplo número de doenças relacionadas ao estresse oxidativo (MORAIS *et al*., 2009; SHAHIDI; AMBIGAIPALAN, 2015).

Desta forma, o corpo produz antioxidantes mesmo estando em pequenas concentrações que as do substrato oxidável, para inibir ou reduzir os danos causados pela ação deletéria dos radicais livres. A ação antioxidante pode ser preventiva, prevenindo a formação dos radicais livres; por varredura, impedindo sua ação; ou por reparo, reconstituindo as estruturas lesadas (RODRIGO; MIRANDA; VERGARA, 2011).

O interesse para a identificação de antioxidantes alimentares de fontes naturais, principalmente de origem vegetal vem aumentando cada vez mais. A caracterização dos compostos fenólicos, capacidade antioxidante, carotenoides e açúcares solúveis totais que estão presentes nos alimentos são de grande valor, devido à proteção desempenhada por esses

compostos no corpo humano contra os efeitos nocivos dos radicais livres, além de retardar o avanço de muitas doenças crônicas e impedir a peroxidação lipídica (GULÇIN, 2012).

A capacidade de um composto inibir a degradação oxidativa espontânea de um substrato é descrita por dois parâmetros distintos: o fato estequiométrico (também denominado por alguns como "capacidade antioxidante"), que é o número de radicais presos por uma molécula antioxidante e a reatividade, o mais importante na determinação da atividade antioxidante, que depende da constante de velocidade da reação entre antioxidantes e os radicais transportadores de cadeias. (ROGINSKY; LISSI, 2005; VALGIMIGLI; PRATT, 2012).

Atividades antioxidantes de óleos essenciais e metabólitos secundários da família Fabaceae, já são descritas na literatura. O equilíbrio celular dos radicais livres é mantido por diferentes antioxidantes. Flavonoides, terpenoides e constituintes fenólicos de EOs exibem efeitos antioxidantes (TOMAINO *et al*., 2005; FERGUSON; PHILPOTT, 2008; MIGUEL, 2010; CAVAR *et al*., 2012; SANCHEZ-VIOQUE *et al*., 2013).

Vasconcelos *et al*. (2014) mostraram que o composto 3-metoxi-[α,β:6,7]-furanoauronol isolado das raízes de *Lonchocarpus obtusus* e foi capaz de sequestrar o radical DPPH e possui potencial em inibir a oxidação do β-caroteno em protegê-lo de radicais livres gerados durante a peroxidação do ácido linoléico.

Awaad *et al*. (2012) analisaram os flavonoides quercetina-6,4'-dimetoxi-3-fructo-rhamnoside e quercetina-4'-metoxi-3-fructo-rhamnoside, isolados do extrato etanolico de *Atriplex lentiformis*, apresentando potenciais antioxidantes ativos sem efeitos colaterais.

Promden *et al*. (2014) avaliaram as atividades antioxidantes de 24 isoflavonoides de *Dalbergia parviflora* através de três sistemas de ensaios *in vitro* antioxidantes complementares: xantina/xantina oxidase, capacidade de absorbância de radicais de oxigênio (ORAC) e 2,2 -difenil-1-picrilidrazilo (DPPH). As isoflavonas exibiram a maiores resultados antioxidante com base nos três ensaios. A presença adicional de um OH no anel B em R3' ou R5' da estrutura básica de R7-OH no anel A, e R4'-OH ou OMe do anel B aumentaram as atividades antioxidantes de todos os subgrupos de isoflavonoides.

Rao *et al*. (2011) analisaram os compostos 3-(4-(3,4,5-trihidroxibenzoiloxi) fenil)-2-ácido aminopropanóico, 3-(4-(4-hidroxi,3-metóxibenzoiloxi) fenil)-2-ácido aminopropanóico e 3-(4 (3,4-dihidroxibenzoiloxi) fenil)-2-ácido aminopropanóico isolados de *Inga laurina* e mostraram atividades pelo método DPPH.

O óleo essencial das folhas de *Myrocarpus frondosus* apresentou atividade antioxidante na inibição de 40,92%, o que pode ser explicado pela presença de terpenos, sendo responsáveis pela atividade (SANTI *et al*., 2017).

Ghahari *et al*. (2017) relatam que o óleo essencial extraído das sementes de *Glycine max* tem capacidade de doação de prótons, servindo como um inibidor ou eliminador de radicais livres e atuando como possível antioxidante. A capacidade de eliminação de radicais livres do óleo essencial foi determinada com um valor IC_{50} de 162,35 μg/mL.

Referências

ABOELSOUD, N. H. Herbal medicine in ancient Egypt. **Journal of Medicinal Plants Research,** v. 4, n. 2, p. 82-86, 2010.

AGRA, M. F. *et al*. Medicinal and poisonous diversity of the flora of "Cariri Paraibano", Brazil. **J Ethnopharmacol,** v. 111, n. 2, p. 383-95, 2007.

AGUIAR, C. L. *et al*. Analysis of isoflavonoids from leguminous plant extracts by RPHPLC/DAD and electrospray ionization mass spectrometry. **International Journal of Food Sciences and Nutrition**. v. 58, p. 116-124, 2007.

AKTAS, Z. *et al*. Resistance to macrolide, lincosamide and streptogramin antibiotics in staphylococci isolated in Istanbul, Turkey. **J Microbiol,** v. 45, n. 4, p. 286-290. 2007.

ALLEN, G.M. *et al*. 50 Common native important plants in Florida's ethnobotanical history. University of Florida. **Circular 1439**, p. 1-21, 2012.

ALVES, C. Q. *et al*. Métodos para determinação de atividade antioxidante *in vitro* em substratos orgânicos. **Química Nova**, v. 33, n. 10, p. 2202-2210, 2010.

ALVES, M. J. *et al*. Phenols, flavonoids and antioxidant and cytotoxic activity of leaves, fruits, peel of fruits and seeds of *Piptadenia moniliformis* Benth (Leguminosae - Mimosoideae). **Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas**, v. 13, n. 5, p. 466-476, 2014.

AMARI; MARQUES; DAVIES. The putative enoyl-coenzyme A hydratase DspI is required for production of the *Pseudomonas aeruginosa* biofilm dispersion autoinducer *cis*-2-decenoic acid. **J. Bacteriol**.,v. 195, p. 4600-4610, 2013.

AN, S. W; DONG, Y. H; ZHANG, L. H. The Impact and molecular genetics of bacterial biofilms. United Kingdom: **Environmental Molecular Microbiology, Caister Academic Press**, p. 211-226, 2009.

ANDERL, J.N.; FRANKLIN, M. J.; STEWART, S. P. Role of Antibiotic Penetration Limitation in *Klebsiella pneumoniae* Biofilm Resistance to Ampicillin and Ciprofloxacin**. Antimicrobial Agents and Chemotherapy**, v. 44, n. 7, p. 1818-1824, 2000**.**

ANDERSEN, Ø. M.; MARKHAM, K. R.; Flavonoids: Chemistry, Biochemistry and Applications. **Taylor & Francis**. Nova Yorque, 2006.

ASKARIAN, M.; MAHMOUDI, H.; ASSADIAN, O. Incidence of Nosocomial Infections in a Big University Affiliated Hospital in Shiraz, Iran: A Six-month Experience. **Int J Prev Med,** v. 4, n. 3, p. 366-72. 2013.

AWAAD *et al*. Novel flavonoids with antioxidant activity from a Chenopodiaceous plant. **Pharmaceutical Biology**, v.50, n.1, p. 99-104, 2012.

AZEVEDO, L.F.P. *et al*., Triagem fitoquímica e atividade antioxidante de *Costus spicatus* (Jacq.) S.w. **Rev. Bras. Pl. Med**., v.16, n.2, p.209-215, 2014.

BARBOSA, K. B. F. *et al*. Estresse oxidativo: conceito, implicações e fatores modulatórios. **Revista de Nutrição**, v. 23, n. 4, p. 629-643, 2010.

BARREIROS, A. L. B. S.; JORGE, M. D**.** Estresse oxidativo: relação entre geração de espécies reativas e defesa do organismo. **Quim. Nova**, v. 29, n. 1, p. 113-123, 2006.

BEECH, B. I.; SUNNER, J. A.; HIRAOKA, K. Microbe-surface interactions in biofouling and biocorrosion processes. **Int. Microbiol**., v. 8, p. 157-168, 2005.

BILLINGS *et al*. The extracellular matrix component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. **PLOS Pathog**., v. 9, n.8, p.1-12, 2013.

BIRBEN, *et al*. Oxidative stress and antioxidant defense, W**orld Allergy Organ. J.** v, 5 1, p, 9-19, 2012.

BJARNSHOLT *et al*. The in vivo biofilm. **Trends in microbiology**. v. 21, p.466-474, 2013.

BOJASE, G. *et al*. Antimicrobial flavonoids from *Bolusanthus speciosus*. **Planta medica**, v. 68, p. 615-620, 2002.

BOLES; HORSWILL. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. **PLoS Pathog**, v.4, n. 4, p. 1-13, 2008.

BORGES, J. S. **Análise comparativa do proteoma e metaboloma de raízes de dois clones de *E. grandis* x *E. camaldulensis*, sendo um tolerante e um susceptível a condições de estresse hídrico**. 2013. 199f. Dissertação (Mestrado em Ciencias)- Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, São Paulo 2013.

BOURGAUD, F. *et al*. Production of plant secondary metabolites: a historical perspective. **Plant Sci.**, v. 161, n. 5, p. 839-851, 2001.

BOWLES, E.J. Chemistry of Aromatherapeutic Oils. Australia: **Allen & Unwin**, 2003.

BRAVO, L. B. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. **Nutr. Rev.**, v. 56, n. 11, p. 317- 333, 1998.

BRESCÓ *et al*. Pathogenic Mechanisms and Host Interactions in *Staphylococcus epidermidis* Device-Related Infection. **Front Microbiol**, v. 2, p. 1-24, 2017.

BUFFET-BATAILLONA, S. *et al*. Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds-a critical review. **Int J Antimicrob Agents**, v. 39, p. 381-389, 2012.

BURT, S. Essential oils: Their antimicrobial properties and potential applications in food- A review. **International Journal of Food Microbiology**, v. 94, p. 223-253, 2004.

BUSH, k. *et al*. Tackling antibiotic resistance. **Nat Rev Microbiol**, v. 9, n. 12, p. 894-896, 2011.

CAI, Y.; SUN, M.; CORKE, H. Antioxidant activity of betalains from plants of the Amaranthaceae. J. Agric. **Food Chem**., v. 51, p. 2288-2294, 2003.

CARTAXO, S. L.; ALMEIDA SOUZA, M. M.; ALBUQUERQUE, U. P. Medicinal plants with bioprospecting potential used in semi-arid northeastern Brazil. **Journal of Ethnopharmacology**, v. 131, n. 2, p. 326-342, 2010.

CARVALHO, *et al*. Potencial bactericida de extratos e substâncias isoladas de *Myroxylon peruiferum* (Cabreúva) frente à micobactérias do trato respiratóio. In: **XVI Congresso de Iniciação Científica**. UFSCar, Sorocaba. 2008.

CAVAR *et al*. Chemical composition and antioxidant and antimicrobial activity of essential oil of Artemisia annua L. from Bosnia. **Ind. Crops. Prod**, v.37, p. 479-485, 2012.

CETIN, E. S. *et al*. Distribution of genes encoding resistance to macrolides, lincosamides and streptogramins among clinical staphylococcal isolates in a Turkish university hospital. **J Microbiol Immunol Infect,** v. 43, n. 6, p. 524-529, 2010.

CHAVES, E. M. F.; BARROS, R. M. F.; ARAÚJO, F. S. Flora Apícola do Carrasco no Município de Cocal, Piauí, Brasil. **Revista Brasileira de Biociências,** v. 5, p. 555- 557, 2007.

CHEN, X. *et al*. Reactive oxygen species regulate T cell immune response in the tumor microenvironment, **Oxid. Med. Cell. Longev**, v. 2016, p. 1-10, 2016.

CHENG *et al*. Contribution of coagulases towards *Staphylococcus aureus* disease and protective immunity. **PLoS pathogens**, v. 6, n. 8, p. 1-18, 2010.

CHEYNIER, V. Polyphenols in foods are more complex than often thought. **Am. J. Clin. Nutr.**, v. 81, n. 1, p. 223S-229S, 2005.

CHIKARA, S, *et al*. Oxidative stress and dietary phytochemicals: Role in câncer chemoprevention and treatment. **Cancer Letters**, n. 413, p. 122-134, 2018.

CHOUHAN, S.; KANIKA, S.; GULERIA, S. Antimicrobial Activity of Some Essential Oils-Present Status and Future Perspectives. **Medicines**, v.4, n. 58, p.1-21, 2017.

COATES; MORAN; HORSBURGH. Staphylococci: colonizers and pathogens of human skin. **Future Microbiol**, v. 9, p. 75-91, 2014.

COLOMBO, A. L. *et al*. Brazilian guidelines for the management of candidiasis -a joint meeting report of three medical societies: Sociedade Brasileira de Infectologia, Sociedade Paulista de Infectologia and Sociedade Brasileira de Medicina Tropical. **The Brazilian Journal of infectious diseases**, v. 17, n. 3, p. 283-312, 2013.

COSTA, R. O. *et al*. Uso do iodeto de potássio na dermatologia: considerações atuais de uma droga antiga. **Anais Brasileiros de Dermatologia**, v. 88, n. 3, p. 401-407, 2013.

COULON *et al*. Chemical analysis of cellular and extracellular carbohydrates of a biofilm-forming strain *Pseudomonas aeruginosa* PA14. **PLoS One**, v. 5, n. 12, 2010.

COWARD, L. *et al*. Genistein, dadzein, and their β-glicoside conjugates: antitumor isoflavones in soybean foods from american and asian diets. **Journal of Agriculture and Food Chemistry**. v. 41, p. 1961-1967, 1993.

CUSTÓDIO, D. L.; VEIGA-JUNIOR, V. F. True and common balsams. **Revista Brasileira de Farmacognosia** v. 22, n. 6, p. 1372–1383, 2012.

DAGLIA M. Polyphenols as antimicrobial agents. **Curr Opin Biotechnol**. v. 23, p.174-181, 2011.

DAVIES, D. Understanding biofilm resistance to antibacterial agents. **Nat. Rev. Drug Discov**, v. 2, p. 114-122, 2003.

DEWICK, P. M. **Medicinal natural products: a biosynthetic approach**. 2. ed. London: John Wiley & Sons, 517p, 2002.

DEWICK, P. M.; **Medicinal Natural Products: A Biosynthetic Approach**, 2ª ed. John Wiley, Chichester, 2002.

DEWICK, P.M. The mevalonate and methylerythritol phosphate pathways: terpenoids and steroids. In: **Medicinal Natural Products: a biosynthetic approach**. 3 ed, Chichester: John Wiley e Sons, cap. 5, p. 187-306, 2009.

DIAS, D. A.; URBAN, S.; ROESSNER, U. A historical overview of natural products in drug discovery. **Metabolites**, v.2, n. 2, p. 303-336, 2012.

DIXON, R. A.; PASINETTI, G. M; Flavonoids and Isoflavonoids: From Plant Biology to Agriculture and Neuroscience. **Plant Physiology**. v. 154, p. 453-457, 2010.

DJILANI, A.; DICKO, A. **The therapeutic benefits of essential oils**. INTECH Open Access Publisher, 2012.

DONLAN, R. M.; COSTERTON, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. **Clinical Microbiology Reviews**, v. 15, p. 167-193, 2002.

DOUGLAS, L. J. *Candida* biofilms and their role in infection. **Trends Microbiol**., v. 11, n. 1, p. 30-36, 2003.

DUNNE, W. M. Bacterial adhesion: Seen any good biofilms lately? **Clin. Microbiol. Rev**., v. 15, n. 2, p. 155-166, 2002.

EDRIS, A. E. Pharmaceutical and Therapeutic Potentials of Essential Oils and Their Individual Volatile Constituents: A Review. **Phytoterapy Research**, v.21, p.308-323, 2007.

ELOUENNASS, M. *et al*. *In Vitro* Activities of Ertapenem and Imipenem against Clinical Extended Spectrum Beta-Lactamase-Producing Enterobacteriaceae Collected in Military Teaching Hospital Mohammed V of Rabat. **Interdiscip Perspect Infect Dis,** v. 2012, p. 6464-80, 2012.

EVARISTO, F. F. V. *et al*. Antibacterial effect on mature biofilms of oral streptococci and antioxidant activity of 3β,6β,16β-trihydroxylup-20(29)-ene from *Combretum leprosum*. **Medicinal Chemistry Research**, v. 1, p. 1, 2017.

EVARISTO, F. F. V. *et al*. Antimicrobial Effect of the Triterpene 3β,6β,16β-Trihydroxylup-20(29)-ene on Planktonic Cells and Biofilms from Gram Positive and Gram Negative Bacteria. **Bio**. **Med. Research International**. 2014.

FACUNDO, V. A. *et al*. "Triterpenes and flavonoids from *Combretum leprosum*, " **Phytochemistry**. v. 32, n 2, p. 411-415, 1993.

FACUNDO, V. A. *et al.* Arjunolic Acid in the Ethanolic Extract of *Combretum leprosum* Root and its Use as a Potential Multi-Functional Phytomedicine and Drug for Neurodegenerative Disorders: Anti-Inflammatory and Anticholinesterasic Activities. **Journal of the Brazilian Chemical Society,** v. 16, n. 6B, p. 1309-1312, 2005.

FAUSTINO, A. R. C. **Preparação de novos Triterpenóides Semi-Sintéticos**. 2015. Dissertação (Mestrado em Química Farmacêutica Industria) - Universidade de Coimbra, Coimbra, 2015.

FAVRE-BONTÉ *et al*. Autoinducer production and *quorum-sensing* dependent phenotypes of *Pseudomonas aeruginosa* vary according to isolation site during colonization of intubated patients. **BMC Microbiol**, v.7, n. 33, 2007.

FERGUSON, L.R., PHILPOTT, M. Nutrition and mutagenesis. **Annu. Rev. Nutr**, v. 28, p. 313-329, 2008.

FILIZOLA, B. C.; SAMPAIO, M. B. Boas Práticas de Manejo para o Extrativismo Sustentável de Cascas. Brasília: **Instituto; Sociedade, População e Natureza**; 2015.

FLEMMING, H. C.; WINGENDER, J. The biofilm matrix. **Nat. Rev. Microbiol**., v. 8, p. 623-633, 2010.

FONTANAY, S. *et al*. Ursolic, oleanolic and betulinic acids: Antibacterial spectra and selectivity indexes. **Journal of Ethnopharmacology**, v. 120, p. 272–276, 2008.

FORMICA, J. V.; REGELSON, W. Review of the biology of quercetin and related bioflavonoids. **Food Chem. Toxicol**, v. 33, n. 12, p. 1061-1080, dez. 1995.

FOSTER *et al*. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. **Nature reviews Microbiology**, v. 12, p.49-62, 2014.

FRANZ, C.; NOVAK, J. Chemistry of Essential Oils. In: BASER, K. Hüsnü Can; BUCHBAUER, Gerhard. **Handbook of essential oils:** science, technology, and applications. CRC Press, p. 39-82, 2010.

FRITSCHE, S.; STEINHART, H. Occurrence of hormonally active compounds in food: a review. **Eurpean Food Research and Technology**. v. 209, p. 153-179, 1999.

GELLATLY; HANCOCK. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. **Pathog. Dis**., v. 67, p. 159-173, 2013.

GHAFOOR; HAY; REHM. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. **Appl. Environ. Microbiol**., v. 77, p. 5238–5246, 2011.

GHAHARI *et al*. Chemical Composition, Antioxidant and Biological Activities of the Essential Oil and Extract of the Seeds of *Glycine max*(Soybean) from North Iran. **Curr Microbiol**, n. 74, p. 522-531,2017.

GIL-CHÁVEZ, J.G. *et al*. Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food Ingredients: An Overview. **Comprehensive Reviews in Food Science and Food Safety**, v. 12, n. 1, p. 5-23, 2013.

GOODMAN; GILMAN'S. **Manual of Pharmacology and Therapeutics**. Nova Iorque: McGraw Hill, 2008.

GORRINI, C., HARRIS, I.S., MAK, T.W. Modulation of oxidative stress as an anticâncer strategy, **Nat. Rev. Drug Discov**, v.12, n.12, p. 931-947, 2013.

GOSSAN, D. P. A. Antibacterial and cytotoxic triterpenoids from the roots of *Combretum racemosum*. **Fitoterapia**, v. 110, p. 89–95, 2016.

GOTTENBOS, B. *et al*. Pathogenesis and prevention of biomaterial centered infections. **Jounal of Materials Science. Materials in Medicine**, v. 13, p. 717-722, 2002.

GUGGENBERGER *et al*. Two distinct coagulase-dependent barriers protect *Staphylococcus aureus* from neutrophils in a three dimensional in vitro infection model. **PLoS pathogens**, v. 8, n. 1, p. 1-17, 2012.

GUIDO, R. C., ANDRICOPULO, A. D. OLIVA, G. Planejamento de fármacos, biotecnologia e química medicinal: aplicações em doenças infecciosas. **Estudos Avançados**, v. 24, n.7, p. 81-98, 2010.

GUIMARAES, D. O; MOMESSO, L. S. PUPO, M. T. Antibióticos: importância terapêutica e perspectivas para a descoberta e desenvolvimento de novos agentes. **Química Nova**, v. 33, n. 3, p. 667-679, 2010.

GULATI, M.; NOBILE, C. J. *Candida albicans* biofilms: development, regulation, and molecular mechanisms. **Microbes and Infection**, v.18, n.5, p.310-21, 2016.

GÜLÇIN, I. Antioxidant activity of food constituents: an overview. **Arch Toxicol**, v. 86, n. 3, p. 345-391, 2012.

HARTMANN, T. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. **Phytochemistry**, v. 68, n. 22-24, p. 2831-2846, 2007.

HARTMANN, T. Global harmonization of herbal health claims **Ent. Exp. Appl.**, v. 80, p. 177-179, 1996.

HAVENSTEEN, B.H. The biochemistry and medical significance of the flavonoids. **Pharmacology and Therapeutics**. v. 96, p. 67-202, 2002.

HAVSTEEN, B. H. The biochemistry and medical significance of the flavonoids, **Pharmacology & Therapeutics**, n. 96, n. 67, 2002.

HERRMANN, M. *et al*. Fibronectin, fibrinogen, and laminin act as mediators of adherence of clinical staphylococcal isolates to foreign material. **The Journal of Infectious Disease**, v. 158, p. 693-701, 1988.

HOIBY, N. *et al*. The clinical impact of bacterial biofilms. **International Journal of Oral Sciences**, v. 3, p. 55-65, 2011.

HORINOUCHI, C.D. *et al*. *Combretum leprosum* Mart. (Combretaceae): potential as an antiproliferative and anti-inflammatory agent. **J. Ethnopharmacol**. v.145, p. 311-319, 2013.

HUBER *et al*. *Pseudomonas aeruginosa* renews its virulence factors. **Environ. Microbiol. Rep.**, v. 8, p. 564–571, 2016.

HURBER, L. S; AMAYA-RODRIGUEZ, D. B; Flavonóides e flavonas. **Alim. Nutr.** v.19, n.1, p. 97-108, 2008.

HURLOW, J. *et al*. Clinical biofilms: a challenging frontier in wound care. **Adv Wound Care**, v. 4, n. 5, p. 295-301, 2015.

IBRAHIM, R.K.; **Flavonoids, encyclopedia of life sciences**, 2001.

JAKOBSEN; TOLKER-NIELSEN; GIVSKOV. Bacterial Biofilm Control by Perturbation of Bacterial Signaling Processes. **International Journal of Molecular Sciences**. v. 18, n. 9, p. 1-27, 2017.

JOO; OTTO. Mechanisms of resistance to antimicrobial peptides in staphylococci. **Biochim Biophys Acta**, v. 1848, p. 3055-3061, 2015.

KAMILOGLU, S. *et al*. Investigating the antioxidant potential of Turkish herbs and spices. **Quality Assurance and Safety of Crops & Foods**, V. 6, n. 2, p. 151-158, 2014.

KARADENIZ, A. *et al*. Antioxidant activity of some Turkish medicinal plants. **Nat. Prod. Res**., v. 29, p. 2308-2312, 2015.

KATERERE, D. R. *et al*. Antimicrobial activity of pentacyclic triterpenes isolated from African Combretaceae. **Phytochemistry**. v. 63, p. 81-88, 2003.

KATZUNG, B. **Farmacologia Básica e Clínica**. Brasil: McGraw Hill. 10ª ed, 2007.

KHORSHIDIAN, N. *et al*. Potential application of essential oils as antimicrobial preservatives in cheese. **Innovative Food Science and Emerging Technologies**, v. 45, p. 62–72, 2018.

KOKARE, C. R. *et al*. Biofilm: Importance and applications. **Indian Journal of Biotechnology**. v. 8, p. 159-168, 2009.

KWIECINSKI *et al.* Staphylokinase control of *Staphylococcus aureus* biofilm formation and detachment through host plasminogen activation**. J Infect Dis**, v. 213. p. 139-148, 2016.

LAGO, J. Mecanismos de Resistência e Selecção de Antibióticos. Lisboa: **Jornadas bioMérieux**, 2011.

LI *et al*. Gram-positive three-component antimicrobial peptide-sensing system. **Proc. Natl. Acad. Sci. U.S.A**, v. 104, p. 9469-9474, 2007.

LIMA, J. R.; MANSANO, V. F. A família Leguminosae na Serra de Baturité, Ceará, uma área de Floresta Atlântica no semiárido brasileiro. **Rodriguésia-Instituto de Pesquisas Jardim Botânico do Rio de Janeiro**, v. 62, n. 3, 2011.

LIMA, T. B. *et al*. Bacterial resistance mechanism: what proteomics can elucidate. **Faseb Journal**, v. 27, n. 4, p. 1291-303, 2013.

LINDE, G. *et al*. Quimiotipos, Extracción, Composición y Aplicaciones del Aceite Esencial de *Lippia alba.* **Revista Brasileira de Plantas Medicinais**, v. 18, n. 1, p. 191-200, 2016.

LISTER; HORSWILL. *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal. **Front Cell Infect Microbiol**, v.4, p. 1-9, 2014.

LLARRULL, L. I. *et al*. The future of the beta-lactams. **Curr Opin Microbiol,** v. 13, n. 5, p. 551-557. 2010.

LONGHI-BALBINOT, D.T. *et al*. Anti-inflammatory effect of triterpene 3beta, 6beta, 16beta-trihydroxylup-20(29)-ene obtained from *Combretum leprosum* Mart & Eich in mice. **J. Ethnopharmacol**. v. 142, p. 59-64. 2012.

LONGHI-BALBINOT, D.T. *et al.* Spinal antinociception evoked by the triterpene 3beta, 6beta, 16beta-trihydroxylup-20(29)-ene in mice: evidence for the involvement of the glutamatergic system via NMDA and metabotropic glutamate receptors. **Eur. J. Pharmacol.** v. 623, p. 30-36, 2009.

LOPES, R.M., OLIVEIRA, T.D., NAGEM, T.J., PINTO A.D.S. **Flavonoides. Biotecnologia Ciência & Desenvolvimento**, v. 3, n. 14, 2010.

LORENZI, H. 1998. **Árvores Brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil**. Nova Odessa, Plantarun.

LUPETTI, A. *et al*. Molecular basis of resistance to azole antifungals. **Trends Mol Med**, v. 8, n.2, p. 76-81, 2002.

MAALEJ, S. M. *et al*. Comparison of disc diffusion, Etest and agar dilution for susceptibility testing of colistin against Enterobacteriaceae. **Lett Appl Microbiol,** v. 53, n. 5, p. 546-51. 2011.

MACEDO, P. M. *et al*. New posology of potassium iodide for the treatment of cutaneous sporotrichosis: study of efficacy and safety in 102 patients. **Journal of the European Academy of Dermatology and Venereology**, v. 29, n. 4, p. 719-724, 2015.

MALAQUIAS, G. *et al*. Utilização na medicina popular, potencial terapêutico e toxicidade em nível celular das plantas *Rosmarinus officinalis* L., *Salvia officinalis* L. e *Mentha piperita* L. (Família Lamiaceae). **Revista Intertox de Toxicologia, Risco Ambiental e Sociedade**, v. 7, n. 3, p. 50-68, 2014.

MARANGONI, M. S. *et al*. Pyricularia blast on white oats -a new threat to wheat cultivation. **Tropical Plant Pathology**, v.38, n.3, 2013.

MARTENS, S.; MITHOFER, A.; Flavones and Flavone synthases. **Phytochemistry Phytochem**. V.66, n. 20, p.2399-2407, 2005.

MATOS, **Francisco José Abreu. Introdução a fitoquímica experimental**. 3. ed. Fortaleza: UFC, 148 p, 2009.

MCADOW *et al.* Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood. **PLoS pathogens**, v. 7, n. 10, p. 1-12, 2011.

MCCONOUGHEY *et al*. Biofilms in periprosthetic orthopedic infections. **Fut Microbiol**, v. 9, p. 987-1007, 2014

MEZZARI, A.; FUENTEFRIA, A. M. **Micologia no laboratório clínico.** Barueri-SP: Manole, 2012.

MILIAUSKAS, G.; VENSKUTONIS, P.R.; VAN BEEK, T.A. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. **Food Chem**., v. 85, p. 231-237, 2004.

MIGUEL, M.G. Antioxidant and anti-inflammatory activities of essential oils: a short review. **Molecules**, v. 15, p. 9252-9287, 2010.

MOGHADDAM; KHODI; MIRHOSSEINI. *Quorum sensing* in bacteria and a glance on *Pseudomonas aeruginosa*. **Clin. Microbial**., v.3, n. 4, p. 1-10, 2014.

Monroe, D. Looking for chinks in the armor of bacterial biofilms, **PLoS Biol**, v. 5, n. 11, p. 2458- 2461, 2007.

MONTANARI, C. A.; BOLZANI, V. S. Planejamento racional de fármacos baseado em produtos naturais. **Química Nova**, v. 24, n. 1, p. 105-111, 2001.

MOORMEIER *et al*. Temporal and stochastic control of *Staphylococcus aureus* biofilm development. **MBio**, v.5, p.1341-1314, 2014.

MOORMEIER *et al.* Use of microfluidic technology to analyze gene expression during *Staphylococcus aureus* biofilm formation reveals distinct physiological niches. **Appl Environ Microbiol**, v.79, p.3413–3424, 2013.

MOORMEIER; BAYLES. *Staphylococcus aureus* biofilm: a complex developmental organism. **Mol Microbiol**, v. 104 p.365-376, 2017.

MORADALI *et al*. Alginate polymerization and modification are linked in *Pseudomonas aeruginosa*. **MBio**, v. 6, 2015.

MORADALI; GHODS; REHM. *Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. **Frontiers in Cellular and Infection Microbiology**, v. 7, p. 1-29, 2017.

MORAIS, S. M. *et al*. Ação antioxidante de chás e condimentos de grande consumo no Brasil. **Revista Brasileira de Farmacognosia**, v. 19, n. 1B, p. 315-320, 2009.

MORAR, M.; WRIGHT, G. D. The genomic enzymology of antibiotic resistance. **Annu Rev Genet,** v. 44, p. 25-51. 2010.

MORGENSTERN, M. *et al*. Antibiotic resistance of commensal *Staphylococcus aureus* and coagulase-negative staphylococci in an international cohort of surgeons: a prospective point-prevalence study. **PLoS ONE**, v. 11, p.1-16, 2016.

MOSQUITO, S. *et al*. Molecular mechanisms of antibiotic resistance in *Escherichia coli*-associated diarrhea. **Rev Peru Med Exp Salud Publica,** v. 28, n. 4, p. 648-56. 2011.

MUKNE, A.P., VISWANATHAN, V., PHADATARE, A.G. Structure pre -requisites for isoflavones as effective antibacterial agents. **Pharmacognosy Review**., v. 5, n.9. p. 13-18, 2011.

MULCAHY; ISABELLA; LEWIS. *Pseudomonas aeruginosa* biofilms in disease. **Microb. Ecol**., v. 68, p.1-12, 2014.

N'DIAYE *et al*. Skin-bacteria communication: involvement of the neurohormone Calcitonin Gene Related Peptide (CGRP) in the regulation of *Staphylococcus epidermidis* virulence. **Sci. Rep**, v. 6, p. 1-15, 2016.

NAMVAR, *et al*. Clinical characteristics of *Staphylococcus epidermidis*: a systematic review. **Hygiene and Infection Control**, v.9, n.3, 2014.

NASCIMENTO, P. G. *et al*. Antibacterial and antioxidant activities of ursolic acid and derivatives. **Molecules**, v. 19, p. 1317-1327, 2014.

NASCIMENTO-NETO, L. G. *et al*. Effect of the triterpene 3β,6 β, 16β-trihydroxylup-20(29)-ene isolated from the leaves of Combretum leprosum Mart. on cutaneous wounds in mice. **Journal of Ethnopharmacology.** v. 117, p. 116-120, 2015.

NAZZARO, F. *et al*. Effect of essential oils on pathogenic bacteria. **Pharmaceuticals**, v. 6, n. 12, p. 1451-1474, 2013.

NELSON, D. L.; COX, M. M. **Princípios de Bioquímica de Lehninger.** 6. ed. Porto Alegre: Artmed, 2014.

NIKAIDO, H. Prevention of drug access to bacterial targets: Permeability barriers and active efflux. **Science**, v. 264, n. 5157, p. 382-388, 1994.

NISNEVITCH, M. Photosensitizers as an Alternative to Antibiotics. **Recent Pat Antiinfect Drug Discov**. 2013.

NOLVACHAI, Y.; MARRIOTT, P. J. GC for flavonoids analysis: Past, current, and prospective trends. **J Sep Sci**., v. 36, v. 1, p. 20-36, 2013.

NUNES, P.H. *et al*. Antiulcerogenic activity of *Combretum leprosum*. **Pharmazie**, v. 64, p. 58–62. 2009.

O'GARA, J. P. Into the storm: Chasing the opportunistic pathogen *Staphylococcus aureus* from skin colonisation to life-threatening infections. **Environmental Microbiology**, v. 19, n. 10, p. 3823–3833, 2017.

OHSAKI, *et al*. Microanalysis of a selective potent anti-*Helicobacter pylori* compound in a Brazilian medicinal plant, *Myroxylon peruiferum* and the activity of analogues. **Bioorganic & Medicinal Chemistry Letters**, v. 9, p.1109-1112, 1999.

OOTANI, Marcio Akio. **Atividade inseticida, antifúngica e herbitóxica dos óleos essenciais de *Eucalyiptus citriodora* e *Cymbopogon nardus***. 2010. 121 f. Dissertação (Mestrado em Produção Vegetal). Universidade Federal do Tocantins, Gurupi, 2010.

OZKAN, G. *et al*. Potential use of turkish medicinal plants in the treatment of various diseases. **Molecules**, v. 21, n. 257, p. 1-32, 2016.

PAIVA, P. M. G. *et al*. Antimicrobial activity of secondary metabolites and lectins from plants. Badajoz: **Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology**, p. 396-406, 2010.

PANKEY, G., SABATH, L. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram positives bacterial infections. **Oxford Journals**, v.38, p. 864-865, 2013.

PARROT, D. *et al.* Review-Lichen-Associated Bacteria as a Hot Spot of Chemodiversity: Focus on Uncialamycin, a Promising Compound for Future Medicinal Applications. **Planta Med.** v. 82, n. 13, p.1143-1152, 2016.

PARVIZI *et al*. Periprosthetic joint infection: the economic impact of methicillin-resistant infections. **J Arthroplasty**, v. 25, p. 103-107, 2010.

PAUL, J. H.; JEFFREY, W. H. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in Vibrio proteolytica. **Appl Environ Microbiol**., v. 50, p. 431-437, 1985.

PAVITHRA, D.; DOBLE, M. Biofilm formation, bacterial adhesion and host response on polymeric implants-issues and prevention. **Biomedical Materials**, v. 3, p. 1-13, 2008.

PERIASAMY *et al*. How *Staphylococcus aureus* biofilms develop their characteristic structure. **Proc Natl Acad Sci**, v. 109, n. 4, p. 1281-1286, 2012.

PETROSILLO, N.; IOANNIDOU, E; FALAGAS, M. E. Colistin monotherapy vs. combination therapy: evidence from microbiological, animal and clinical studies. **Clin Microbiol Infect,** v. 14, n. 9, p. 816-27, 2008.

PIERCE, C. G. *et al*. Antifungal therapy with an emphasis on biofilms. **Current Opinion in Pharmacology**, v. 13, p.1-5, 2013.

PIETROVSKI, E.F. *et al*. Antinociceptive properties of the ethanolic extract and of the triterpene 3beta,6beta,16beta-trihidroxilup-20(29)-ene obtained from the flowers of *Combretum leprosum* in mice. **Pharmacol. Biochem. Behav**. v.83, p. 90-99, 2006.

PROMDEN *et al*. Structure and antioxidant activity relationships of isoflavonoids from *Dalbergia parviflora*. **Molecules**, v. 19, p. 2226–2237, 2014.

QUITANS-JÚNIOR, L. *et al*. Antinociceptive Action and Redox Properties of Citronellal and Essential Oil Present in Lemongrass. **J Med Foot**. v.14, n.6, p.630- 639, 2011.

RAI, R. V; JAMUNA, B. A. Nanoparticles and Their Potential Application as Antimicrobials, Science against Microbial Pathogens: Communicating Current Research and Technological Advances. In: Méndez-Vilas, A., Ed., **Formatex, Microbiology Series**, v. 1, n. 3, p. 197-209, 2011.

RAO *et al*. Synthesis and antioxidant activity of galloyltyrosine, derivatives from young leaves of *Inga laurina*. **International Journal of Pharma and Bio Sciences**, v. 2, n. 4, p. 39-44, 2011.

RASAMIRAVAKA *et al*. The formation of biofilms by *Pseudomonas aeruginosa*: A review of the natural and synthetic compounds interfering with control mechanisms. **BioMed Res. Int.**, v. 2015, 2015.

RAUT, J. S.; KARUPPAYIL, S. M. A status review on the medicinal properties of essential oils. **Industrial Crops and Products**, v. 62, p. 250-264, 2014.

REZENDE, F. M. *et al*. Vias de síntese de metabólitos secundários em plantas. **VII Botânica no Inverno**. São Paulo: Instituto de Biociências da Universidade de São Paulo, Departamento de Botânica, p. 332, 2017.

RHEE, S.G., Cell signaling. H_2O_2, a necessary evil for cell signaling, **Science**, v.312, n. 5782, p. 1882-1883, 2006.

RIBEIRO *et al*. New frontiers for anti-biofilm drug development. **Pharmacol Ther**, v. 160, p. 133-144, 2016.

RIBEIRO; MONTEIRO; FERRAZ. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. **Biomatter**, v. 2, p. 176–194, 2012.

ROCHFORD, E.T.; RICHARDS, R.G.; MORIARTY, T.F. Influence of material on the development of device-associated infections. **Clinical Microbiology and Infection**, v. 18, p. 1162-1167, 2012.

RODRIGO, R.; MIRANDA, A.; VERGARA, L. Modulation of endogenous antioxidant system by wine polyphenols in human disease. Clinica chimica acta. **International journal of clinical chemistry**, v. 412, n. 5-6, p. 410-424, 2011.

ROGINSKY, V.; LISSI, E. A. Review of methods to determine chainbreaking antioxidant activity in food. **Food Chemistry,** v. 92, p. 235-254, 2005.

RYBTKE *et al*. *Pseudomonas aeruginosa* Biofilm infections: community structure, anti-microbial tolerance and immune response. **J. Mol. Biol**., v. 427, p. 3628-3645, 2015.

SAAD, N. Y.; MULLER, C. D.; LOBSTEIN, A. Major bioactivities and mechanism of action of essential oils and their components. **Flavour Frag. J.**, v. 28, p. 269-279, 2013.

SANCHEZ-VIOQUE *et al*. Polyphenol composition and antioxidant and metal chelating activities of the solid residues from the essential oil industry. **Ind. Crops Prod**. v. 49, p.150-159, 2013.

SANDHAR, H.K. *et al*. A Review of Phytochemistry and Pharmacology of Flavonoids. **Internationale Pharmaceutica Sciencia**. v.1, n.1, p. 25-41, 2011.

SANTOS, R. I. Metabolismo básico e origem dos metabólitos secundários. In: SIMÕES, C. M. O. et al. (Org.). **Farmacognosia: da planta ao medicamento**. 5. ed. rev. ampl. Florianópolis: Ed. da UFSC; Porto Alegre: Ed. da UFRGS, p. 403-434, 2004.

SANTI *et al*. Chemical Composition, Antioxidant and Antimicrobial Activity of the Oil and Plant Extract *Myrocarpus frondosus* Allemão. American Journal of Plant Sciences, v.8, p.1560-1571,2017.

SARTORI, A.L.B. *Myroxylon* in Lista de Espécies da Flora do Brasil. **Jardim Botânico do Rio de Janeiro**. Available in: Acesso em: 16 nov. 2017.

SARTORI, A.L.B. **Revisão taxonômica e estudos morfológicos, *Myrocarpus Allemão, Myroxylon* L.f. e *Myrospermum* Jacq**. 2000.162f.Tese (doutorado em Biologia Vegetal). Instituto de Biologia. Universidade Estadual de Campinas, Campinas, São Paulo. 2000.

SEMWAL *et al*. From arrow poison to herbal medicine-The ethnobotanical, phytochemical and pharmacological significance of *Cissampelos* (Menispermaceae). **J Ethnopharmacol**, v.155, n. 2, p. 1011-1028, 2014.

SENA, L.A., CHANDEL, N.S. Physiological roles of mitochondrial reactive oxygen species, **Mol. Cell**, v. 48, n. 2, p.158-167, 2012.

SHAHIDI, F.; AMBIGAIPALAN, P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects-A review. **Journal of Functional Foods**, v. 18, p. 820-897, 2015.

SHARMA *et al*. *Pseudomonas aeruginosa* biofilm: potential therapeutic targets. **Biologicals**, v.42, p. 1-7, 2014.

SILVA, M. I. G. *et al*. Bioactivity and potential therapeutic benefits of some medicinal plants from the Caatinga (semi-arid) vegetation of Northeast Brazil: a review of the literature. **Revista Brasileira de Farmacognosia**, v. 22, n. 1, p. 193-207, 2012.

SILVA, M. J. D. *et al*. Ethanolic extract of *Mimosa caesalpiniifolia* leaves: Chemical characterization and cytotoxic effect on human breast cancer MCF-7 cell line. **South African Journal of Botany**, v. 93, p. 64-69, 2014.

SILVA, S. *et al. Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. **FEMS Microbiology Reviews**, v. 36, n. 2 p. 288-305, 2012.

SILVA, S. M. F. Q. *et al*. Atividade *in vitro* de extratos brutos de duas espécies vegetais do cerrado sobre leveduras do gênero *Candida*. **Revista Ciência & Saúde Coletiva**, v. 17, n. 6, p. 1649-1656, 2012.

SIMOES, M.; BENNETT, R. N.; ROSA, E. A. S. Understanding antimicrobial activities of phytochemicals against multidrug resistantbacteria and biofilms. **Natural Product**, v.26, p.746-757, 2009.

SIMOES, M.; SIMOES, L. C.; VIEIRA, M. J. A review of current and emergent biofilm control strategies. **Lwt-Food Science and Technology,** v. 43, n. 4, p. 573 583, 2010.

SONG *et al*. Clinical and economic impact of methicillin-resistant *Staphylococcus aureus* colonization or infection on neonates in intensive care units. **Infect Control Hosp Epidemiol**, v.31, p.177-182, 2010.

SOTO, S.M. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. **Virulence**, v. 4, n. 3, p. 223-229, 2013.

SOUZA, R. K. D.; MENDONÇA, A. C. A. M.; SILVA, M. A. P. Ethnobotanical, phytochemical and pharmacological aspects Rubiaceae species in Brazil. **Revista Cubana de Plantas Medicinales**, v. 18, n. 1, p. 140-156, 2013.

STREMPEL *et al*. Human host defense peptide LL-37 stimulates virulence fator production and adaptive resistance in *Pseudomonas aeruginosa*. **PLoS ONE 8**, v. 8, n. 12, p. 1-12, 2013

SUNTRES, Z. E., COCCIMIGLIO, J., ALIPOUR, M. The bioactivity and toxicological actions of carvacrol. **Critical Reviews in Food Science and Nutrition**, v. 55, n.3, p. 304 318, 2015.

TA, C. A. K; ARNASON, J. T. Mini Review of Phytochemicals and Plant Taxa with Activity as Microbial Biofilm and Quorum Sensing Inhibitors. **Molecules,** v. 21, n.1, p. 21- 29, 2015.

TAIZ, L.; ZEIGER, E. **Fisiologia Vegetal**, 3. ed. Porto Alegre: Artmed, 2004.

TAIZ, L.; ZEIGER, E. **Fisiologia vegetal**. 4.ed. Porto Alegre: Artmed, 2009. 848p.

TAPWAL, A. *et al*. In Vitro antifungal potency of plant extracts against five phytopathogens. **Brazilian Archives of Biology and Technology**, v.54, n.6, p.1093-1098, 2011.

TECHATHUVANAN, C. *et al*. Efficacy of comercial natural antimicrobials alone and in combinations against pathogenic and spoilage microorganisms. **Journal of Food Protection**, v. 77, n. 2, p. 269–275, 2014.

TEHRANI, F., SADEGHI, E. Effect of mint essential oil on growth of *Listeria monocytogenes* during the ripening and storage of Iranian white brined cheese. **Journal of Applied Environmental and Biological Sciences**, v. 5, p. 150-154, 2015.

THAMMAVONGSA *et al*. Staphylococcal manipulation of host immune responses. **Nature reviews Microbiology**, v.13, p.529-543, 2015.

THOMER *et al.* Antibodies against a secreted product of *Staphylococcus aureus* trigger phagocytic killing. **The Journal of experimental medicine**, v. 213, p. 293-301, 2016.

TIELEN *et al*. Alginate acetylation influences initial surface colonization by mucoid *Pseudomonas aeruginosa*. **Microbiol. Res**., v.160, p. 165-176, 2005.

TOLKER-NIELSEN, T. *Pseudomonas aeruginosa* biofilm infections: From molecular biofilm biology to new treatment possibilities. **APMIS Suppl**, v. 122, p. 1-51, 2014.

TOLMACHEVA, A. A.; ROGOZHIN, E. A.; DERYABIN, D. G. Antibacterial and quórum sensing regulatory activities of some traditional Eastern-European medicinal plants. **Acta Pharmaceutica,** v. 64, n. 2, p. 173-186, 2014.

TOMAZONI, E. Z. *et al*. Atividade antifúngica do óleo essencial de *Cinnamomum zeylanicum* Ness sobre fungos fitopatogênicos do tomateiro (*Lycopersicon esculentum* Mill.). In: VIII CONGRESSO BRASILEIRO DE AGROECOLOGIA, 2013, Porto Alegre. **Anais eletrônicos**. Porto Alegre: Universidade de Caxias do Sul: 2013. Disponível em: <www.abaagroecologia.org.br/revistas/index.php/cad/article/download/13846/9464>. Acesso em 18 nov. 2017.

TOMAINO *et al*. Influence of heating on antioxidant activity and the chemical composition of some spice essential oils. **Food Chem**, v. 89, p. 549-554, 2005.

TRENTIN, D. S. *et al*. Tannins possessing bacteriostatic effect impair *Pseudomonas aeruginosa* adhesion and biofilm formation. **PLoS One**, v. 8, p. 1-13, 2013.

TSAO, R.; YANG, R.; YOUNG, J. C. Antioxidant isoflavones in osage orange, maclura pomifera (Raf.) schneid. **Journal Agricultural and Food Chemistry**. v. 51, p. 6445-6451, 2003.

VAARA, M. Agents that increase the permeability of the outer membrane. **Microbiological Reviews**, v. 56, n.3, p. 395-411, 1992.

VALGIMIGLI, L. PRATT, D. A. Antioxidants in Chemistry and Biology. **Encyclopedia of Radicals in Chemistry, Biology and Materials**, 2012.

VASCONCELOS *et al*. Antibacterial and antioxidant activities of aerriobtusone A isolated from *Lonchocarpus obtusus*. **Biomed res Int**, v. 2014, p. 1-9, 2014.

VILLARES, A. *et al*. Content and Profile of Isoflavones in Soy-Based Foods as a Function of the Production Process. **Food Bioprocess Technology**. v. 4, p. 27-38, 2011.

WAHID, A. *et al*. Heat tolerance in plants: An overview. **Environ. Exp. Bot.**, v. 61, n. 3, p. 199-233, 2007.

WALKER, T. D. The medicines trade in the Portuguese Atlantic World: acquisition and dissemination of healing knowledge form Brazil (c. 1580-1800). In: **Social History of Medicine Advanced Access**. Oxford: Oxford University Press, 2013.

WANG, Q., WANG, H., XIE, M. Antibacterial mechanism of soybean isoflavone on S*taphylococcus aureus*. **Arch Microbiol**., v.192, p. 893-898, 2010.

WEI; MA. Biofilm matrix and its regulation in *Pseudomonas aeruginosa*. **Int. J. Mol. Sci**., v. 14, n. 10, 2013.

WRIGHT, G. D. Bacterial resistance to antibiotics: enzymatic degradation and modification. **Adv Drug Deliv Rev,** v. 57, n. 10, p. 1451-1470. 2005.

YARWOOD *et al*. *Quorum sensing* in *Staphylococcus aureus* biofilms. **J Bacteriol**, v.186, p. 1838-1850, 2004.

YIN, H.; XU, L.; PORTER, N.A. Free Radical Lipid Peroxidation: Mechanisms and Analysis. **Chem. Rev**., v. 111, n. 10, p. 5944–5972, 2011.

ZAPOTOCZNA *et al.* An essential role for coagulase in *Staphylococcus aureus* biofilm development reveals new therapeutic possibilities for device-related infections. **J Infect Dis**, v.212, p. 1883-1893, 2015.

ZHENG, W.; WANG, S.Y. Antioxidant activity and phenolic compounds in selected herbs. **J. Agric. Food Chem.** v. 49, p. 5165-5170, 2001.

ZORE, G. *et al*. Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. **Phytomed**, v.18 p.1181–1190, 2011.

ZWENGER, S.; BASU, C. Plant terpenoids: applications and future otentials. **Biotechnol. Mol. Biol**., v. 3, p.1-7, 2008.

3 PROPRIEDADES QUÍMICAS E FARMACOLÓGICAS DO GÊNERO *Myroxylon* L. f. (FABACEAE): UMA REVISÃO DE LITERATURA

RESUMO

Compostos químicos com atividades biológicas isolados de espécies vegetais e usados pela população por meio de extratos e óleo essencial são promissoras fontes de prospecção de novos fármacos. O gênero *Myroxylon* L. f. apresenta elevado potencial farmacológico, sendo usado na medicina popular para o tratamento de diversas enfermidades, como, por exemplo, feridas, catarros, úlceras, dor de cabeça e de ouvido. Este artigo de revisão apresenta as principais características fitoquímicas e farmacológicas das espécies do gênero *Myroxylon* (*M. peruiferum* L. f., *M. balsamum* e *M. pereirae*), cujo estudo de atividades biológicas são promissores.

Palavras-chave: Etnofarmacologia. Fitoquímica. Fabaceae. Papilionoideae. Potencial biológico.

ABSTRACT

Chemical compounds with biological activities isolated from plant and used by the population through extracts and essential oils are promising sources of prospection for new drugs. The genus *Myroxylon* L. f. showed high pharmacological potential being used in popular medicine for the treatment of various diseases such as wounds, colds, ulcers, headache and ear infections. This review article presents the main phytochemical and pharmacological characteristics of the species from *Myroxylon* (*M. peruiferum* L. f., *M. balsamum* and *M. pereirae*), wich study of biological activities are promising.

Keywords: Ethnopharmacology. Phytochemistry. Fabaceae. Papilionoideae. Biological potential.

Introdução

Os vegetais têm sido utilizados com fins medicinais desde o início da civilização humana para a finalidade de prevenção, tratamento e cura de diversas doenças (De Carvalho *et al*. 2013). A evolução do conhecimento científico intensificou os estudos sobre plantas medicinais, relacionando a sua composição química com os seus efeitos, confirmando, muitas vezes, a sua utilização popular (Cavalcante *et al*. 2013).

Várias espécies vegetais apresentam constituintes na sua composição química, compostos possuidores de várias atividades biológicas, dentre as quais podemos destacar as atividades antimicrobianas, antioxidantes e anticâncer.

A família Fabaceae, também denominada Leguminosae, pertence à ordem Fabales, classe Magnoliopsida e divisão Magnoliophyta (Roskoy *et al*. 2007). É composta por aproximadamente 19.000 espécies, distribuídas em 695 gêneros (Giulietti *et al*. 2005). Essa família é formada por árvores, arbustos, lianas e ervas e tem distribuição cosmopolita (Di Stasi & Hiruma-Lima 2002). O Brasil apresenta cerca de 2.827 espécies, distribuídas em 222 gêneros, sendo que 1.524 espécies e 16 gêneros são endêmicos (BFG 2015).

É uma das maiores e mais importantes famílias botânicas, devido ao grande número de espécies vegetais utilizadas como fonte de produtos alimentares, medicinais, ornamentais, madeireiros e fornecedores de forragem, fibras, corantes, gomas, resinas e óleos (Di Stasi & Hiruma-Lima 2002; Watson & Dallwitz 2009). A família Fabaceae é dividida em três subfamílias: Faboideae, Caesalpinioideae e Mimosoideae (Souza & Lorenzi 2008).

Dentre seus principais gêneros das leguminosas, destaca-se o *Myroxylon*, cujos estudos fitoquímicos e biológicos de extratos, óleos essenciais e/ou metabólitos secundários estão sendo relatados tanto na medicina cientifica quanto na medicina popular. Apenas duas espécies são encontradas no Brasil: *M. peruiferum* L. f., amplamente distribuída no país, e *M. balsamum*, esta última apenas é encontrada em território brasileiro no estado do Acre (Sartori *et al*. 2015).

Entretanto, a importância de realização de estudos sobre plantas medicinais não reside apenas na caracterização química e física, mas sim na possibilidade de vincular os conteúdos apresentados com determinadas propriedades funcionais bioativas.

Nesse contexto, este artigo tem como objetivo apresentar um levantamento bibliográfico do potencial fitoquímico e farmacológico das espécies do gênero *Myroxylon*: *M. peruiferum* L. f., *M. balsamum* (L.) Harms e *M. pereirae* (Royle) Klotzsch.

Material e Métodos

As pesquisas foram realizadas entre 02 de novembro de 2015 a 02 de agosto de 2017, utilizando as seguintes palavras-chave de busca: "atividade antimicrobiana de *Myroxylon"*, "atividade antibacteriana e antifúngica do gênero *Myroxylon*", "atividade anticâncer do gênero *Myroxylon*", "avaliação antioxidante do gênero *Myroxylon*", "composição química do gênero *Myroxylon*", "óleo essencial do gênero *Myroxylon*" e "medicina tradicional do gênero *Myroxylon*". As pesquisas foram realizadas utilizando bases de dados em periódicos, incluindo a PubMed, Science Direct, Scopus, Scielo, periódicos Capes e Google acadêmico.

Resultados e Discussão

Família Fabaceae

Fabaceae constitui uma das famílias botânicas de grande importância econômica e medicinal, destacando-se algumas espécies dessa família no tratamento de doenças devido às suas propriedades curativas e terapêuticas. Muitas delas são usadas tanto na farmacologia quanto na medicina popular (Gomes *et al*. 2008).

Loiola e colaboradores (2010) destacam o uso de plantas da família Fabaceae como recurso medicinal em várias comunidades rurais da caatinga, mencionando a utilização dessas plantas em rituais religiosos, além de enfatizarem a influência desse grupo de plantas na cultura sertaneja.

As espécies dessa família são ricas em flavonoides e compostos biossinteticamente relacionados, como o rotenóides e isoflavonoides. Alcalóides, terpenóides e esteroides são exemplos de outras classes de substâncias que ocorrem em muitos exemplares da família, já os taninos têm frequência muito baixa se comparados aos flavonoides (Rocha e Silva *et al.* 2007).

Deste modo, o estudo fitoquímico e farmacológico tem se tornado imprescindível para auxiliar na identificação de substâncias bioativas, mostrando o uso correto das mesmas, seja na medicina popular ou no direcionamento para estudos biológicos específicos.

Gênero *Myroxylon*

O gênero *Myroxylon* foi instituído pelo cientista Linnaeus Filuis (1781), com relação às amostras provenientes da América do Sul, coletadas por Mutis. Já Willdenow (1799) ampliou a circunscrição do gênero *Myroxylon*, adicionando como sinônimo a *M. frutescens*

Jacq. e *M. pedicellatum* Lam. Além disso, considerou *Toluifera* como gênero monotípico. As espécies do gênero *Myroxylon* são denominadas, popularmente, por diferentes nomes nas áreas de ocorrências, como Chuchupate (Linares & Bye 1987), Guatemala (Léon & Alain 1951), Bálsamo, Cabreúva-vermelha, Pau-de-incenso, Caboreiba-vermelha, Cabreúva e Pau-vermelho (Lorenzi 1992).

O bálsamo terapêutico mais utilizado é do Peru e do Tolu, obtidos das espécies *M. pereirae* e *M. balsamum*, respectivamente. Algumas diferenças são abordadas entre as espécies produtoras do bálsamo, tanto nos aspectos morfológicos, quanto no seu aroma. É descrito que essas espécies do gênero *Myroxylon* têm uso na medicina popular desde o início da civilização, sendo usada contra acarinos e nos tratamentos de feridas e vias respiratórias (Inenami *et al*. 1984).

De acordo com Fluckiger e Hanbury (1874), é no caule desse gênero que são encontrados os principais constituístes que são utilizados como ingredientes para a fabricação de pomadas cicatrizantes, aromatizantes de xarope e de sabão. Linares e Bye Jr. (1987) acrescentam ainda a utilização como medicamento para congestão nasal, dores estomacais e reumáticas, e para fabricação de perfumes. Entretanto, trabalhos relacionados a atividades farmacológicas do gênero *Myroxylon* são escassos na literatura.

***Myroxylon peruiferum* L. f**

Descrição geográfica

É uma planta de origem nativa, não endêmica no Brasil, com distribuição geográfica confirmadas no Nordeste (Bahia, Ceará, Paraíba), Centro-oeste (Distrito Federal, Goiás, Mato Grosso do Sul, Mato Grosso), Sudeste (Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo) e Sul (Paraná). Pode ser encontrada no Cerrado e na Mata Atlântica (Sartori 2015).

Conhecimento popular

Myroxylon peruiferum L.f. (Fabaceae) é conhecida popularmente como cabriúva, pau-de-bálsamo e bálsamo. É uma planta decídua, heliófita, possuindo cerca de 10 a 20 metros de altura. Suas folhas são compostas pinadas, de ocorrência em quase toda a extensão do país, e a madeira é adequada para mobiliário, revestimentos, construção civil, dentre outras atribuições, por causa da sua alta resistência ao apodrecimento (Lorenzi 1998).

Em relação ao conhecimento popular, Lorenzi &Matos (2002) relatam que a utilização das folhas, frutos e resina do *M. peruiferum* L.f. é ideal para combater feridas e úlceras. Lorenzi & Matos (2008) acrescentam ainda a recomendação do uso para asma,

reumatismo, catarro, feridas externas, dor de cabeça, torcicolo e tuberculose. Sandoval & companheiro (1996) mostraram a utilização do extrato aquoso do fruto para aliviar a dor de ouvido. De acordo com Nogueira (1977), todas as partes da planta são consideradas medicinais (a casca, a entrecasca, a madeira, as folhas e o óleo).

Propriedades químicas

O estudo da composição química do caule de *M. peruiferum* L.f mostrou a presença de compostos predominantes como o nerolidol, vanilina e flavonoides, sendo relatada pela primeira vez na literatura. (Maranduba *et al.* 1979). Inenami (1984) identificou a presença de cumarina (2h-1-benzo-piran-2-ona) endógena nas sementes de *M. peruiferum* L.f, atuado, provavelmente, como um inibidor alelopático.

Akisue (1971,1972a e 1972b) descreveu vários experimentos com a finalidade de encontrar melhor rendimento da espécie estudada. Esse mesmo autor efetuou a caracterização física e química com diversas reações e análises químicas quantitativas através de cromatografia em camada delgada. Nesse estudo, fracionou o bálsamo em ácidos fortes e fracos, fenóis fortes e fracos, lactonas, aldeídos, cetonas, álcoois primários e secundários, entre outros compostos. Entre os componentes, foram demonstrados os ácidos benzoicos e cinâmico, vanilina, benzoato de benzila, cinamato de benzila, nerolidol e o álcool benzílico.

Já o óleo essencial do *M. peruiferum* L.f., obtido por hidrodestilação, apresentou as mesmas estruturas citadas, como a presença do fernesol, contribuindo com as pesquisas feitas por Marandura e colaboradores (1979). Além disso, na sua fração resinosa foram encontrados monoterpenoides, sesquiterpenoides, fenilpropanoides, álcoois e derivados de fenilpropanoides (Oliveira *et al.* 1978, Maranduba et al. 1979).

Maranduba e colaboradores (1979) realizaram análise do extrato etanolico e benzênico do caule de *M. peruiferum* L.f e encontraram onze flavonoides, dois deles sendo descritos pela primeira vez na literatura: 2'-hidroxi-7,3', 4-trimetoxiflavona e 2'-hidroxi-7,3', 4 trimetoxi-isoflavonona.

Propriedades farmacológicas

Os compostos 3',4',7-trimetoxiisoflavonas, 6-hidróxi-4',7-metoxi-isoflavona e germacreno D foram isolados a partir das folhas de *M. peruiferum* L.f e apresentaram atividade antimicrobiana sobre *Mycobacterium tuberculosis*, *Mycobacterium avium* e *Mycobacterium kansasii* (Carvalho *et al*. 2008).

Estudos biológicos dessa espécie são raramente encontrados na literatura. Um estudo realizado por Gonçalves *et al*. (2005 e 2007) mostrou que o extrato hidroalcoólico da casca de *M. peruiferum* L.f apresenta atividade antimicrobiana pelo método de difusão em ágar contra isolados clínicos das espécies *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Providencia* spp, *Proteus mrabilis, Pseudomanas aeruginosa, Staphylococus aureus e Salmonella Typhimurium,* e sensíbilidade para *Streptococcus pyogenes, Shigella flexneri, Shigella sonnei, Staphylococcus epidermidis* e *Staphylococcus* spp.

Corroborando com esses achados, Matos Neto (2013) demonstrou que o extrato etanólico da casca de *M. peruiferum* L.f. apresenta atividade antimicrobiana sobre cepas isoladas a partir de contaminação em alimentos, dentre estas: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae*, *Aeromonas caviae*, *Pseudomonas aeruginosa, Víbrio parahaemolyticus*, e *Escherichia coli*. A cabreuvina é o composto mais importante do extrato acetato de etila de *M. peruiferum*, com atividade antibiótica comprovada contra *Helicobacter pylori* (Ohsaki *et al*.1999).

A ação de compostos bioativos sobre fatores de virulência de determinados microrganismos é uma das alternativas mais promissoras para combater microrganismos patogênicos. A partir do extrato aquoso das folhas de *M. peruiferum*, Trentin *et al*. (2011), constataram a atividade antimicrobiana desse vegetal na formação do biofilme de *Staphylococcus epidermidis* ATCC 35984.

Muñoz *et al*. (2000) mostraram que o extrato etanólico do fruto apresentou inibição de 100 μg/mL em atividade antimalárica *in vitro*, cerca de 44% de inibição. Relataram ainda a atividade *in vivo* em ratos com dose de 1000 mg/kg, com 35% de inibição.

O óleo essencial da casca de *M. peruiferum* apresentou inibição frente à *Leishmania amazonensis* de IC_{50} 162, 25 μg/mL. Já para a citotoxicidade contra células monocamadas de L6, o óleo essencial apresentou concentração capaz de paralisar 50% (CC_{50}) das células após 24h de tratamento de 160, 80, apresentando baixa toxicidade (Andrade *et al*. 2016). Para atividade antioxidante, Silva-Júnior *et al*. (2015) demonstraram claramente, pela primeira vez, que os extratos metanólico e aquoso das folhas de *M. peruiferum* apresentam forte atividade antioxidante nos ensaios *in vitro* realizados.

Myroxylon balsamum (L.) Harms

Distribuição geográfica

É uma planta de origem nativa, não endêmica no Brasil, com distribuição geográfica confirmada apenas no Acre (Sartori 2015). Bálsamo do Tolu é obtido a partir *M. balsamum*,

uma árvore da família Fabaceae, nativa da Colômbia e Venezuela. Ele também cresce nas Antilhas, principalmente em Cuba. O Bálsamo é produzido fazendo incisões na casca das árvores, sob a forma de canais (Bährle-Rapp 2007), e foi incluso na Farmacopeia Britânica em 1820, com indicação para o tratamento de bronquite, laringite, diarreia e leucorreia, bem como um aromatizante alimentar.

Conhecimento popular

É amplamente utilizado como uma loção para o tratamento de feridas, úlceras e sarna, condicionadores capilares, produtos anticaspa, desodorantes, sabões, cremes, loções, pulverizações e comprimidos para resfriado comum (Taylor, 1998, BBaylor, 19, 2007, Heinrich *et al*. 2008).

Propriedades químicas

M. balsamum apresenta na sua fração resinosa aproximadamente 70% a 80% de composto ésteres cinâmico e benzoico de um óleo resinoso conhecido como toluresinotanol. O bálsamo tem 35% de ácidos livres, como o ácido cinâmico, de 12% a 15%, e 8% de ácido benzóico. A fração volátil (7,5%) é composta por cinamato de benzilo, benzoato de benzilo, vanilina, farnesol e terpenos (Costa 1994). Banerji (1995) identificou nas sementes de *M. balsamum* dez ácidos graxos e várias proteínas com o potencial de aditivos alimentares.

A fração hexânica obtida do extrato etanólico de *M. balsamum* tem sido considerada a fração mais aromática, com uma mistura de substâncias terpênicas e aromáticas, incluindo os ácidos benzóico e cinâmico, os álcoois e aldeídos, o etilo, ésteres de benzilo e de cinamilo, cinamato de metilo, estireno, eugenol, vanilina, ácido ferúlico, benzilo isoferúlico, ferúlico, e 1,2-difeniletano, todos identificados por Wahlberg *et al*. (1971) e por Boelens *et al*. (1982).

Colaborando com esses achados, Naves (1949), Gottlieb & Magalhães (1959), Harborne *et al*. (1963), Cheng *et al*. (1996) e Simas *et al*. (2004) identificaram os seguintes constituintes: Cabreuva, farnesol, terpenos, nerolidol, α e β-pineno, carvona, geraniol, mentol, citronelal, linalol, os fenilpropanoides cinamaldehído, eugenol e safrol a partir da fração obtida por hexano do extrato etanólico da casca do vegetal.

No entanto, a resina desse vegetal contém aproximadamente 80% de ésteres cinâmicos e benzoicos do álcool resinoso, conhecido como tolu-resinotannol, que é essencialmente composto de benzoato de benzilo e cinamato de benzilo (Custódio *et al.* 2012).

Em relação a constituintes mais complexo, Oliveira *et al.* (1978) isolaram os flavonoides (±) -7-hidróxi-4'-metoxi-isoflavona, (±) -7,3'-dihidroxi-4'- metoxi-isoflavona e 2-(2', 4'-di-hidroxifenil)-5,6-metilbenzofurano provenientes dos extratos etanólico e benzênico do tronco da madeira de *M. balsamum*. Do seu tronco, foram isolados isoflavonóides, flavononas e isoflavononas, enquanto nas folhas foi caracterizado um triperpeno pentacíclico. (Mathias 2000).

Propriedades farmacológicas

O extrato etanólico, assim com as frações hexânicas, e clorofórmico da casca de *M. balsamum* apresentou atividades sobre bactérias resistentes, especialmente a *Staphylococcus aureus, resistente à meticilina*, e *Pseudomonas aeruginosa* isoladas de infecções hospitalares, revelando a sua possível aplicação clínica. O bioensaio com o extrato levou à identificação da isoliquiritigenina calcona, um composto antimicrobiano, apresentando atividade sobre *S. aureus, S. epidermidis* e *S. haemolyticus* (Machado *et al*. 2005).

Estudos realizados por Jeong-Hyun & Lee (2010) demostraram o potencial antifúngico da resina de *M. balsamum* frente à *Malassezia furfur*. Por sua vez, Sales *et al*. (2016) mostraram em ensaios *in vitro* que a tintura mãe de *M. balsamum* apresentou zonas de inibição de crescimento de 12 e 11 mm de diâmetro para *Chalara paradoxa* e *Fusarium guttiforme*, respectivamente. Os ensaios foram realizados pelo método de difusão em ágar. As tinturas maternas apresentaram alta difusão no meio de crescimento devido ao seu caráter hidrofílico (Talibi *et al*. 2012), tornando os grupos químicos ativos disponíveis para os fungos analisados, o que representa uma característica importante na avaliação de novos compostos.

Já Simas e colaboradores (2004) utilizaram a fração hexânica do extrato etanólico da casca de *M. balsamum* e foi caracterizada uma substância ativa, denominada de sesquiterpeno *E*-nerolidol, mostrando-se atividade larvicida sobre o *A. aegypti*, com uma CL_{50} de 17 ppm. Em trabalho anterior, Chantraine *et al.* (2002) relataram atividade larvicida para a mesma fração, apresentando uma CL_{50} de 9,0 ppm.

Jankowsky (2005), em seus experimentos sobre atividade anticâncer, descreve que o extrato diclorometano e etanólico da casca de *M. balsamum* apresentou atividade citostática e citocida para as seguintes linhagens celulares: melanoma, carcinoma mamário, carcinoma mamário resistente e carcinoma de grande célula, leucemia, adenocarcinoma ovariano, prostático, colorretal e de células renais.

No que se refere à toxicidade, Popova e colaboradores (2001) demostraram que os extratos etanólicos, éter de petróleo, Cinnamato de benzilo, Benzoato de benzilo e butanol de

M. balsamum apresentaram toxicidade sobre *Artemia salina* com IC_{50} 1,8, 1,7,0,4, 0,4 e 23, respectivamente.

Em relação à atividade antioxidade, estudos realizados por Saleh *et al*. (2010) mostraram que o óleo essencial de *M. balsamum* apresentou mais de 90% de inibição do radical livre 2,2-difenil-1-picril-hidrazil (DPPH) nas concentrações de 100 mg/mL, 25 mg/mL e 5 mg/mL. Produtos naturais estão em crescente demanda para a fabricação de alimentos, cosméticos e produtos farmacêuticos.

O Bálsamo do Tulo é um agente bloqueador solar bem conhecido na qual apresenta um fator de proteção solar (FPS) adequado, quando comparado com o ácido aminobenzóico (El-Shaer 2006).

Myroxylon pereirae (Royle) Klotzsch

Distribuição geográfica

M. pereirae é uma espécie nativa da América Central. (Robbers *et al*. 1996).

Conhecimento popular

Tradicionalmente, é utilizada no tratamento contra câncer (Hartwell 1970). Essa espécie produz um bálsamo denominado de Bálsamo do Peru, assim chamado porque um dos primeiros lugares que foi vendido foi no Porto, no Peru (Robbers *et al*. 1996). As folhas e caule foram usados por tribos indígenas do México e América Central para o tratamento de asma, catarro, reumatismo e feridas externas (Taylor 1998).

Propriedades químicas

A composição química do Bálsamo do Peru é complexa, com algumas diferenças em relação ao Bálsamo do Tolu (*M. balsamum*). Foram identificados dois hidrocarbonetos, o estireno e estilbeno, e apenas um sesquiterpeno, δ-cadineno. Outras substâncias também foram identificadas em sua composição, tais como o álcool benzílico, álcool de cinamilo, elemol, vanilina, farnesol, (+) -nerolidol e ácidos benzóico e cinâmico (Boelens *et al*. 1982).

A resina *M. pereirae* é uma resina natural, obtida do tronco da árvore após incisões da casca (Tanaka *et al*. 2004). É constituída por várias substâncias, e apenas 60-70% já foram identificadas com precisão atualmente. Outra substância resinosa chamada de peruresinotanol foi observada tanto livre quanto na forma de ésteres de ácidos benzóico e cinâmico, sendo estes os principais constituintes da fração resinosa (28%) (Costa 1994).

Seo et al. (2012) identificaram na composição química do óleo essencial da resina de *M. pereirae* o benzoato de benzilo, seguido por cinamato de benzilo, ácido benzóico, e -nerolidol (E), sendo esses os componentes majoritários.

Propriedades farmacológicas

M. pereirae possui propriedades antissépticas, antibacterianas e antiparasitárias (especialmente para a sarna) (Blumenthal & Klein 1995). O Bálsamo do Peru tem sido vastamente estudado como um agente alergênico em muitas áreas, e as suas substâncias são encontradas em muitos produtos utilizados na sociedade, tais como alimentos e produtos de higiene, como sabonetes, desodorantes, perfumes e outros. Farnesol, uma substância que está presente no Bálsamo do Peru, mostrou uma elevada percentagem das alergias de contato (Schnuch *et al*. 2004).

De fato, *M. pereirae* pode causar dermatite de contato alérgica, contato urticária, reação fototóxica (Kroon 1983), reações de contato não imunológico (Tanaka *et al*. 2004, Katsarou *et al*. 1999), assim como a susceptibilidade a uma reação imediata.

Atualmente, essa espécie é considerada um marcador de alergia em perfumes (Hjorth1961, Larsen 1985), sendo que a presença de muitos alérgenos em perfumes ocorre pela possível presença de componentes presentes no Bálsamo do Peru ou estão intimamente relacionados a eles (Avalos-Peralta *et al*. 2005).

M. pereirae é uma planta amplamente utilizada em preparações tópicas para o tratamento de feridas, cicatrização de enxerto de pele, úlceras indolentes, sarna, assaduras, hemorroidas, prurido anal, escaras, intertrigo, eczema e outros; em tônico capilar e preparações anticaspa, sprays de higiene feminina e como um fixador ou fragrância (Opdyke 1974, Carson *et al*.2003).

O óleo essencial e os seus constituintes apresentaram atividade larvicida contra *A. aegypti,* apresentando grandes vantagens, tais como um efeito mínimo de degradação sobre o ecossistema com pouco resíduo na água (Seo *et al*.2012).

Em relação à atividade antioxidante, estudos realizados por Lin e seus companheiros (2009) mostraram que o óleo essencial de *M. pereirae* apresentou atividade antioxidante nos testes de inibição do radical livre DPPH.

Conclusão

Apesar do crescente uso de plantas medicinais na medicina popular, é importante à comprovação do seu efeito farmacológico. Os resultados desta revisão confirmam o grande potencial desse gênero para o desenvolvimento de novos medicamentos. Contudo, é extremamente necessária uma investigação cientifica mais aprofundada das espécies do gênero *Myroxylon* partir de seus extratos brutos, suas frações e óleos essenciais com o objetivo de utilizá-las especificadamente no tratamento de doenças que vêm acometendo o planeta.

Referências

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. I Morfologia, histologia de órgãos secretores e obtenção de bálsamo. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 9, p.115-133, 1971.

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. II Caracterização física do bálsamo e análise qualitativa de alguns componentes. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 10, p. 73-96, 1972a

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. III Caracterização física e química do óleo essencial do bálsamo e análise qualitativa de alguns componentes. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 10, p. 151-165, 1972b.

ANDRADE, M. A. *et al*. Essential oils: in vitro activity against *Leishmania amazonensis*, cytotoxicity and chemical composition. **BMC Complementary and Alternative Medicine**, v.16, p. 1-8, 2016.

AVALOS-PERALTA, P., GARCÍA-BRAVO, B., Camacho, F. M. Sensitivity to *Myroxylon pereirae* resin (balsam of Peru). A study of 50 cases. **Contact Dermatitis**, v. 52, p. 304-306, 2005.

BÄHRLE-RAPP, M. *Myroxylon balsamum* var. pereirae. In: Springer Lexikon Kosmetik und Körperpflege. Heidelberg: **Springer Medizin Verlag**, 2007.

BFG. Growing knowledge: an overview of Seed Plant diversity in Brazil. **Rodriguésia** v. 66, p. 1085-1113, 2015.

BLUMENTHAL, J.A. *et al*. Mental stress induced ischemia in the laboratory and ambulatory ischemia during daily life: Associatio and hemodynamic features. **Circulation,** v. 92, p. 2102-2108, 1995.

BOELENS, H.M., RIJKE, D; HARING, H.G..Studies of some balsamics in perfumery. **Perf Flavor** v. 6, p. 7-14, 1982.

CARSON, S.N. *et al*. Using a Castor Oil-Balsam of Peru-Trypsin Ointment to Assist in Healing Skin Graft Donor Sites. **Ostomy Wound Manage**, v. 49, p. 60-64, 2003.

CARVALHO, T.A. *et al.* Potencial bactericida de extratos e substâncias isoladas de *Myroxylon peruiferum* (Cabreúva) frente à micobactérias do trato respiratóio. In: **XVI Congresso de Iniciação Científica**. UFSCar, Sorocaba, 2008.

CAVALCANTE, G.M. *et al.* Atividade antimicrobiana de *Artocarpus heterophyllus* Lam. (Moraceae) sobre o desenvolvimento de *Streptococcus pneumoniae* e *Escherichia coli*. **Scientia Plena**, v. 9, p. 1-7, 2013.

CHANTRAINE, J.M. *et al*. Insecticidal activity of essential oils on *Aedes aegypti* larvae. **Phytotherapy Research**, v. 12, p. 350-354, 1998.

CHENG, B. *et al*. Study on the plant resources of nerolidol and their uses. **Linchan Huaxue Yu Gongye**, v. 16, p. 22-28, 1996.

COSTA, A.F. Farmacognosia. Lisboa: **Calouste Gulbenkian**, 1994.

CUSTÓDIO, D.L.VEIGA-JUNIOR, V.F. True and common balsams. Rev. Bras. **Farmacogn**. v. 22, p. 1372-1383, 2012.

DE CARVALHO, R.B.F. *et al.* Composição química e atividade anticolinesterásica de uma fração ativa do extrato de folhas de *Citrus limon* (L.) Burm. **Quimica Nova**, v, 36, p. 1375-1379, 2013.

DI STASI, L.C. HIRUMA-LIMA, C.A. Plantas medicinais na Amazônia e na Mata Atlântica. 2 ed. **Revista e ampliada**, São Paulo, 2002.

EL-SHAER, N.S. Evaluation of some natural products and plant acids against UVB radiation *(in vitro*). **Alex J Pharm Sci**, v. 20, p. 7-11, 2006.

FLUCKIGER, F. A. HANBURY, D. Balsamum tolunatum. **Pharmacographia**, v. 15, p.177-184, 1874.

GIULIETTI, A.M. *et al*. Biodiversidade e conservação das plantas no Brasil. **Megadiversidade**, v. 1, p. 52-60, 2005.

GOMES, E. C. S. *et al*. Plantas da Caatinga de uso terapêutico: Levantamento Etnobotânico. **Engenharia Ambiental, Espírito Santo do Pinhal**, v.5, p.74-85, 2008.

GONÇALVES, A.L, FILHO, A.A. MENEZES, H. Estudo comparativo da atividade antimicrobiana de extratos de algumas árvores nativas. **Arquivos do Instituto Biológico**, v. 72, p. 353-358, 2005.

GONÇALVES, A.L. Estudo da atividade antimicrobiana de algumas árvores medicinais nativas com potencial de conservação/recuperação de florestas tropicais. **Tese de doutorado**, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rio Claro, 2007.

GOTTLIEB, O.R. MAGALHAES, M.T. The chemistry of Brazilian Leguminosae. I. Isolation of 3',4',7-trimethoxyisoflavone (cabreuvin) from *Myroxylon balsamum* and *Myrocarpus fastigiatus*. **An Assoc Bras Quim**, v.18, p.89-97, 1959.

HARBORNE, J.B., GOTTLIEB, O.R. MAGALHAES, M.T. Occurrence of the isoflavone afromosin in cabreuva wood. **J Org Chem**, v. 28, p. 881-882, 1963.

HARTWELL, J.L. Plants used against cancer. A survey. **Lloydia**, v. 33, p. 97-194, 1970.

HEINRICH, M. *et al*. Fundamentals of Pharmacognosy and Phytotherapy. Edinburgh: **Churchill Livingstone**, 2008.

HJORTH, N. Eczematous allergy to balsams, allied perfumes and flavouring agents, with special reference to balsam of Peru. **Acta Derm Venereol (Stockh)**, v. 41, p. 1-216, 1961.

INENAMI, T. O. Estudo do inibidor de germinação em sementes de *Myroxylon peruiferum* L.f. (Cabriúva)- Leguminosae. **Tese de Doutorado**, Universidade Estadual de Campinas, Campinas, 1984.

JANKOWSKY, L. Atividade farmacológica de extratos obtidos de a partir de resíduos madeireiros. **Tese de doutorado**, Universidade Estadual de Campinas, Campinas, 2005.

KATSAROU, A. *et al.* Frequency of immediate reactions to the European standard series. **Contact Dermatitis**, v. 41, p. 276-279, 1999.

KROON, S. Standard photopatch testing with Waxtar1, paraaminobenzoic acid, potassium dichromate and balsam of Peru. **Contact Dermatitis**, v. 9, p. 5-9, 1983.

LARSEN, W. G. Perfume dermatitis. **J Am Acad Dermatol**, v.12, p.1-9, 1985.

LEE, J-H; LEE J-S. Chemical Composition and Antifungal Activity of Plant Essential Oils against *Malassezia furfur*. **Journal of Microbiology and Biotechnology**, v. 38, p. 315-321, 2010.

LEON, H.; H. ALAIN. Dicotiledoneas: Casuarinaceas a Meliaceas. **Fl. Cuba**, v, 2p. 1-456, 1951.

LIN, C. W. *et al.* DPPH Free-Radical Scavenging Activity, Total Phenolic Contents and Chemical Composition Analysis of Forty-Two Kinds of Essential Oils. **Journal of Food and Drug Analysis**, v. 17, p. 386-395, 2009.

LINARES, E; BYE JR, R. A. A study of four medicinal plant complexes of Mexico and adjacente United States. Journal of ethnopharmacology, v. 19, p. 153-183, 1987.

LINNAEUS FILIUS, C. *Myroxylon peruiferum*. **Supplementum Plantarum** v. 34, p. 232-234, 1781.

LOIOLOA, M. I. B. *et al.* Leguminosas e seu potencial de uso em comunidades rurais de São Miguel do Gostoso-RN. **Revista Caatinga**, v. 23p. 59-70, 2010.

LORENZI, H; MATOS, F.J. A..Plantas Medicinais no Brasil: nativas e exóticas. Nova Odessa, **Plantarum**, 2008.

LORENZI, H. Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil. Nova Odessa: Ed. **Plantarium**, 1992.

LORENZI, H. Árvores Brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil. Nova Odessa, **Plantarun**, 1998.

LORENZI, H.E; MATOS, F.J. A. Plantas medicinais no Brasil/ Nativas e exóticas. Nova Odessa, **Instituto Plantarum**, 2002.

MACHADO, T.B. *et al.* Brazilian phytopharmaceuticals-evaluation against hospital bacteria. **Phytother Res**, v. 19p. 519-525, 2005.

MARANDUBA, A. *et al.* Isoflavonoids from *Myroxylon peruiferum*. **Phytochemistry** v, 18, p. 815-817, 1979.

MATHIAS, L. *et al.* A New Pentacyclic Triterpene Isolated from *Myroxylon balsamum* (syn. *Myroxylon peruiferum*). Journal of the Brazilian **Chemical Society**, v. 11p. 195-198, 2000.

MATOS NETO, F.A. Abordagem etnobotânica de plantas medicinais usadas para tratamento de lesões de pele associado, ou não, a bactérias ou fungos no município de Itapajé, Ceará, Brasil. **Trabalho de conclusão de curso**, Universidade Estadual Vale do Acaraú, Sobral, 2013.

MUÑOZ, V. *et al*. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach Part III. Evaluation of the antimalarial activity of plants used by Alteños Indians. **Journal of Ethnopharmacology**, v. 71p. 123-131, 2000.

NAVES, Y.R. Presence of nerolidol in papilionaceous essential oils. **Helv Chim Acta** v. 32, p. 2181-2185, 1949.

OHSAKI, A. *et al*.Microanalysis of a selective potent anti-*Helicobacter pylori* compound in a Brazilian medicinal plant, *Myroxylon peruiferum* and the activity of analogues. **Bioorganic & Medicinal Chemistry Letters**, v. 9, p. 1109-1112, 1999.

OLIVEIRA, A.B. *et al.* Isoflavonoids from *Myroxylon balsamum*. **Phytochemistry**, v. 17, p. 593-595, 1978.

OPDYKE, D.L.J. Monographs on fragrance raw materials: fennel oil, bitter. **Food Cosmet Toxicol**, v. 14, p. 309-310, 1976.

POPOVA, M. *et al.* A scientific note on the high toxicity of propolis that comes from *Myroxylon balsamum* trees. **Apidologie**, v. 33, p.87-88, 2002.

ROBBERS, J.E; SPEEDIE, M.K; TYLER, V.E. Pharmacognosy and Pharmacobiotechnology. Baltimore: **Williams & Wilkins**, 1996.

ROCHA E SILVA, H. *et al*. Constituintes químicos das cascas do caule de *Cenostigma macrophyllum*: ocorrência de colesterol. **Química Nova**, v. 30, p. 1877-1881, 2007.

ROSKOY, Y.R. *et al*. International Legume Database and Information Service (ILDIS). Disponível em http://www.ildis.org (acesso em 10-IV-2017), 2007.

SAHOO, S.; SINGH, S.; NAYAK, S. Chemical composition, antioxidant and antimicrobial activity of essential oil and extract of *Alpinia malaccensis* Roscoe (Zingiberaceae). **International Journal of Pharmacy and Pharmaceutical Sciences**, v. 6, p. 183-188, 2014.

SALES, M. D. C. *et al.* Antifungal activity of plant extracts with potential to control plant pathogens in pineapple. **Asian Pacific Journal of Tropical Biomedicine**, v. 6, p. 26-31, 2016.

SANDOVAL, P; CHOQUE, J; URIONA, P. Cartilla popular sobre las plantas utiles de los Alteños de Mizque-Cochabamba. **Fonama-Cibe-Umsa**, Cochabamba, Bolivia, 1996.

SARTORI, A.L.B. *et al*. A revision of the genus *Myroxylon* (Leguminosae: Papilionoideae). **Kew Bulletin**, v. 70, p. 1-12, 2015.

SCHNUCH, A. *et al*. Contact allergy to fragrances: frequencies of sensitization from 1996 to 2002. Results of the IVDK. **Contact Dermatitis**, v. 50, p. 65-76, 2004.

SEO, S-M.; PARK, H-M;PARK, IL-K. Larvicidal Activity of Ajowan (*Trachyspermum ammi*) and Peru Balsam (*Myroxylon pereira*) Oils and Blends of Their Constituents against Mosquito, Aedes aegypti, Acute Toxicity on Water Flea, *Daphnia magna*, and Aqueous Residue. **Journal of Agricultural and Food Chemistry**, v. 60, p. 5909-5914, 2012.

SILVA-JÚNIOR, C. A. *et al*. *Myroxylum peruiferum*: antioxidant activity and phenolic content. **Current Topics in Phytochemistry**, v.12, p.21-25, 2015.

SIMAS, N.K**.** *et al.* Produtos naturais para o controle da transmissão da dengue- Atividade larvicida de *Myroxylon balsamum* (óleo vermelho) e de terpenóides e fenilpropanóides. **Quim Nova**, v.27, p.46-49, 2004.

SOUZA, V. C; LORENZI, H. Botânica Sistemática: guia ilustrado para identificação das famílias de fanerógamas nativas e exóticas no Brasil, baseado em APGII. 2 ed. Nova Odessa, SP: **Instituto Plantarum**, 2008.

TALIBI, I. *et al.* Antifungal activity of some Moroccan plants against *Geotrichum candidum*, causal agent of postharvest citrus sour rot. **Crop Protection**, v.35, p.41-46, 2012.

TANAKA, S. *et al.* Immediate contact reactions to fragrance mix constituents and *Myroxylon pereirae* resin. **Contact dermatites**, v.51, p. 20-21, 2004.

TAYLOR, L. Herbal secrets of the rainforest: the healing power of over 50 medicinal plants you should know about. **Rocklin: Prima Healh**, 1998.

TRENTIN, D. S. *et al*. Potential of medicinal plants from the Brazilian semi-arid region (Caatinga) against *Staphylococcus epidermidis* planktonic and biofilm lifestyles. **Journal of Ethnopharmacology**, v.137, p.327-335, 2011.

WAHLBERG, I. *et al*. Constituents of commercial Tolu balsam. **Acta Chem Scand**, v. 25, p. 3285-3295, 1971.

WATSON, L; DALLWITZ, M.J. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Disponível em http://delta-intkey.com (acesso em 10-V-2017), 2009.

WILLDENOW, K. Species plantarum, 4 ed. Impensis G. C. **Nauk, Berolin**,1799.

Tabela 1. Potencial farmacológico de espécies do Gênero *Myroxylon*: *Myroxylon peruiferum* L.f., *Myroxylon balsamum* (L.) Harms e *Myroxylon pereirae* (Royle) Klotzsch.

Espécie	Parte usada	Preparação	Atividade Biológica	Referências
	Folhas	Extrato etanólico	Atividade antimicrobiana sobre *Mycobacterium tuberculosis*, *Mycobacterium avium* e *Mycobacterium kansasii*	Carvalho *et al*. 2008
	Casca	Extrato hidroalcoólico	Atividade antibacteriana frente à *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Providencia* spp, *Proteus mrabilis, Pseudomanas aeruginosa, Staphylococus aureus e Salmonella Typhimurium*, e sensíbilidade para *Streptococcus pyogenes, Shigella flexneri, Shigella sonnei, Staphylococcus epidermidis* e *Staphylococcus* spp.	Gonçalves *et al*. 2005 e 2007
***Myroxylon peruiferum* L.f.**	Casca	Extrato etanolico	Atividade antibacteriana contra *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae*, *Aeromonas caviae, Pseudomonas aeruginosa, Víbrio parahaemolyticus*, e *Escherichia coli*.	Matos Neto 2013
	Tronco da madeira	Extrato acetato de etila	Atividade antibiótica contra *Helicobacter pylori*	Ohsaki *et al*.1999
	Folhas	Extrato aquoso	Atividade antimicrobiana na formação do biofilme de *Staphylococcus epidermidis* ATCC 35984	Trentin *et al*. 2011
	Fruto	Extrato etanolico	Inibição de 100 µg/mL em atividade antimalárica *in vitro* e dose de 1000 mg/kg em atividade *in vivo* em ratos.	Muñoz *et al*. 2000

	Casca	Óleo essencial	Apresentou inibição frente à *Leishmania amazonensis* de IC_{50} 162, 25 μg/mL e citotoxicidade contra células monocamada de L6 na concentração de 50% (CC_{50}) das células após 24 h de tratamento de 160, 80	Andrade *et al*. 2016
	Folha	Extrato metanólico e aquoso	Apresentou atividade antioxidante nos ensaios in vitro.	Silva-Júnior *et al*. 2015
Espécie	**Parte usada**	**Preparação**	**Atividade Biológica**	**Referências**
	Casca	Extrato etanólico e frações hexânica e clorofórmico	Apresentaram atividades sobre bactérias resistentes, especialmente, *Staphylococcus aureus resistente à meticilina* e *Pseudomonas aeruginosa, S. epidermidis* e *S. haemolyticus.*	Machado *et al*. 2005
	Casca	Resina	Potencial antifúngico frente à *Malassezia furfur*	Jeong-Hyun & Lee (2010)
	Casca e folha	Tintura mãe	Atividade antifúngica para *Chalara paradoxa* e *Fusarium guttiforme*	Sales *et al*. (2016)
Myroxylon balsamum (L.) Harms	Casca	Fração hexânica do extrato etanólico	Atividade larvicida sobre o *A. aegypti*, com uma CL_{50} de 17 ppm. Atividade larvicida apresentando uma CL_{50} de 9,0 ppm.	Simas *et al*. (2004), Chantraine *et al.* (2002).
	Casca	Extrato diclorometano e etanolico	Atividade anticâncer, demonstrando ação citostática e citocida para as linhagens celulares: melanoma, carcinoma mamário, carcinoma mamário resistente e carcinoma de grande célula, leucemia, adenocarcinoma ovariano, prostático, colorretal e de células renais.	Jankowsky (2005)
		Extratos	Apresentaram toxicidade sobre *Artemia salina*	Popova *et al*. (2001)

		etanolico, éter de petróleo, Cinnamato de benzilo, Benzoato de benzilo e butanol		
	Folha	Óleo essencial	Inibição do radical livre 2,2-difenil-1-picril-hidrazil (DPPH) nas concentrações de 100 mg/mL, 25 mg/mL e 5 mg/mL.	Saleh *et al*. (2010)

Espécie	**Parte usada**	**Preparação**	**Atividade Biológica**	**Referências**
Myroxylon pereirae (Royle) Klotzsch	Resina	Óleo essencial	Atividade larvicida contra *A. aegypti*.	Seo *et al*.2012)
	Resina	Óleo essencial	Atividade antioxidante nos testes de inibição do radical livre DPPH.	Lin *et al*. (2009)

Fonte: elaborada pelo autor.

4 EVALUATION OF THE ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF ISOFLAVONE 7-HYDROXY-4', 6-DIMETHOXY-ISOFLAVONE AND ESSENTIAL OIL OF *Myroxylon peruiferum* L.f

ABSTRACT

This study evaluated the antibacterial, antifungal, and antioxidant effect of 7-hydroxy-4',6-dimethoxy-isoflavone and essential oil of *Myroxylon peruiferum* L.f. The fractions (F'202-277) of the chloroform fraction resulting from the extraction in 8:2 chloroform/ethyl acetate were pooled by similarity, yielding 20 mg of a whitish material which was purified and analyzed by Nuclear Magnetic Resonance of ^{1}H and ^{13}C. The chemical composition of essential oil was determined by gas chromatography using mass spectrometry detector (GC-MS). To evaluation of antimicrobial activity, the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) ware determinated. In addition, for analysis of antioxidant activity, DPPH radical scavenging tests, iron chelating assay (FIC), antioxidant reducing power assay (FRAP) and β-carotene bleaching assay (BCB) were performed. The compound was identified as 7-hydroxy-4', 6-dimethoxy-isoflavone. For the essential oil ware identified 24 organized compounds having as ~~the~~ main constituents Germacrene D (17.15%), α-pinene (14.85%) and E-caryophyllene (10.78%). The results showed that isoflavone and essential oil showed antibacterial activity and antifungal activity against Gram-positive bacteria and filamentous fungi but not against Gram-negative bacteria and yeasts. The isoflavone and the essential oil also presented antioxidant activity in all the tests, mainly on inhibition of the oxidation of β-carotene test. In conclusion, isoflavone and essential oil of *M. peruiferum* L.f may be a potential antimicrobial alternative against Gram-positive bacteria and dermatophyte fungi as well as a potential antioxidant.

Key words Antimicrobial activity. Antioxidant activity. Isoflavone. Essential oil. *Myroxylon peruiferum* L.f

Introduction

Plants have been used for medicinal purposes since the beginning of human civilization for prevention, treatment and cure of several diseases (De Carvalho et al., 2013). The evolution of scientific knowledge has intensified studies with medicinal plants relating their chemical composition to its effects, often confirming its popular use (Cavalcante et al., 2013). In fact, several studies have investigated the presence of chemical compounds with biological potential in plant extracts and essential oils, commonly in plants used in folk medicine by communities (Guimarães et al., 2014).

Studies of novel products of plant origin with antimicrobial activity have increasing interest, since emergence of resistant bacteria and fungi to conventional antimicrobials agents, aggravating this problem of global public health (Silva et al., 2012). Thus, extracts or compounds isolated from plant species used in folk medicine can be promising sources for the investigation of new antimicrobial agents (Al-Fatimi et al., 2007).

Myroxylon peruiferum L.f. belongs to the Fabaceae family, popularly known as “cabriúva” or “bálsamo”. It is a deciduous plant, heliophytic, having about 10 to 20 meters of height. Their leaves are composed of pinnacles, occurring almost throughout the country. Their wood is suitable for furnitures, claddings and civil constructions because of its high resistance to rot (Lorenzi 1998).

Regarding popular knowledge, Lorenzi and Matos (2002) report the use of leaves, fruits and resin of *M. peruiferum* L.f. like ideal for fighting wounds and ulcers. According Lorenzi and Matos (2008), leaves, fruits and resin can be used for treating asthma, rheumatism, phlegm, external wounds, headache, torticollis and tuberculosis. França et al. (2008), in an extensive consultation on medicinal literature, states that *Myroxylon toluifera* resin is used to treat coughing caused by bronchitis. Santos et al. (1988) describes that *Myroxylon balsamum* (L.) Harms, popularly known as "Bálsamo-to-tolú", is an effective antiseptic of the urinary tract.

Thus, the aim of this study was to describe the isolation the isoflavone and define the chemical composition of the essential oil from the leaves of *Myroxylon peruiferum* L.f. In addition, evaluate the antimicrobial and antioxidant activities of isolated compounds and essential oil.

Material and methods

Plant material

Leaves and stem (1000 g) of *Myroxylon peruiferum* L.f were collected in July 2016 at the Uruburetama massif, located in the Soledade district of Itapagé, under coordinates 03º37'86" S and 39º35'33" W, located around 800 m above sea level. The plant authentication was performed by Professor E. B. Souza and a voucher specimen (N. 19240) has been deposited at the herbarium Francisco José de Abreu Matos of the University Acaraú Valley (Sobral, Brazil).

Extraction and analysis of the chemical compound

The stem (1000 g), dried at room temperature, was triturated and subjected to cold extraction with ethanol for three days. The obtained solution was distilled under reduced pressure resulting in 105.52 g of the ethanolic extract. Then, the ethanol extract was adsorbed onto silica gel and subjected to the chromatographic column using increasing eluents: hexane (100%), chloroform (100%), ethyl acetate (100%) and methanol (100%). The obtained fractions were distilled under reduced pressure and provide the following fractions: hexane (1.12 g), chloroform (19.59 g), ethyl acetate (17.46 g) and methanol (18.23 g).

The chloroform fraction was subjected to silica gel column chromatography using the eluents in increasing order of polarity.: hexane (100%), hexane/chloroform (9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9), chloroform (100%), chloroform/AcoEt (9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9), AcoEt/methanol (9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9) and methanol (100%), resulting in 499 fractions with 5 mL each. Fractions were analyzed by thin layer chromatography (TLC).

The chloroform fraction was subjected to column chromatography using the eluents in increasing order of polarity resulted in 499 fractions. Fractions (F'202-277), resulted from extraction in chloroform/ethyl acetate 8:2, were grouped by similarity, yielding an off-white material of 20 mg that was purified and analyzed.

The single and two-dimensional ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopic data required for the structural determination of the isolated constituents were obtained at the Northeastern Center for the Application and Use of Nuclear Magnetic Resonance, Federal University of Ceará. Single and two-dimensional Nuclear Magnetic Resonance spectra were obtained by Bruker DRX-300 and DPX-500 (^{1}H: 300 e 500 MHz; ^{13}C: 75 e 125 MHz) spectrometers using deuterated solvents and TMS as the internal standard.

Extraction and analysis of essential oil

From the leaves, the essential oil was extracted by the hydrodistillation method using the Clevenger type apparatus for 2 hours. At the end of the extraction, the oil volume was measured and stored in an amber glass recipient with under refrigerated conditioning for analysis. The chemical composition of the essential oil was determined by gas chromatographic analysis using a mass spectrometry detector (GC-MS), model QP-2010. It was used a silica capillary column with 30 m in length, 0.25 mm ~~of~~ i.d, 0.25 μm film and helium gas with a linear velocity of 1 mL/min, 250°C injector, 250°C interface and 250°C detector. The initial temperature was 35°C to 180°C at 4°C per minute, then 180°C to 280°C at 17°C per minute, electron impact of 70 eV.

Microorganisms

In this study the following strains were used: the bacteria *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 11303, and *Pseudomonas aeruginosa* ATCC 10145; the yeasts: *Candida albicans* ATCC 90028, *Candida tropicalis* LABMIC 0110, *Candida parapsilosis* LABMIC 0123, and *Candida krusei* LABMIC 0124; and the dermatophytic fungi: *Trichophyton rubrum* 0207, *Trichophyton rubrum* 0208, *Trichophyton rubrum* 0209, and *Trichophyton rubrum* 0210.

Culture conditions

Bacteria were grown in Tryptone Soy Agar (TSA; Himedia, India) and incubated at 37°C for 24 hours. After growth, an isolated colony was removed and inoculated into 5 mL of *Tryptic* Soy *Broth* (TSB; Himedia, India) and incubated at 37°C for 18 hours. Then, the bacterial cells concentration of each inoculum was adjusted to $2x10^6$ cells/mL by turbidimetry (620 nm) and calibration curves previously determined for each bacterium. Fragments of *Trichophyton rubrum* and *Candida* spp. were transferred to tubes containing 9 mL of saline to obtain a turbidity equivalent to standard 5 x 10^4 mL^{-1} or 0.5 McFarland scale. The suspensions were diluted to 1:2 for *Candida* spp. and 1:5 for *T. rubrum,* both with RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Sigma Chemical Co., St. Louis, Mo.), buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.), to obtain inoculum concentrations of approximately 2.5 – 5 x 10^3 CFU/mL for *Candida* spp. and 5 x 10^4 CFU/mL for *T. rubrum.*

Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antibacterial effect of compound isolated and the essential oil was determined by the broth microdilution method according to the guidelines from the National Committee for Clinical Laboratory Standards, M7-A6 (NCCLS 2003), with some modifications. Earlier, the compound was diluted to 10 mg/mL in TSB medium containing 4% DMSO and 10% essential oil was diluted in TSB medium containing 10% Tween 20. The concentrations of the compound were used in a ranged from 5 to 0.07 mg/mL and the essential oil from 5.0% to 0.07%. The substances were added (100 µL) with bacterial suspensions previously adjusted (2 x 10^6 cells/mL). The plates were then incubated at 37°C for 24 hours. The minimum inhibitory concentration (MIC) was considered the lower dilution of the compound that does not show visible bacterial growth. For Minimum Bactericidal Concentration (MBC), 10 µL of wells that having no visible microbial growth were inoculated onto Petri dishes with TSA medium. MBC was considered as the lower concentration capable of completely inhibiting microbial growth in plaques.

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The minimum inhibitory concentration was determined by the method described by Clinical and Laboratory Standards Institute-CLSI (CLSI M27-A2 2008), while the minimum fungicidal concentration (CFM) was according to Fontenelle et al. (2007). The compound and the essential oil were prepared in DMSO and mineral oil, respectively. 100 µL of sterile RPMI 1640 was inoculated into each well of the microdilution plate followed by 100 µL of sample added to the first microwell column and serially diluted until column 11; the concentration range was of 5 to 0.009 mg/mL. Subsequently, 100 µL of the inoculum was added to all wells making a final volume of 200 µL per well. The MIC was defined by the smallest test concentration capable of inhibiting visually fungal growth. The MFC was defined by the lowest concentration which resulted in non-growth after 2 days for *Candida* spp. and 5 days for *T. rubrum* spp. after transferring 100 µL solution from wells without turbidity to agar dextrose 28 (Fontenelle et al., 2007; 2008).

Antioxidant Activity

Determination of DPPH radical scavenging activity

The ability of the compound and the essential oil to scavenge the radicals DPPH was measured according to Duan et al. (2006). Test samples consisted of a methanolic solution containing 0.16 mM DPPH added to different concentrations of the isoflavone (400 to 6.25 μg/mL) and the essential oil (2.5 to 7.8%). The control was DPPH solution without sample, and the blank consisted of sample without DPPH solution. Samples, controls and blanks were incubated in the dark at 25°C for 30 minutes and the optical density was measured at 517 nm using a microplate reader (Biochrom Asys UVM 340). Ascorbic acid was used as a positive control. The percentage of the DPPH radical scavenging effect was calculated by the following equation:

$$\text{DPPH scavenging (\%)} \quad \left(1- \frac{(O.D_{sample} - O.D_{blank})}{O.D_{control}} \right) \; x\; 100\%$$

Iron chelating activity (FIC)

The iron ion chelating activity assay was performed according to Wang et al. (2009). The samples were prepared using deionized water to obtain concentrations equals to previous test where 2 mM iron chloride ($FeCl_2$) and 5 mM ferrozine were added. Blank and control were prepared with distilled water replacing ferrozine and samples, respectively. Samples were incubated at 25°C for 10 minutes and the optical density was measured at 562 nm using a microplate reader (Biochrom Asys UVM 340). EDTA (ethylenediamine tetraacetic acid) was used as a positive control. The FIC activity was calculated by the following equation:

$$\text{Iron chelating activity (\%)} = \frac{[O.D_{control} - (O.D_{sample} - O.D_{blank})]}{O.D_{control}} \; x\; 100\ \%$$

Iron reduction method (FRAP)

The antioxidant capacity of iron reduction of the sample was determined according to the method described by Ganesan et al. (2008). Initially, the isoflavone at concentrations ranging from 400 to 6.25 μg/mL and the essential oil from 2.5 to 7.8% were mixed with 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide. Samples were incubated at 50°C for 20 minutes. After cooling, 10% trichloroacetic acid was added. An aliquot was mixed with 0.1% iron hydrochloride ($FeCl_3$) and distilled water. After 10 minutes, the optical density of this mixed was measured at 700 nm using a microplate reader (Biochrom Asys UVM 340).

Butylhydroxyanisole (BHA) was used as a positive control. Higher the optical density, greater the compound's ability to reduce iron.

Oxidation inhibition with β-carotene and linoleic acid (BCB)

The ability of the compound and the essential oil to inhibit the oxidation of β-carotene and linoleic acid was determined by a combination of the methods described by Chew et al. (2008) and Dhongade and Chandewar (2013). In brief, the reagent for this assay consists of 3 mL of 0.1 mg/mL *β*-carotene dissolved in chloroform, 40 mg linoleic acid and 400 mg Tween 40. The chloroform was evaporated using a rotary evaporator and then ultra-pure water (Milli-Q) was added to the solution. The samples were adjusted to the same concentrations as before. The optical density of the solutions was initially measured at 470 nm using a microplate reader (Biochrom Asys UVM 340), then again after 3 hours of incubation at 50°C. BHA was used as a positive control for this assay. The antioxidant activity was calculated by the following equation:

$$\text{Antioxidant activity (\%)} = \left(\frac{O.D_{\text{final}}}{O.D_{initial}} \right) \; x\ 100\ \%$$

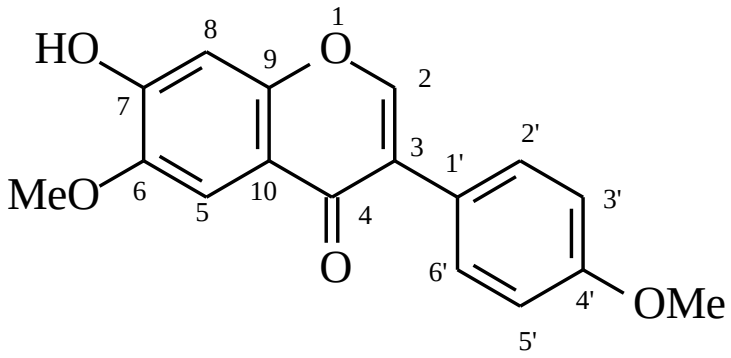
Statistical analysis

For the antioxidant assays, the percent values obtained in the DPPH, FIC and BCB assay for each of the concentrations tested were converted to absolute values, submitted to angular transformation and compared by Student's t-test for independent values. The optical density values obtained in the FRAP test were also analyzed using Student's t-test for independent values. Values of $p < 0.05$ were considered statistically significant.

Results and Discussion

RMN ^{13}C spectra showed 17 carbons signals, being two signals of methoxy groups. The isolated compound of the species was identified as 7-hydroxy-4', 6-dimethoxy-isoflavone whose RMN ^{1}H, RMN ^{13}C spectral data are according to literature values (Figure 14). This compound was isolated by Jha, Zilliken and Breitmaier (1980), from the desoxybenzoin obtained by condensation of phenols with derivatives of arylacetic acids or aliphatic acids. Fernandes et al. (2009) isolated the isoflavone from suspension cell cultures and callus of *Dipteryx odorata*, popularly known as afrormosin or castanin.

Figure 14: Chemical structure of the 7-hydroxy-4', 6-dimethoxy-isoflavone isolated from the ethanol extract of *Myroxylon peruiferum* L.f bark.



Fonte: elaborada pelo autor.

The essential oil yield was 2.0% (w/w) dry weight. The chemical composition of the volatile constituents of the essential oil of *M. peruiferum* L.f (EOMP) and their respective percentages are set out in Table 2. The results of gas chromatography coupled to mass spectrometry (CG/MS) showed the identification and quantification of 24 compounds organized by elution order in column DB-5 corresponding to 85.50% of the chemical composition of the oil, having the Germacrene D (17.15 %), α-pinene (14.85 %) and E-caryophyllene (10.78 %) as the main.

Studies of chemical composition and biological activity of EOMP are scarce. The literature presents as their main components (E) and (Z)-nerolidol, α-bisabolol and (E, E)-farnesol (Wanner et al., 2010), but these compounds were not identified in the present study.

The study of the chemical composition of *M. peruiferum* L.f showed a predominantly presence of nerolidol and a variety of flavonoids being reported for the first time in the literature (Maranduba et al., 1979). Through the chemical analysis of the essential oil of bark and of the essential oil of trunk of *M. peruiferum* L.f several phenolic substances were found. Furthermore, in its resinous fraction were found monoterpenoids, sesquiterpenoids, phenylpropanoids, alcohols and phenylpropanoids derivatives (Akisue 1971; 1972a; 1972b, Oliveira et al., 1978, Maranduba et al., 1979).

Variations in the chemical composition of essential oils, secondary metabolism products of plant, occur due to changes in abiotic factors as the geographic location of the plant, season and even time of day when the plants are exposed (Dudareva et al., 2004, Gobbo-Neto and Lopes, 2007).

Substances like 3',4',7-trimethyl isoflavones, 6-hydroxy-4',7-methoxy-isoflavone and germacrene D isolated of *M. peruiferum* leaves show antimicrobial activity against *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium kansasii* (Carvalho et al., 2008). Lattanzio et al. (2007) isolated three substances from *M. peruiferum* stem; these were luteolin, cabreuvine and an isoflavone, described for the first time in the literature.

The results of the antibacterial activity (Table 3) showed that isoflavone was able to inhibit the bacterial growth of *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228 with MIC values of 2000 μg/mL for both strains. OEMP showed MIC at 2.5% and MBC at 5.0% against *S. aureus* ATCC 25923 and MIC at 5.0% against *S. epidermidis* ATCC 12228, however, without MBC. The isoflavone and OEMP have not shown activity against *Escherichia coli* ATCC 11303 and *Pseudomonas aeruginosa* ATCC 10145.

Corroborating with our study, Gonçalves et al. (2005) reported that *M. peruiferum* L.f ethanol extract showed activity against *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Providencia* spp., *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Streptococcus pyogenes*, *Shigella flexneri* and *Shigella sonnei*; all microorganisms obtained from clinical infections.

Matos Neto (2013) describes that the ethanol extract from *M. peruiferum* L.f bark has antimicrobial activity against strains of *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae*, *Aeromonas caviae*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* and *Escherichia coli*, all pathogenic microorganisms contaminants of foods.

The isoflavone isolated from the ethyl acetate extract, the most predominant compound of the species, shows a proven antibiotic activity against *Helicobacter pylori* (Ohsaki et al., 1999).

The ethanolic extract as well as the hexanic and chloroform fractions of the shell of *M. balsamum* showed activities against resistant bacteria, especially *S. aureus* resistant to methicillin and *P. aeruginosa* isolated from hospital infections, revealing their medicinal use. The bioassay with the extract identified the isoliquiritigenin calzone, an antimicrobial compound that has potential against *S. aureus*, *S. epidermidis* and *S. haemolyticus* (Machado et al., 2005).

Gram-positive bacteria were more susceptible to the action of isoflavone and EOMP than Gram-negative bacteria because their cell wall is composed of an external membrane, constituting a second lipid bilayer.

The results in the antifungal assays showed MIC and MFC values for the isoflavone of 156 and 312 μg/mL against *T. rubrum* LABMIC 0207; 312 and 625 μg/mL against *T. rubrum* LABMIC 0208; 625 and 1250 μg/mL against *T. rubrum* LABMIC 0209; and 1250 and 2500 μg/mL against *T. rubrum* LABMIC 0210, respectively. The EOM showed MIC and MFC on *T. rubrum* LABMIC 0207, *T. rubrum* LABMIC 0208 and *T. rubrum* LABMIC 0209 at concentrations ranging from 5000 to 1250 μg/mL (Table 3). It was observed that the EOMP did not present inhibition effectiveness against the yeast strains tested.

The isoflavonoids acquired considerable importance for exhibiting diverse biological activities like antioxidant, antifungal, bactericidal, anti-inflammatory, estrogenic and contraceptive (Jang et al., 2003). Furthermore, essential oils have been described for their antibacterial and antifungal properties, whose use may represent an advance against resistance mechanisms that inactivate standard antifungal (Castro 2010; Saad et al., 2010; Tempone et al., 2008). Thus, the discovery of natural products with antibacterial and antifungal activity may represent a new therapy through the production of phytotherapics (Khan et al., 2012; Mendes 2011; Silva et al., 2009).

Antimicrobial studies of isolated isoflavone are rarely found in literature. The isoflavone 6,7,4'-trihydroxy-isoflavone showed antibacterial activity against *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228 and *Streptococcus pyogenes* ATCC 19615 at concentrations of 32, 128, 16, 32 and 64 μg/ml (Hummelova et al., 2014).

The formononetine (7-hydroxy, 4'methoxy-isoflavone) has several pharmacological properties such as antimicrobial, healing potential, anti-lipid effect, antioxidant, hyperlipidemic, cardio-protective activity, antitumor, antidiabetic and neuroprotective activity (Vishnuvathan et al., 2016).

Zhu (2014) showed that formononetine has antifungal effects for *Candida albicans* Y0109 and *C. albicans* SC5314 with MIC values of 8 μg/mL. Li et al. (1990) isolated isoflavone from tobacco roots and demonstrated antibacterial effect (over 90% inhibition) against *Proteus vulgaris*, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*.

The EOMP showed MIC and MFC of 1.25 and 2.5 μL/mL for *C. albicans* URM-6543 and *C. tropicalis* URM-6741, 1.25 μL/mL for MIC and MFC for *C. glabrata* URM-6393 and 0.625 and 1.25 μL/mL for *C. krusei* URM-6391 and *C. parapsilosis* URM- 6557 (Costa et al., 2017).

Several studies have evaluated the antibacterial and antifungal activity and reported different types of action mechanisms owing to large variety in the chemical composition of essential oils (Burt 2004; Di Pasqua et al., 2010; Greay and Hammer, 2011; Qiu et al., 2011).

Certain authors consider the lipophilicity of the constituents of essential oils as a property that explains the antimicrobial activity, which allows bacterial inhibition through the permeability and depolarization of the cytoplasmic membrane. With the permeability of membrane, increase the proton evasion of cells and the modification of electrical potential of the membrane, indications of membrane damage and consequently cell death (Cowan et al., 2011; Rua et al., 2011).

Regarding antioxidant activity, in the DPPH scavenging assay (Figures 15A and 16A), the isoflavone and the EOMP showed antioxidant activities at all concentrations tested with percentages of approximately 60%. The ascorbic acid (positive control) presented significantly higher values than the samples in all concentrations tested, with percentage values of 70-100%. The ability to bind to metal ions, specifically to the iron ion (Figures 15B and 16B.), the compound showed dose-dependent chelating action with percentages of 10-20% in the concentrations of 6.25 to 400 μg/mL, while the EOMP showed a percentage of 40-100% in concentrations of 0.078 to 2.5%. The positive control (EDTA) showed values significantly from 10 to 100% when compared to compound and essential oil.

The samples were also tested for their ability to reduce Fe^{3+} to Fe^{2+}. The results obtained showed that the optical density remained stable at the concentrations of isoflavone; but the optical density increased in accordance with the concentrations for the EOMP when compared to the positive control (BHA) (Figures 15C and 16C).

Finally, it was evaluated the ability of the samples to inhibit β-carotene oxidation. Both the essential oil and the isoflavone showed a high oxidizing activity in up to 60 hours of storage varying from 80 to 100% and inhibiting β-carotene oxidation in values close to these found in the positive control (BHA) (Figures 15D and 16D).

In this study, we have isolated the compound 7-hydroxy-4', 6-dimethoxy-isoflavone and described the chemical composition of essential oil of *M. peruiferum* L.f. Furthermore, the isoflavone and essential oil exhibits antifungal e antibacterial, mainly on Gram-positive bacteria and dermatophytes fungi. Moreover, in the antioxidant assays the samples showed a potential antioxidant action in different trial. Thus, the essential oil and the isoflavone isolated of *M. peruiferum* can be considered as potential antimicrobial agents against infections and contaminations caused by bacteria and fungus. In addition, they can be used against oxidative stress present in infections by these microorganisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Reference

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. I Morfologia, histologia de órgãos secretores e obtenção de bálsamo. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 9, p.115-133, 1971.

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. II Caracterização física do bálsamo e análise qualitativa de alguns componentes. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 10, p. 73-96, 1972a

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. III Caracterização física e química do óleo essencial do bálsamo e análise qualitativa de alguns componentes. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 10, p. 151-165, 1972b.

AL-FATIMI, M. *et al*. Antioxidant, antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. **Journal of Ethnopharmacology**, v. 111, p 657-666, 2007.

BURT, S. Essential oils: their antibacterial properties and potential applications in foods-a review. **Int J Food Microbiol**, v. 94, p. 223-253, 2004.

CARVALHO T.A. *et al*. Potencial bactericida de extratos e substâncias isoladas de *Myroxylon peruiferum* (cabreúva) frente a micobactérias do trato respiratório. **In: XVI Congresso de Iniciação Científica**. UFSCar, Sorocaba, 2008.

CASTRO, R. D. Atividade antifúngica do óleo essencial de *Cinnamomum zeylanicum* Blume (canela) e de sua associação com antifúngicos sintéticos sobre espécies de *Candida*. **Tese de doutorado**. Universidade Federal da Paraíba, 2010.

CAVALCANTE, G.M. *et al*. Atividade antimicrobiana de *Artocarpus heterophyllus* Lam. (Moraceae) sobre o desenvolvimento de *Streptococcus pneumoniae* e *Escherichia coli*. **Scientia Plena**, v.9, p. 1-7, 2013.

CHEW Y.L. *et al*. Antioxidant activity of three edible seaweeds from two areas in South East Asia. **Food Science and Technology**, v. 41, p. 1067-1072, 2008.

COSTA, M.C.M.F. *et al*. Essential oils from leaves of medicinal plants of Brazilian flora: chemical composition and activity against *Candida* species. **Medicines**, v. 27, p. 1-8, 2017.

COWAN, M.M. Plant products as antimicrobial agents. **Clin Microbiol Ver**, v. 12, p. 564-582, 1999.

DE CARVALHO, R.B.F. Composição química e atividade anticolinesterásica de uma fração ativa do extrato de folhas de *Citrus limon* (L.) Burm. **Quim. Nova**, v. 36, p.1375-1379, 2013.

DHONGADE, H.J; CHANDEWAR, A.V. An *in-vitro* investigation of the antioxidant activity of *Phyllanthus amarus*. **International Journal of Biomedical and Advance Research** v, 4, p. 435-439, 2013.

DI PASQUA, R. *et al*. Changes in the proteome of *Salmonella enterica* serovar Thompson as stress adaptation to sublethal concentrations of thymol. **Proteomics**, v.10, p. 1040-1049, 2010.

DUAN, X.J. *et al*. Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata.* **Food Chemistry**, v. 95, p. 37-43, 2006.

DUDAREVA, N; PICHERSKY, E; GERSHENZON, J. Biochemistry of plant volatiles. **Plant Physiol**, v. 135, p.1893-1902, 2004.

FERNANDES, R.S. *et al*. Validação do método de extração e quantificação de 7-hidróxi- 4',6 dimetóxi-isoflavona em culturas de células em suspensão e calos de *Dipteryx odorata.* **Eclética Química**, v.34, p.13-18, 2009.

FONTENELLE, R.O.S. *et al*. Antifungal activity of essential oils of *Croton* species from the Brazilian Caatinga biome. **Journal of Applied Microbiology**, v.104, p. 1383-1390, 2008.

FONTENELLE, R.O.S. *et al*. Chemical composition, toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham. **J Antimicrob Chem**, v. 59, p. 934-940, 2007.

FRANÇA, I.S.X. *et al*. Medicina popular: benefícios e malefícios das plantas medicinais. **Revista Brasileira de Enfermagem**, v. 61, p. 201-208, 2008.

GANESAN, P; KUMAR, C.S; BHASKAR, N. Antioxidant properties of methanol extract an its solvent fractions obtained from selected Indian red seaweeds. **Beoresource Techonology, v.** 99, p. 2717-2723, 2008.

GARCIA, R.G; MALO, A.L; PALOU, E. Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against *Listeria innocua*. Jo**urnal of Food Science**, v.76, p. 95-100, 2011.

GOBBO-NETO, L; NORBERTO, P.L. Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. **Química Nova**, v.30, p. 374-381, 2007.

GONÇALVES, A.L; FILHO, A.A; MENEZES, H. Estudo comparativo da atividade antimicrobiana de extratos de algumas árvores nativas. **Arq. Inst. Biol**, v. 72, p. 353-358, 2005.

GREAY, S.J; HAMMER, K.A. Recent developments in the bioactivity of mono- and diterpenes: anticancer and antimicrobial activity. **Phytochemistry Reviews**, v.14, p.1-6, 2011.

GUIMARÃES, A.G; SERAFINI, M.R; QUINTANS-JUNIOR, L.J. Terpenes and derivatives as a new perspective for pain treatment: a patent review. E**xpert Opinion on Therapeutic Patents**, v. 24, p. 243-265, 2014.

HUMMELOVA, J. *et al*. The relationship between structure and *in vitro* antibacterial activity of selected isoflavones and their metabolites with special focus on antistaphylococcal effect of demethyltexasin. **Applied Microbiology**, v. 60, p. 242-247, 2014.

JANG, D.S. et al. Potential cancer chemopreventive constituents of the seeds of *Dipteryx odorata* (tonka bean). **J. Nat. Prod**, v. 66, p. 583-587, 2003.

JHA, H.C; ZILLIKEN, F; BREITMAIER, E. Carbon-13 chemical shift assignments of chromones and isoflavones. **Can. J. Chern**, v. 58, p.1211-1219, 1980.

KHAN, S.M.A; MALIK, A; AHMAD, I. Anti-candidal activity of essential oils alone and in combination with amphotericin B or fluconazole against multi-drug resistant isolates of *Candida albicans*. **Medical Mycology**, v.50, p. 33-42, 2012.

LATTANZIO, N.A. *et al*. Busca de inibidores da Transtirretina (TTR) humana em extratos e substâncias isoladas de *Myroxylon peruiferum* (Fabaceae). **In: Sociedade Brasileira de Química** (SBQ), Águas de Lindóia-SP, 2007.

LI, T. *et al*. Isoflavonoid compound from tobacco rhizome and preparation and application thereof. Patent CN102351827A, filled Aug 16, 2011 issued Feb 15, 2012.

LORENZI, H. Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil. Nova Odessa: **Plantarun**, 1998.

LORENZI, H.E; MATOS, F.J.A. Plantas Medicinais no Brasil: nativas e exóticas. Nova Odessa: **Instituto Plantarum**, 2002.

LORENZI, H.E; MATOS, F.J.A. Plantas Medicinais no Brasil: nativas e exóticas. Nova Odessa: **Plantarum**, 2008.

MACHADO, T.B. *et al*. Brazilian phytopharmaceuticals-evaluation against hospital bacteria. **Phytother Res**, v.19, p.519-525, 2005.

MARANDUBA, A. *et al*. Isoflavonoids from *Myroxylon peruiferum*. **Phytochemistry**, v. 18, p.815-817, 1979.

MATOS NETO, F.A. Abordagem etnobotânica de plantas medicinais usadas para tratamento de lesões de pele associado, ou não, a bactérias ou fungos no município de Itapajé, Ceará, Brasil. **Trabalho de conclusão de curso**, Universidade Estadual Vale do Acaraú, Sobral, 2013.

MENDES, J.M. Investigação da atividade antifúngica de óleo essencial de *Eugenia coryophyllata* Thunb. sobre cepas de *Candida tropicalis*. **Dissertação**, Universidade Federal da Paraíba, 2011.

National Committee Clinical Laboratory Standards (NCCLS) M27A. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Approved Standard, M27-A3; USA: Wayne, 2008.

National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6, 6th ed. National Committee for Clinical Laboratory Standards, Wayne, Pa, 2003.

OHSAKI, A. *et al*. Microanalysis of a selective potent anti-*Helicobacter pylori* compound in a Brazilian medicinal plant, *Myroxylon peruiferum* and the activity of analogues. **Bioorganic & Medicinal Chemistry Letters**, v.9, p. 1109-1112, 1999.

OLIVEIRA, A.B. *et al*. Isoflavonoids from *Myroxylon balsamum.* **Phytochemistry**, v.17, p. 593-595, 1978.

QIU, J. *et al.* Subinhibitory concentrations of perilla oil affect the expression of secreted virulence factor genes in *Staphylococcus aureus*. **PLoS ONE**, v. 6, p. 1-8, 2011.

RÚA, J. *et al*. Antibacterial activity against foodborne *S. aureus* and antioxidant capacity of various pure phenolic compounds. **Foodborne Pathogen and Diseases**, v. 8, p. 149-157, 2011.

SAAD, A. *et al*. Anticandidal activity of the essential oils of *Thymus maroccanus* and *Thymus broussonetii* and their synergism with amphotericin B and fluconazol. **Phytomedicine**, v. 17, p.1057-1060, 2010.

SANTOS CADE, M; TORRES, K.R; LEONART, R. Plantas medicinais/herbarium, flora et scientia. São Paulo. **Ícone**, 1988.

SILVA, F.M; PAULA, J.E; ESPINDOLA, L.S. Evaluation of the antifungal potential of Brazilian cerrado medicinal plants. **Mycoses**, v. 52, p.511-517, 2009.

SILVA, M. J. *et al*. Avaliação da atividade antioxidante e antimicrobiana dos extratos e frações orgânicas de *Mimosa caesalpiniiflolia* Benth. (Mimosaceae). R**ev Ciên Farm Básica Apl**, v.33, p. 267-274, 2012.

TEMPONE, A.G. *et al*. Brazilian flora extracts as source of novel antileishmanial and antifungal compounds. **Memórias do Instituto Oswaldo Cruz**, v. 103, p.443-49,2008.

VISHNUVATHAN, V.J; LAKSHMI, K.S; SRIVIDYA, A.R. Medicinal uses of formononetin - a review. **The Journal of Ethnobiology and Traditional Medicine**, v. 126, p.1197-1209, 2016.

WANG, T; JÓNSDÓTTIR, R; ÓLAFSDÓTTIR, G. Total phenolic compounds, radical scavenging and metal chelation of extracts from *Icelandic seaweeds*. **Food Chemistry**, v. 116, p. 240-248, 2009.

WANNER, J. *et al*. Chemical composition and antibacterial activity of selected essential oils and some of their main compounds. **Nat Prod Commun**, v.5, p.1359-1364, 2010.

ZHU, M. Application of 7-hydroxy-4'-methoxy-isoflavone in preparation of antifungal drugs. Patent CN103565794A, filled Nov 08, 2013 issued Feb 12, 2014.

Table 2 - Chemical composition of the leaves of the essential oil from *Myroxylon peruiferum* L.f *(*EOMP).

Constituintes	**IK**	**%**
2E-hexenol	862	0.4
α-tujeno	930	1.5
α-pinene	**939**	**14.8**
β-pinene	979	4.8
Mirceno	990	0.1
δ-2-carene	1002	0.5
β-felandren	1029	5.8
Limonene	1029	2.9
γ--terpinene	1059	0.1
Terpinolene	1088	0.1
4-terpineol	1177	2.6
Nerol	1229	0.3
α-copaene	1376	1.5
E- caryophyllene	**1419**	**10.8**
γ-elemene	1436	6.5
Germacrene D	**1485**	**17.1**
δ-cadinene	1522	0.3
δ -cadinene	1523	2.0
α-humulene	1554	3.4
Germacrene B	1561	3.5
Espatulenol	1578	1.8
Oxide caryophyllene	1583	1.1
Ledol	1602	0.3
α-cadinol	1654	0.3
Total		85.5

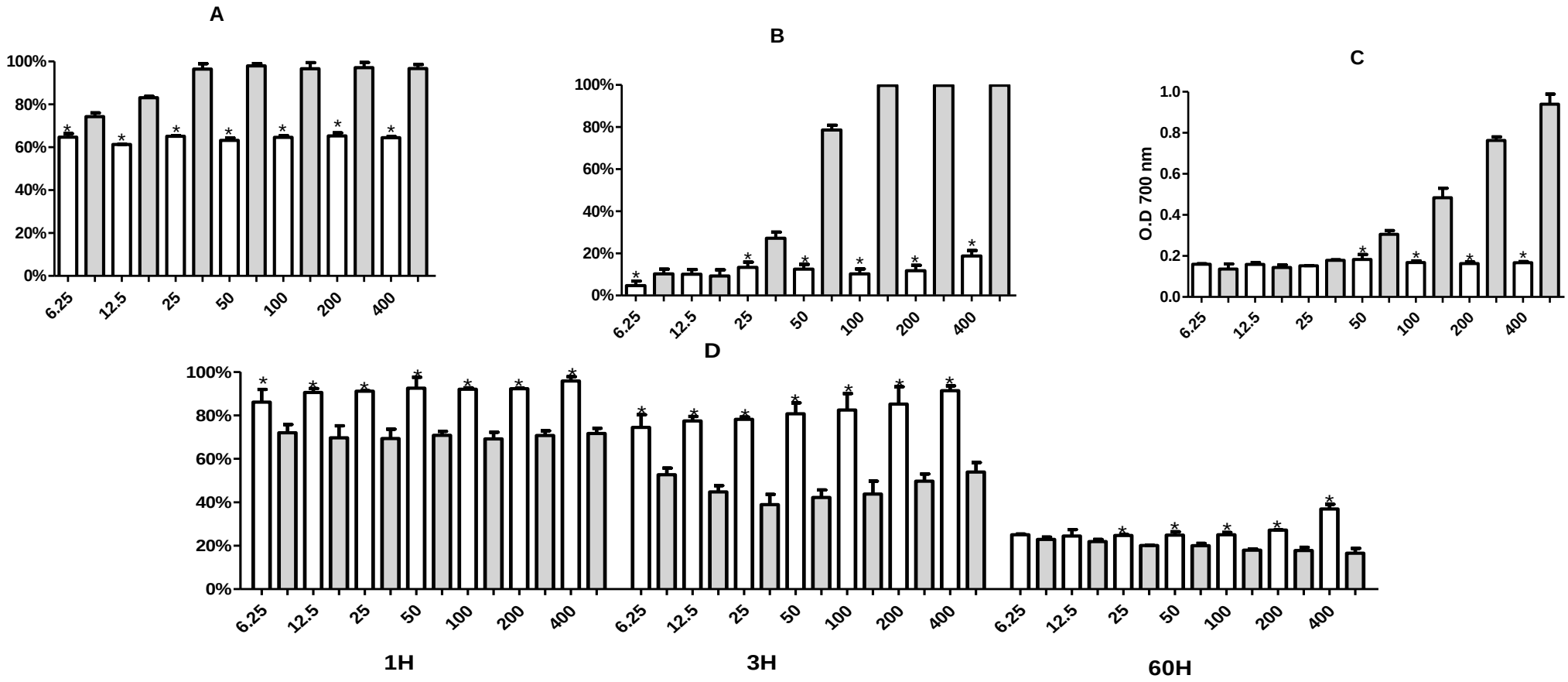
Fonte: elaborada pelo autor.

Table 3. Inhibition of planktonic growth of Gram-negative and Gram-positive bacteria and filamentous and yeasts fungi.

MICRORGANISMS	**ISOFLAVONE**		**ESSENTIAL OIL**	
BACTERIA	MIC (µg/mL)	MBC (µg/mL)	MIC (%)	MBC (%)
Staphylococcus aureus ATCC 25923	2000	N.I	2.5	5.0
S*taphylococcus epidermidis* ATCC 12228	2000	N.I	5.0	N.I
Escherichia coli ATCC 11303	N.I	N.I	N.I	N.I
Pseudomonas aeruginosa ATCC 10145	N.I	N.I	N.I	N.I
FUNGI	MIC (µg/mL)	MFC (µg/mL)	MIC (%)	MFC (%)
Trichophyton rubrum LABMIC 0207	156	312	1.25	2.5
Trichophyton rubrum LABMIC 0208	312	625	2.5	5.0
Trichophyton rubrum LABMIC 0209	625	1250	2.5	5.0
Trichophyton rubrum LABMIC 0210	1250	2500	N.I	N.I
Candida albicans ATCC 90028	N.I	N.I	N.I	N.I
Candida tropicalis LABMIC 0110	N.I	N.I	N.I	N.I
Candida parapsilosis LABMIC 0123	N.I	N.I	N.I	N.I
Candida krusei LABMIC 0124	N.I	N.I	N.I	N.I

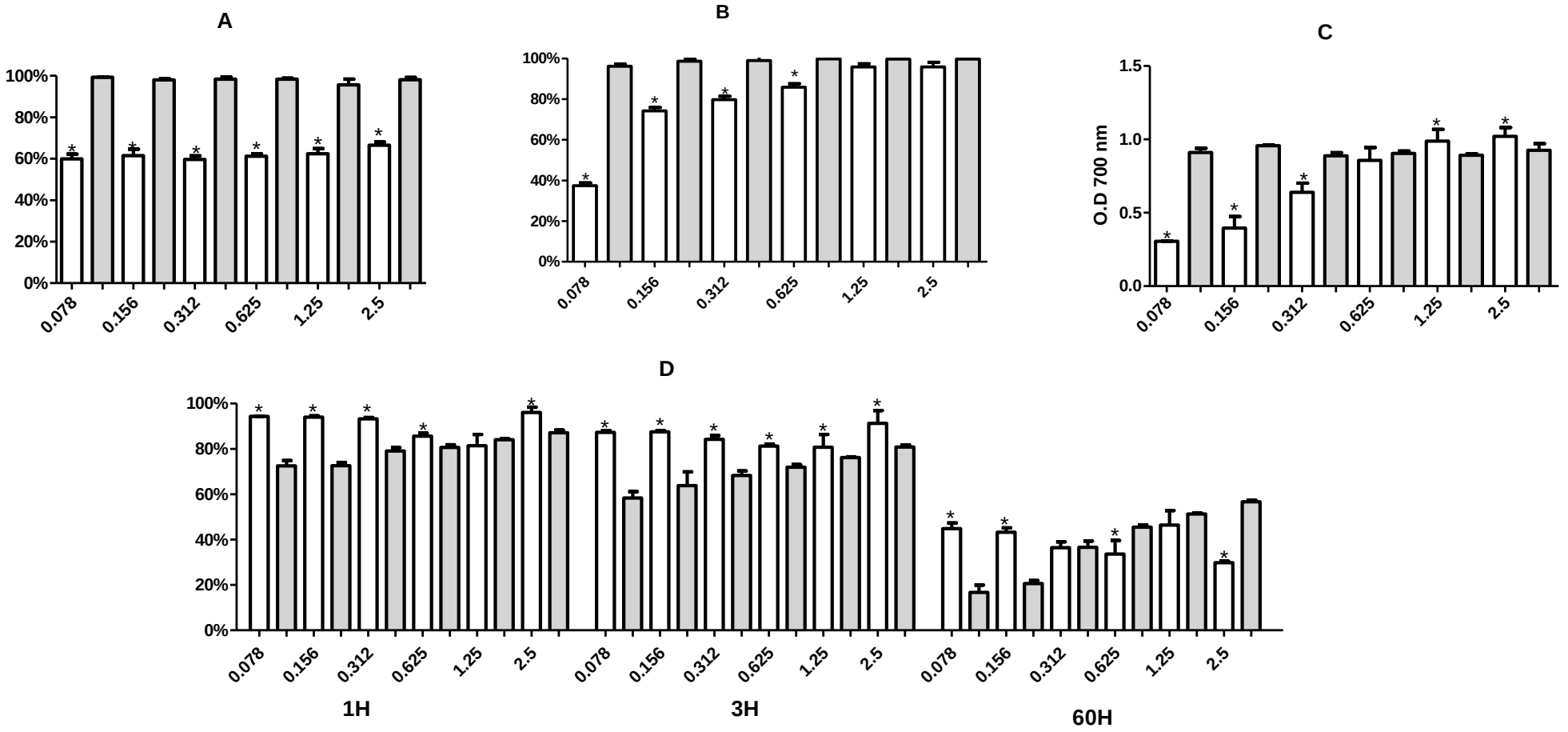
LABMIC- Microbiology Laboratory. ATCC- American Type Culture Collection. Fonte: elaborada pelo autor.

Figure 15. Antioxidant activity of the isoflavone from *Myroxylon peruiferum* L.f



Antioxidant effect of isoflavone at test DPPH (A), FRAP (B), FIC (C) and BCB (D). White column: sample; Gray column: positive control.***** Statistically significant in relation to the positive control, when $p \leq 0.05$. Fonte: elaborada pelo autor.

Figure 16. Antioxidant activity of the essential oil from *Myroxylon peruiferum* L.f



Antioxidant effect of the essential oil of *M. peruiferum* L.f at test DPPH (A), FRAP (B), FIC (C) and BCB (D). White column: sample; Gray column: positive control.

***** Statistically significant in relation to the negative positive, when $p \leq 0.05$. Fonte: elaborada pelo autor.

5 AVALIAÇÃO DA ATIVIDADE ANTIMICROBIANA E ANTIBIOFILME DO TRITERPENO ISOLADO DO EXTRATO ETANÓLICO DE FOLHAS DE *Combretum leprosum* E SEU EFEITO SINÉRGICO COM OS ANTIBIÓTICOS AMPICILINA E TETRACICLINA

RESUMO

A resistência aos antibióticos em bactérias geralmente é um fenômeno natural para adaptação a agentes antimicrobianos. Na literatura, pesquisas têm mostrado o potencial antibacteriano de espécies do gênero *Combretum. C. leprosum* é uma planta arbustiva e que apresenta em sua constituição o composto bioativo 3β-6β-16β-trihidroxilup-20(29)-eno (CLF1), capaz de promover atividades antibiofilme e antibacteriana. Deste modo, o objetivo do trabalho foi avaliar o efeito antibacteriano, antibiofilme e sinergismo do triterpeno 3β,6β,16β-trihidroxilup-20(29)-eno com antibióticos comuns no mercado, isolado das folhas de *C. leprosum*. A Concentração Inibitória Mínima e a Concentração Bactericida Mínima foram determinadas pelo método de microdiluição em caldo. A atividade moduladora foi avaliada pelo método de *checkerboard* com a interação das drogas comerciais, ampicilina e tetraciclina. Já a formação do biofilme foi avaliada através da quantificação de biomassa pelo método de coloração com cristal de violeta e enumeração de células viáveis, enquando que a atividade metabólica foi analisada pelo método XTT. As espécies bacterianas tiveram o seu crescimento planctônico comprometido por CLF1 numa concentração inibitória de 1,9 µg/mL e numa concentração bactericida entre 3,9 a 15,6 µg/mL. O triterpeno mostrou uma redução progressiva tanto na biomassa produzida pelos biofilmes quanto ao número de células viáveis. O CLF1 apresentou efeito sinérgico com ampicilina para *S. aureus* ATCC 25923, *S. aureus* ATCC 700698 e *S. epidermidis* ATCC 35984 e *S. epidermidis* ATCC 12228 e com tetracilina para *S. aureus* ATCC 25923, *S. aureus* ATCC 700698 e *S. epidermidis* ATCC 35984, já para *S. epidermidis* ATCC 12228 apresentou efeito antagônico. Em relação ao método XTT, as estirpes *S. aureus* ATCC 25923 e *S. aureus* ATCC 700698 apresentaram maiores reduções metabólicas do que *S. epidermidis* ATCC 12228 e *S. epidermidis* ATCC 35984. Deste modo, podemos considerar que o triterpeno 3β,6β,16β-trihidroxilup-20(29)-eno apresentou ação antibacteriana, antibiofilme e sinergismo. Com base nos resultados obtidos, o CLF1 pode ser considerado promissor no desenvolvimento de novos fármacos na prevenção de infecções associadas a bactérias Gram-positivas.

Palavras chaves: *Combretum leprosum*, triterpeno, sinergismo, biofilmes.

ABSTRACT

Resistance to antibiotics in bacteria is often a natural phenomenon for adaptation to antimicrobial agents. In the literature, research has shown the antibacterial potential of species of the genus *Combretum*. *Combretum leprosum* which is a shrubby plant and has the bioactive compound 3β-6β-16β-trihydroxylup-20(29)-eno (CLF1), capable of promote antibiofilm and antibacterial activities. Thus, the objective of the present study was to evaluate the antibacterial, antibiofilm and synergism effect of triterpene 3β, 6β, 16β-trihydroxyilup-20(29)-ene with commercially available. The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration were determined by broth microdilution method. The modulating activity was evaluated by the *checkerboard* method with the interaction of commercial drugs, ampicillin and tetracycline. The biofilm formation was evaluated through the quantification of biomass by the crystal violet staining (CV) and enumeration of viable cells (CFU), whereas the metabolic activity was analyzed by the XTT method. The bacterial species had their planktonic growth compromised by CLF1 at an inhibitory concentration of 1.9 μg/mL and at a bactericidal concentration of 3.9 to 15.6 μg/mL. The triterpene showed a progressive reduction in both the biomass produced by biofilms and the number of viable cells. CLF1 showed synergistic effect with ampicillin for *S. aureus* ATCC 25923, *S. aureus* ATCC 70069, *S. epidermidis* ATCC 35984 and *S. epidermidis* ATCC 12228 and with tetracylin for *S. aureus* ATCC 25923, *S. aureus* ATCC 700698 and *S. epidermidis* ATCC 35984, whereas for *S. epidermidis* ATCC 12228 presented antagonistic effect. In relation to the XTT method, strains *S. aureus* ATCC 25923 and *S. aureus* ATCC 700698 showed higher metabolic reductions than *S. epidermidis* ATCC 12228 and *S. epidermidis* ATCC 35984. Thus, we can consider that triterpene 3β, 6β, 16β -trihidroxilup-20(29)-eno showed antibacterial, antibiofilm and synergism action. Based on the results obtained, CLF1 may be considered a promising in the development of new drugs in the prevention of infections associated with gram-positive bacteria.

Keywords: *Combretum leprosum*. Triterpene. Synergism. Biofilms.

Introdução

A resistência aos antibióticos em bactérias geralmente é um fenômeno natural para adaptação a agentes antimicrobianos. Uma vez que as bactérias se tornam resistentes a algum antibiótico, elas passam essa característica para sua progênie através de transferência horizontal ou vertical. O uso indiscriminado e irracional de antibióticos nos dias de hoje levou

à evolução de novas estirpes resistentes de bactérias que são um pouco mais letais em comparação com a cepa parental (CHANDRA *et al*., 2017).

Nas últimas décadas, os estudos sobre as comunidades microbianas têm chamado a atenção para a análise das complexas interações que surgem entre microrganismos da mesma espécie e espécies distintas durante a formação de comunidades microbianas elaboradas denominadas biofilmes (PARROT *et al*., 2016). Biofilmes são comunidades microbianas caracterizadas por células aderidas a um substrato abiótico ou biótico e incrustadas numa matriz de substâncias poliméricas extracelulares (SPE), produzida pelos próprios microrganismos (HURLOW *et al*., 2015).

Além de auxiliar os microrganismos em sua adesão, a matriz é capaz de conferir-lhes maior resistência, provendo-lhes mecanismos adicionais de adaptação a fatores de estresse (presença de antibióticos, desenvolvimento da resposta imunológica do hospedeiro e a dessecação da comunidade microbiana) (PAUL; JEFFREY, 1985; STOODLEY *et al*., 2002; BEECH; SUNNER; HIRAOKA, 2005; FLEMMING; WINGENDER, 2010).

O uso de produtos naturais para propriedades medicinais é um mercado cada vez maior e, atualmente, bilhões de pessoas em paises em desenvolvimento do mundo estão utilizando plantas medicinais e outros produtos naturais como meio de tratamento de doenças infecciosas. (VUUREN; HOLL, 2017).

Combretum leprosum Mart. é uma planta arbustiva localizada nas regiões Norte e Nordeste do Brasil, conhecida popularmente de “mufumbo” ou “cipoaba”. Representante da família Combretaceae são compostas por 18 gêneros, sendo os de maiores representatividades o gênero *Combretum*, com certa de 370 espécies, (MCGAW *et al*., 2001; KATERERE *et al*., 2003). Usada na medicina popular como expectorante, sedativo, cicatrizante, agente anti-hemorrágico e antimicrobiano (LIRA *et al*., 2002; NUNES *et al*., 2009).

Facundo *et al*. (1993) isolaram e caracterizaram o metabólito secundário 3β,6β,16β-trihidroxilup-20(29)-eno obtido das flores de *C. leprosum* como sendo um triterpeno pentacíclico da classe dos lupanos. Extratos e compostos isolados a partir de *C. leprosum* têm demonstrado atividades antinociceptiva, antiulcerogênica, anticancerígena, antimicrobiana, leishmanicida e cicatrizante (PIETROVSKI *et al*., 2006; NUNES *et al*., 2009; VIAU *et al*., 2014; EVARISTO *et al*., 2014; 2017; TELES *et al*., 2015; NASCIMENTO-NETO *et al*., 2015).

Deste modo, o objetivo do trabalho foi avaliar o efeito antimicrobiano, antibiofilme e seu efeito sinérgico com os antibióticos ampicilina e tetraciclina do triterpeno isolado do extrato etanólico das folhas de *C. leprosum*.

Materiais e Métodos

Material vegetal e isolamento de CLF1

Folhas frescas de *C. leprosum* foram coletadas na localidade Salgado dos Machados, Sobral, CE. A exsicata da planta encontra-se arquivada no Herbário Prof. Francisco José de Abreu Matos da Universidade Estadual Vale do Acaraú, autenticada pelo Dr. E. B. Souza sob o tombo N° 4573. Para a obtenção do metabólito secundário 3β-6β-16β-trihidroxilup-20(209)-eno (CLF1), realizou-se as etapas previamente descritas por Evaristo *et al*. (2014).

Atividade antibacteriana

Microrganismos

Neste estudo foram utilizadas cepas de *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 700698, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus epidermidis* ATCC 35984, *Escherichia coli* ATCC 11303 e *Pseudomonas aeruginosa* ATCC 10145.

Condições de cultivo

As bactérias foram cultivadas em Agar Triptona de Soja (TSA; Liofilchem, Itália) e incubadas a 37 °C durante 24 horas. Após o crescimento uma colônia isolada foi removida e inoculada em 5 mL de Caldo de Triptona de Soja (TSB; Liofilchem, Itália) e incubada a 37 °C por 18-24 horas. Em seguida, a concentração de células de cada inóculo foi ajustada para 2 x 10^6 células/mL, através de turbidimetria (620 nm) e curvas de calibração previamente determinada para cada bactéria.

Concentração inibitória mínima (CIM) e Concentração Bactericida Mínima (CBM)

O efeito do CLF1 sobre o crescimento planctônico de bactérias foi determinado pelo método de microdiluição em caldo, utilizando placas de poliestireno de 96 poços. Anteriormente ao ensaio, o composto foi solubilizado em uma emulsão contendo TSB mais dimeltilsulfóxido 4% originando concentrações que variavam na placa de 31,2 a 0,45 µg/mL. Primeiramente, foram adicionados 100 L de meio TSB contendo as várias concentrações do CLF1 na microplaca e em seguida adicionado 100 µL de cada um dos microrganismos separadamente (2 x 10^6 células/mL). As placas foram então incubadas a 37 °C durante 24 horas. O crescimento dos microrganismos foi mensurado através da densidade óptica do conteúdo de cada poço, a um comprimento de onda de 620 nm, usando uma leitora de microplacas. A concentração inibitória mínima (CIM) foi considerada a menor diluição do

composto que não apresentou crescimento visível dos microrganismos. Para determinação do CBM, foram inoculados 10 µL dos poços que não houve um crescimento microbiano visível em placas de Petri com meio TSA. Foi considerado CBM a menor concentração capaz de inibir completamente o crescimento microbiano nas placas.

Sinergismo com antibiótico

O efeito do composto combinado com o antimicrobiano padrão foi determinado pela técnica do *checkerboard,* sendo este método utilizado para determinar a interação das drogas por meio do cálculo do Índice de Concentração Inibitória Fracional (ICIF). O ICIF é calculado pela adição da Concentração Inibitória Fracional (FIC) para cada composto testado, sendo definida como a adição dos valores de CIM de cada fármaco na combinação e CIM do mesmo produto sozinho (WHITE *et al*., 1996).

Nas soluções foram utilizados os produtos testados nas concentrações dos seus respectivos valores de CIM. Inicialmente 50 µL do meio TSB foram adicionados em todos os 96 poços da placa de microdiluição. Adicionaram-se então, na primeira linha 100 µL do composto e realizado a diluição seriada verticalmente. Na primeira coluna foram colocados 50 µL da ampicilina e tetraciclina em diferentes concentrações de acordo com o CIM. Finalmente, 100 µL do inóculo foram colocados em todos os poços. Foi utilizado como controle negativo o meio TSB com o inóculo. As placas foram incubadas a 37 ºC durante 24h.

De acordo com os resultados obtidos, os valores de ICIF $\leq$ 0,5 serão indicativos de efeito sinérgico, valores de ICIF > 0,5 e $\leq$ 1,0 serão indicativos de efeitos aditivos e valores de ICIF > 1,0 serão indicativos de efeito antagônico (LECHARTIER; HARTKOORN; COLE, 2012; ROSATO *et al*., 2007).

Atividade antibiofilme

Ensaio de inibição da formação de biofilme e biofilmes pré-formados

O ensaio foi realizado baseado nos testes de microdiluição seriada, coloração com cristal de violeta e unidades formadoras de colônias em placas de polietireno de 96 poços segundo Stepanovic *et al*. (2000) com algumas modificações de acordo com Vasconcelos *et al*., (2014). A preparação das placas para os testes foi similar ao procedimento usado no ensaio de CIM. Para avaliar a ação de CLF1 sobre biofilmes pré-formados, cada poço da placa de poliestireno foi preenchido com 100 µL de bactérias mais 100 µL de TSB em suspensão na concentração de $2x10^6$ UFC.mL^{-1} e incubadas a 37°C por 24h. Após o tempo de

incubação, o sobrenadante foi removido e substituído por 200 µL de meio de CLF1 nas diferentes concentrações por mais 24h.

Quantificação da biomassa

A quantificação da biomassa dos biofilmes foi determinada pelo método de coloração com cristal de violeta (CV). Após as 24 horas de incubação as placas foram lavadas com água destilada estéril por três vezes para a retirada das células planctônicas aderidas. Posteriormente os poços foram preenchidos com 200 µL de metanol por 5 minutos para fixação dos biofilmes. Adicionou-se, então, 200 µL de cristal de violeta 1% por mais 5 minutos. Em seguida, o excesso de corante foi removido e as placas lavadas com água destilada. O corante remanescente foi removido com ácido acético a 33% e então a biomassa foi quantificada através da medição da densidade óptica a 590nm (DO_{590}) com o auxílio de um leitor de microplacas (SpectraMax i3).

Enumeração de células viáveis dos biofilmes

Após a formação dos biofilmes, os sobrenadantes foram removidos e as placas foram lavadas três vezes com água destilada estéril para remoção das células planctônicas aderidas. Posteriormente, cada poço foi preenchido com 200 µL de água destilada estéril e submetido ao banho ultrassônico (Cristófoli/EQM-CF) por 5 minutos para o desprendimento das células incrustadas nos biofilmes. O conteúdo dos poços foi retirado e diluições seriadas de cada suspensão obtida foram plaqueadas em TSA. As placas foram então incubadas por 24 horas a 37 °C e o número de unidades formadoras de colônias (UFC) por unidade de área dos poços da placa de microtitulação foram enumerados ($logUFC/cm^2$).

Viabilidade celular da inibição dos biofilmes

A atividade metabólica da inibição dos biofilmes na presença e ausência das substâncias foi determinada através da aplicação do corante XTT (2,3-bis-[2-methoxy-4-nitro-5- sulfophenyl]-2H-tetrazolium-5-carboxanilide). Para tal, foram adicionados 100 µL de meio TSB contendo as várias concentrações do CLF1 na microplaca e em seguida adicionado 100 µL de cada um dos microrganismos separadamente (2×10^6 células/mL). As microplacas foram então incubadas a 37 °C durante 24 horas. Após este período, as placas foram lavadas duas vezes com água destilada e adicionados 200 µL de XTT e incubadas a 37 ºC na ausência

de luz durante 3h. Após este tempo, a absorbância dos poços foi mensurada a um comprimento de onda de 490 nm utilizando espectrofotômetro.

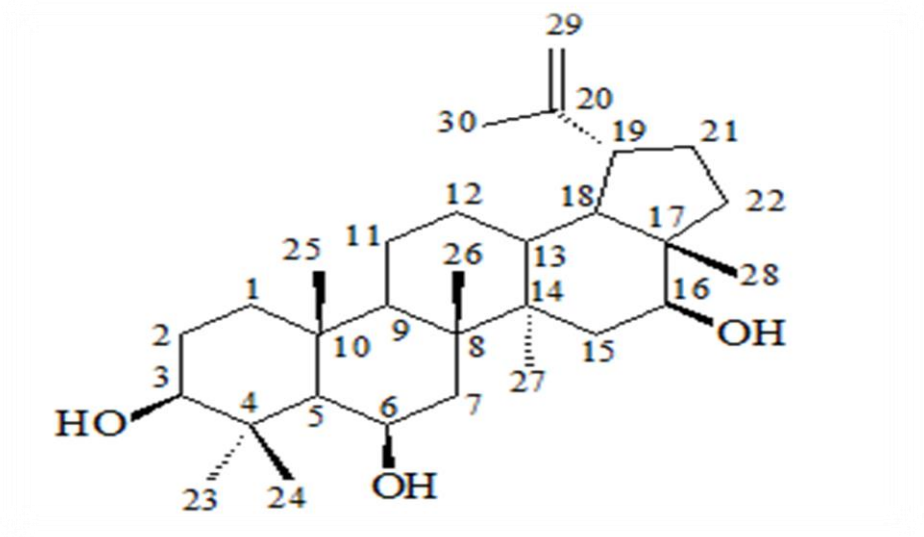
Análise estatística

Todos os testes foram realizados em triplicatas e com nível de significância $p<0,01$. Para os ensaios antimicrobianos a diferença entre as médias das triplicatas foi verificada através da aplicação do teste One-way ANOVA com Bonferroni pós-teste, executados com o auxílio do programa GraphPad Prism versão 5.0 para Windows (San Diego, California, USA).

Resultados e Discussão

De acordo com os dados obtidos e com base na análise comparativa dos dados de RMN ^{13}C registrados na literatura, a substância isolada do extrato etanólico das folhas de *C. leprosum*, tratava-se do triterpeno de esqueleto lupano de nome 3β-6β-16β-trihidroxilup-20(29)-eno. (Figura 17)

Figura 17: 3β-6β-16β-trihidroxilup-20(29)-eno isolado do extrato etanolico da folha de *C. leprosum.*



Fonte: Facundo *et al*., (1993)

Os resultados da atividade antibacteriana estão apresentados na tabela 4 e mostraram que o CLF1 foi capaz de inibir o crescimento microbiano de *S. aureus* ATCC 25923, *S. aureus* ATCC 700698, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 35984 com concentração inibitória mínima de 1,9 µg/mL. Em relação aos resultados de CBM, CLF1 demonstrou atividade sobre *S. aureus* ATCC 25923 (3,9 $\mu g.mL^{-1}$), *S. aureus* ATCC 700698 (15,6 $\mu g.mL^{-1}$), *S. epidermidis* ATCC 12228 (7,8 $\mu g.mL^{-1}$) e *S. epidermidis* ATCC 35984

(15,6 μg.mL^{-1}). Em relação às bactérias Gram-negativas *E. coli* ATCC 11303 e *P. aeruginosa* ATCC 10145 não se mostraram susceptíveis a ação biológica da substância.

A membrana exterior presente em bactérias Gram-negativas pode constituir uma barreira física contra a permeabilidade de metabólitos secundários, inibindo assim o efeito antimicrobiano do composto testado. Por este motivo, os demais experimentos foram realizados apenas com as bactérias Gram-positivas.

Vale ressaltar que para a ampicilina e tetraciclina, a concentração de CIM e CBM de *S. aureus* ATCC 700698 foi maior do que a do composto mostrando assim, que o CLF1 foi mais eficaz do que a própria droga comercializada.

De acordo com Evaristo *et al*., (2017) o CLF1 apresentou CIM de 3,9 μg/mL e CBM de 15,6 μg/mL para ambas as cepas de *Streptococcus mutans* ATCC 700610 e *Streptococcus parasanguinis* ATCC 903, concentrações maiores do que encontradas neste trabalho.

Estudo realizado por Angeh *et al*. (2007), com os triterpenos pentacíclicos 1α,3β-dihidroxi-12-oleanen-29-oico,1-hidroxi-12-olean-30-oico, 3,30-dihidroxil-12-oleanen-22-ono, 1,3,24-trihidroxyl-12-olean-29-oico e o 1α,3β,23-trihidroxi-12-oleanen-29-oico isolados das folhas de *C. imberbe*, demostraram atividade antibacteriana sobre *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 e *Enterococcus faecalis* ATCC 29212 com concentrações que variaram de 16 a ≥250 μg/mL.

Já Gossan *et al*., (2016) isolaram um novo triterpenoide pentacíclico 28-O-β-Dglucopiranosil-2α,3β,21β,23-tetra-hidroxioleano-18-en-28-oato das raízes de *C. racemosum*, exibindo atividade antibacteriana contra *S. aureus*, *E. coli* e *E. faecalis* (CIMs nas concentrações de 64 e 256 μg/mL).

O ácido ursólico e seus derivados, o ácido 3p-acetoxi-urs-12-en-28-oic e o ácido 3p-formiloxi-urs-12-en-28-oic apresentaram atividade antibacteriana sobre diferentes bacterianas de *Staphylococcus aureus* (ATCC 12692, 12624 e 6538), *Bacillus cereus* ATCC 33018, *Escherichia coli* (ATCC 25922 e 27), *Pseudomonas aeruginosa* ATCC 15442, *Aeromonas caveae* ATCC 15468, *Klebsiella pneumoniae* ATCC 10031, *Shigella flexneri* ATCC 12022, *Vibrio colareae* ATCC 15748 e *Listeria monocytogenes* ATCC 19117 com concentrações inibitórias mininas de 32 a ≥1024 μg/mL (NASCIMENTO *et al*., 2014).

A associação de agentes antimicrobianos pode ser usada para aumentar o espectro de ação, para precaver a emergência de mutantes resistentes e promover o sinergismo entre duas ou mais drogas. Quando se retrata sobre a atividade sinérgica, os resultados estão dispostos nas tabelas 5 e 6. O ensaio de combinação do CLF1 com os antibióticos padrões ampicilina e tetraciclina mostrou que houve uma redução nos valores de CIM em todas as cepas testadas e

também dos CIMs dos antibióticos. A partir dos valores, foi possível calcular o índice de concentração inibitória fracionária (ICIF) na qual mostrou um efeito sinérgico para *S. aureus* ATCC 25923, *S. epidermidis* ATCC 35984 e *S. aureus* ATCC 700698 e efeito antagônico para *S. epidermidis* ATCC 12228.

Wang *et al*., (2016) analisaram os triterpenos ácido oleanólico e ácido ursólico na presença da combinação com ampicilina e tetraciclina, apresentando efeito sinérgico para as cepas de *Bacillus cereus* (ATCC 9139), *S. aureus* sensível à meticilina (MSSA, ATCC 29213), *S. aureus* resistente à meticilina (MRSA, ATCC 43300) e *Listeria monocytogenes* (ATCC 7644) apenas para ácido ursólico com tetraciclina, e efeito indiferente para *E. faecalis* (ATCC 29212).

Kurek *et al*., (2012) relatamram-se que o ácido ursólico aumentou a susceptibilidade de *Staphylococcus aureus*, *Staphylococcus epidermidis* e *Listeria monocytogenes* aos antibióticos β-lactâmicos, ampicilina e oxacilina, mas não há nenhum relató na literatura sobre este composto agindo sinergicamente com os antibióticos aminoglicosídeos neomicina, amicacina, canamicina e gentamicina. (NASCIMENTO *et al*., 2014)

A associação do ácido ursólico e seus derivados, o ácido 3p-acetoxi-urs-12-en-28-oic e o ácido 3p-formiloxi-urs-12-en-28-oic juntamente com os antibióticos neomicina, amicacina, canamicina e gentamicina apresentaram atividade sinergica sobre diferentes cepas de *Staphylococcus aureus* (ATCC 12692 e 6538), *Bacillus cereus* ATCC 33018, *Escherichia coli* (ATCC 25922 e 27), *Pseudomonas aeruginosa* ATCC 15442, *Aeromonas caveae* ATCC 15468, *Klebsiella pneumoniae* ATCC 10031, *Shigella flexneri* ATCC 12022, *Vibrio colareae* ATCC 15748 e *Listeria monocytogenes* ATCC 19117 (NASCIMENTO *et al*., 2014)

Como mostrado na figura 18 sobre a inibição da formação dos biofilmes, CFL1 reduziu significativamente a biomassa dos biofilmes de todas as bactérias testadas neste estudo. As concentrações variaram de 31,2 a 0,45 µg/mL na qual o metabolito secundário reduziu a quantidade de biomassa a valores entre 88 a 100%. Corroborando com os resultados da quantificação de biomassa, os resultados de enumeração de células viáveis dos biofilmes demonstraram que o CFL1 reduziu as células bacterianas dos biofilmes de todas as espécies entre 1 a 4,2 Log_{10}UFC/mL.

Em relação à quantificação de biomassa dos biofilmes pré-formados, houve redução de 70 a 100% nos biofilmes de *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228 e *S. epidermidis* ATCC 35984. Já para *S. aureus* ATCC 700698 não houve redução significativa. Quando se refere ao número de células viáveis dos biofilmes, o composto reduziu o número de UFC's entre 0,6 a 3,3 Log_{10}UFC/mL. (Figura 19)

Em relação ao método colorimétrico baseado no XTT, que mede a atividade metabólica dos biofilmes formados, as estirpes de *S. aureus* ATCC 25923 e *S. aureus* ATCC 700698 apresentaram maiores reduções metabólicas na presença do CLF1 do que *S. epidermidis* ATCC 12228 e *S. epidermidis* ATCC 35984 chegando a inibição de quase 100 % (Figura 20).

Este método colorimétrico basea-se na redução do sal XTT pelas células metabolicamente ativas e tem sido empregado para determinar o efeito das drogas sobre células em biofilme, já que este métoto possibilita quantificar apenas as células viáveis (PIERCE *et al*., 2008; MELO *et al*., 2011; MAIOLO *et al*., 2014).

Os mecanismos de ação com metabólitos bioativos, flavonoides e triterpenos, mostram que a atividade antibacteriana pode estar relacionada a danos na membrana plasmática, (perfuração e/ou redução na fluidez na membrana), inibição da síntese dos ácidos nucléicos (inibição da topoisomerase) e inibição do metabolismo energético (causado por inibição da redutase e NADH-citocromo c) (CUSHNIE; LAMB, 2005; PLAPER *et al*., 2003; TSUCHIYA; IINUMA, 2000; BERNARD *et al*., 1997; PARK; AHN; KOOK, 2015). Na literatura, diversos relatórios têm mostrado ação inibitória de triterpenos pentaciclicos sobre as enzimas topoisomerases I e II de células eucariontes (MORIARITY *et al*., 1998; MA *et al*., 2000; WADA *et al*., 2001).

Evaristo *et al*., (2017) mostraram o mecanismo de ação do triterperno CLF1 sobre a membranda celular das bactérias planctônicas e em forma de biofilmes. Outros ensaios realizados para determinação do mecanismo de ação do ácido ursólico e ácido oleanólico, mostraram que estes triterpenos podem comprometer a síntese de peptidioglicanos através da produção de proteínas envolvidas na parede celular, na hidrólise ou em alterações que permitam diminuir a ligação cruzada do peptidiglicano otimizando sua desestabilização e portanto, ocasionando um comprometimento da integridade da parede celular (KORSAK; POPOWSKA; MARKIEWICZ, 2005; KUREK *et al*., 2010).

Conclusão

Com base nos dados obtidos neste trabalho, constatou-se que CLF1 apresenta atividade antibacteriana, antibiofilme e sinergismo com os antibióticos padrões ampicilina e tetraciclina sobre espécies do gênero *Staphylococcus*, responsáveis pelo desenvolvimento de patologias em humanos. Considerando-se dessa forma, como um possível insumo biotecnológico, com potencial terapêutico no tratamento de patologias associadas a biofilmes formados por *S. aureus* e *S. epidermidis.*

REFERÊNCIAS

ANGEH, J. E. *et al*. Antimicrobial and anti-inflammatory activity of four known and one new triterpenoid from *Combretum imberbe* (Combretaceae). **J. Ethnopharmacol**. v. 1, n. 1, p. 56-60, 2007.

BEECH, B. I.; SUNNER, J. A.; HIRAOKA, K. Microbe-surface interactions in biofouling and biocorrosion processes. **Int. Microbiol**., v. 8, p. 157-168, 2005.

BERNARD *et al*. Gly-cosylated flavones as selective inhibitors of topoisomerase IV. **Antimicrob. Agents Ch**., v. 41, n. 5, p. 992-998,1997.

CHANDRA *et al*. Antimicrobial Resistance and the Alternative Resources with Special Emphasis on Plant-Based Antimicrobials-A Review. **Plants (Basel)**, v. 6, n.2, p.1-11, 2017.

CUSHNIE, T. P. T.; LAMB, A. J. Antimicrobial activity of flavonoids. **International Journal of Antimicrobial Agent**. v. 26, n. 5, p. 343–356, 2005.

EVARISTO, F. F. V. *et al*. Antibacterial effect on mature biofilms of oral streptococci and antioxidant activity of 3β,6β,16β-trihydroxylup-20(29)-ene from *Combretum leprosum*. **Medicinal Chemistry Research**, v. 1, p. 1, 2017.

EVARISTO, F. F. V. *et al*. Antimicrobial Effect of the Triterpene 3β,6β,16β-Trihydroxylup-20(29)-ene on Planktonic Cells and Biofilms from Gram Positive and Gram Negative Bacteria. **Bio**. **Med. Research International**, v, 2014, p. 1-7, 2014.

FACUNDO, V. A. *et al*. "Triterpenes and flavonoids from *Combretum leprosum*, " **Phytochemistry**, v. 32, n 2, p. 411-415, 1993.

FLEMMING, H. C.; WINGENDER, J. The biofilm matrix. **Nat. Rev. Microbiol**., v. 8, p. 623-633, 2010.

GOSSAN, D. P. A. Antibacterial and cytotoxic triterpenoids from the roots of *Combretum racemosum*. **Fitoterapia**, v. 110, p. 89-95, 2016.

HURLOW, J. *et al*. Clinical biofilms: a challenging frontier in wound care. **Adv Wound Care**, v. 4, n. 5, p. 295-301, 2015.

KATERERE, D. R. *et al*. Antimicrobial activity of pentacyclic triterpenes isolated from African Combretaceae. **Phytochemistry**, v. 63, p. 81-88, 2003.

KORSAK, D.; POPOWSKA, M.; MARKIEWICZ, Z. Analysis of the murein of *Listeria monocytogenes* EGD mutant lacking functional penicillin binding protein 5 (PBP5). **Pol. J. Microbiol**., v. 54, p. 339-342, 2005.

KUREK, A. *et al*. Modulation of antibiotic resistance in bacterial pathogens by oleanolic acid and ursolic acid. **Phytomedicine**, v.19, p. 515-519, 2012.

KUREK, A. *et al*. Oleanolic acid and ursolic acid affect peptidoglycan metabolism in *Listeria monocytogenes*. **Antonie van Leeuwenhoek**, v. 97, p. 61-68, 2010.

LECHARTIER, B.; HARTKOORN, R. C.; COLE, S. T. *In vitro* combination studies of be nzothiazinone lead compound BTZ043 against *Mycobacterium tuberculosis*. **Antimicrobial agents and chemotherapy**, v. 56, n. 11, p. 5790-5793, 2012.

LIRA, S. R. D. *et al*. Preliminary studies on the analgesic properties of the ethanol extract of *C. leprosum*. **Pharm Biol**., V. 40, p. 213-5, 2002.

MA, Z. Z. *et al*. Three New Triterpenoids from *Peganum nigellastrum*. **Nat. Prod**., v. 63, p. 390-392, 2000.

MAIOLO, E. M. Activities of fluconazole, caspofungin, anidulafungin, and amphotericin B on planktonic and biofilm *Candida* species determined by microclorimetry. **Antimicrobial Agents and Chemotherapy**. v.58, n.5, p.2709-2717, 2014.

MELO, A. S. *et al*. Biofilm production and evaluation of antifungal susceptibility amongst clinical *Candida* spp. Isolates, including strains of the *Candida parapsilosis* complex. **Medical Mycology**. v.49, p.253-262, 2011.

MORIARITY, D. M. *et al*. Lupeol is the cytotoxic principle in the leaf extract of *Dendropanax* cf. querceti. **Planta Med**., v. 64, p. 370-372, 1998.

NASCIMENTO, P. G. *et al*. Antibacterial and antioxidant activities of ursolic acid and derivatives. **Molecules**, v. 19, p. 1317-1327, 2014.

NASCIMENTO-NETO, L. G. Effect of the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene isolated from the leaves of *Combretum leprosum* Mart. on cutaneous wounds in mice. **Journal of Ethnopharmacology**, v. 171, p. 116-120, 2015.

NUNES, P. H. M.; CAVALCANTI, P. M. S.; GALVAO, S. M. P.; MARTINS, M. C. C. "Antiulcerogenic activity of *Combretum leprosum*." **Pharmazie**, v. 64, n. 1, p. 58-62, 2009.

PARROT, D.; *et al.* Review-Lichen-Associated Bacteria as a Hot Spot of Chemodiversity: Focus on Uncialamycin, a Promising Compound for Future Medicinal Applications. **Planta Med.** v. 82, n. 13, p.1143-1152, 2016.

PARK, S. N; AHN, S. J; KOOK, J. K. Oleanolic acid and ursolic acid inhibit peptidoglycan biosynthesis in *Streptococcus mutans* UA159. **Brazilian Journal of Microbiology**, v. 46, p. 613-617, 2015.

PAUL, J. H.; JEFFREY, W. H. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in Vibrio proteolytica. **Appl Environ Microbiol**., v. 50, p. 431-437, 1985.

PIERCE, C. G. A simple and reproducible 96 well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. **Nature Protocols**. v.3, n.9, 1494-1500, 2008.

PIETROVSKI, E. F. *et al*. Antinociceptive properties of the ethanolic extract and of the triterpene 3β,6β,16β -trihidroxilup-20(29)-ene obtained from the flowers of *Combretum leprosum* in mice. **Pharmacology, Biochemistry and Behavior**, v. 83, p. 90-99, 2006.

PLAPER, A. *et al*. Characterization of quercetin binding site on DNA gyrase. **Biochem. Biophys. Res. Commun**., v. 306, n. 2, p. 530-336, 2003.

STOODLEY, P. *et al*. Biofilm material properties as related to shear-induced deformation and detachment phenomena. **J. Ind. Microbiol. Biotechnol**, v. 29, n. 6, p. 361-367, 2002.

TSUCHIYA, H.; IINUMA, M. Reduction of membrane fluidity by antibacterial sophoraflavanone G isolated from *Sophora exigua*. **Phytomedicine. Stuttgart**. v. 7, n. 2, p. 161-165, 2000.

VASCONCELOS *et al*. Antibacterial and antioxidant activities of aerriobtusone A isolated from *Lonchocarpus obtusus*. **Biomed res Int**, v. 2014, p. 1-9, 2014.

VIAU. C. M. *et al*. The natural triterpene 3β,6β,16β-trihydroxy-lup-20 (29)-ene obtained from the flowers of *Combretum leprosum* induces apoptosis in MCF-7 breast cancer cells. **BMC Complementary and Alternative Medicine**, v. 14, n. 280, p. 1-12, 2014.

VUUREN; HOLL, Antimicrobial natural product research: A review from a South African perspective for the years 2009-2016. **Journal of Ethnopharmacology**, n. 208, p. 236–252, 2017.

WADA, S-I.; IIDA, A.; TANAKA, R. Screening of Triterpenoids Isolated from *Phyllanthus flexuosus* for DNA Topoisomerase Inhibitory Activity. **Nat. Prod**,. v. 64, p. 1545-1547, 2001.

WANG. *et al*. Antibacterial and Synergistic Activity of Pentacyclic Triterpenoids Isolated from *Alstonia scholaris*. **Molecules**, v. 21, n.139, p. 1-11, 2016.

WHITE, R.L.; BURGESS, D.S.; MANDURU, M.; BOSSO, J.A. Comparasion of three different in vitro methods of detecting synergy: Time-kill, *checkerboard* and E-test. **Antimicrobial agents and chemotherapy.** v. 40, p.1914-1918, 1996.

Tabela 4. Concentração Inibitória Mínima (MIC) e Concentração Bactericida Mínima (CBM) do CLF1 e da ampicilina e tetraciclina.

Bactérias	**CLF1 (µg/mL)**		**ANTIBIÓTICO (µg/mL)**			
			Tetraciclina		Ampicilina	
	CIM	CBM	CIM	CBM	CIM	CBM
S. aureus ATCC 25923	1,9	3,9	0,39	3,12	0,19	-
S. aureus ATCC 700698	1,9	15,6	50	50	25	25
S. epidermidis ATCC 35984	1,9	15,6	0,78	12,5	1000	1000
S. epidermidis ATCC 12228	1,9	7,8	0,19	3,12	1,56	1,56
E. coli ATCC 11303	-	-	0,39	25	0,39	0,78
P. aeruginosa ATCC 10145	-	-	-	-	-	-

-sem CIM e/ou CBM. Fonte: elaborada pelo autor.

Tabela 5. Sinergismo do CLF1 com a ampicilina sobre as bactérias Gram- positivas

Microrganismo	**COMPOSTO**		**ANTIBIÓTICO**			**EFEITO**
	CLF1		**Ampicilina**			
	CIM µg/mL (sozinho)	CIM µg/mL (combinado)	CIM µg/mL (sozinho)	CIM µg/mL (combinado)	ICIF	
S. aureus ATCC 25923	1,9	0,0009	0,19	0,023	0,12	Sinergismo
S. aureus ATCC 700698	1,9	0,0009	25	12,5	0,50	Sinergismo
S. epidermidis ATCC 35984	1,9	0,00005	1000	250	0,25	Sinergismo
S. epidermidis ATCC 12228	1,9	0,003	1,56	0,39	0,25	Sinergismo

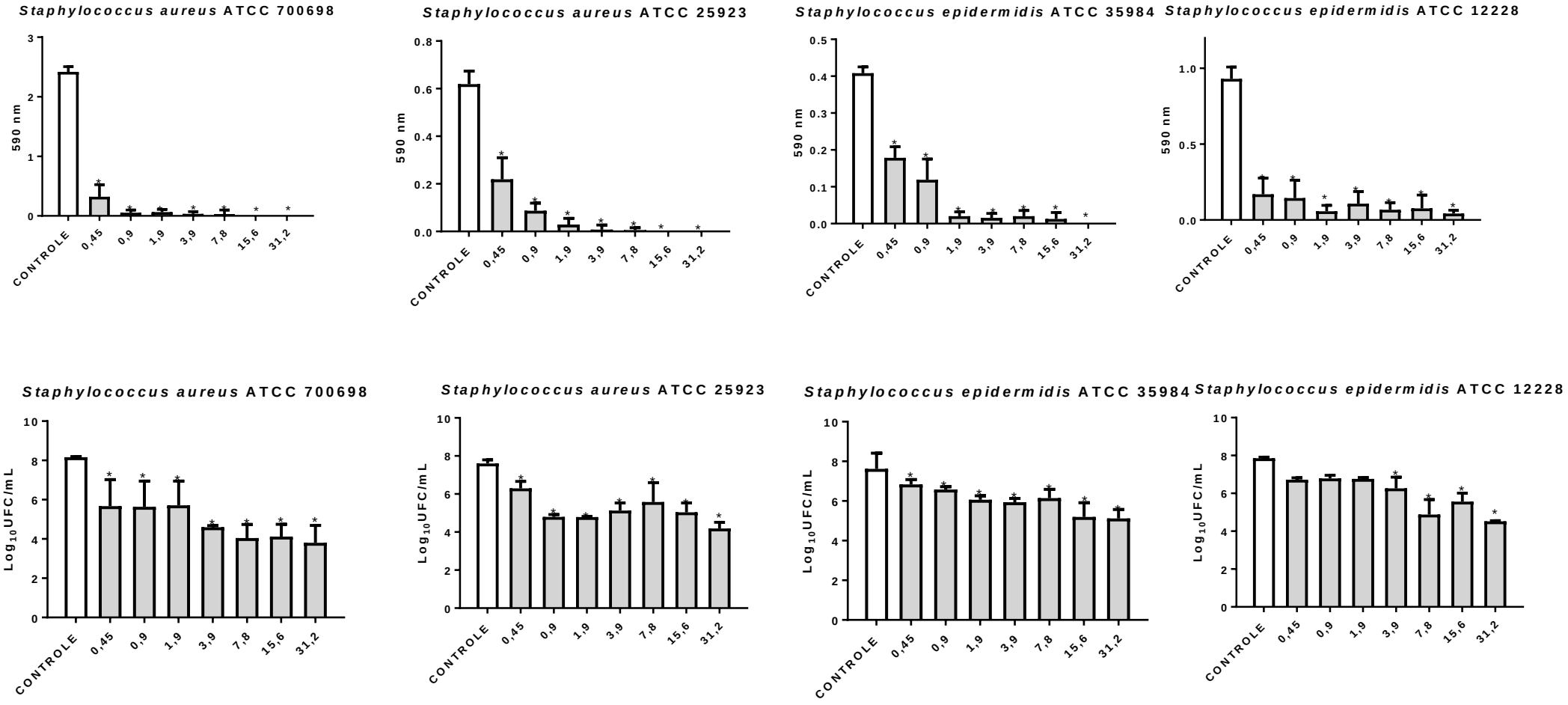
Fonte: elaborada pelo autor.

Tabela 6. Sinergismo do CLF1 com a tetraciclina sobre as bactérias Gram- positivas

Microrganismo	**COMPOSTO**		**ANTIBIÓTICO**			**EFEITO**
	CLF1		**Tetraciclina**			
	CIM µg/mL (sozinho)	CIM µg/mL (combinado)	CIM µg/mL (sozinho)	CIM µg/mL (combinado)	ICIF	
S. aureus ATCC 25923	1,9	0,0004	0,39	0,19	0,48	Sinergismo
S. aureus ATCC 700698	1,9	0,0004	50	25	0,50	Sinergismo
S. epidermidis ATCC 35984	1,9	0,0004	0,78	0,39	0,50	Sinergismo
S. epidermidis ATCC 12228	1,9	0,029	0,19	0,19	1,1	Antagônico

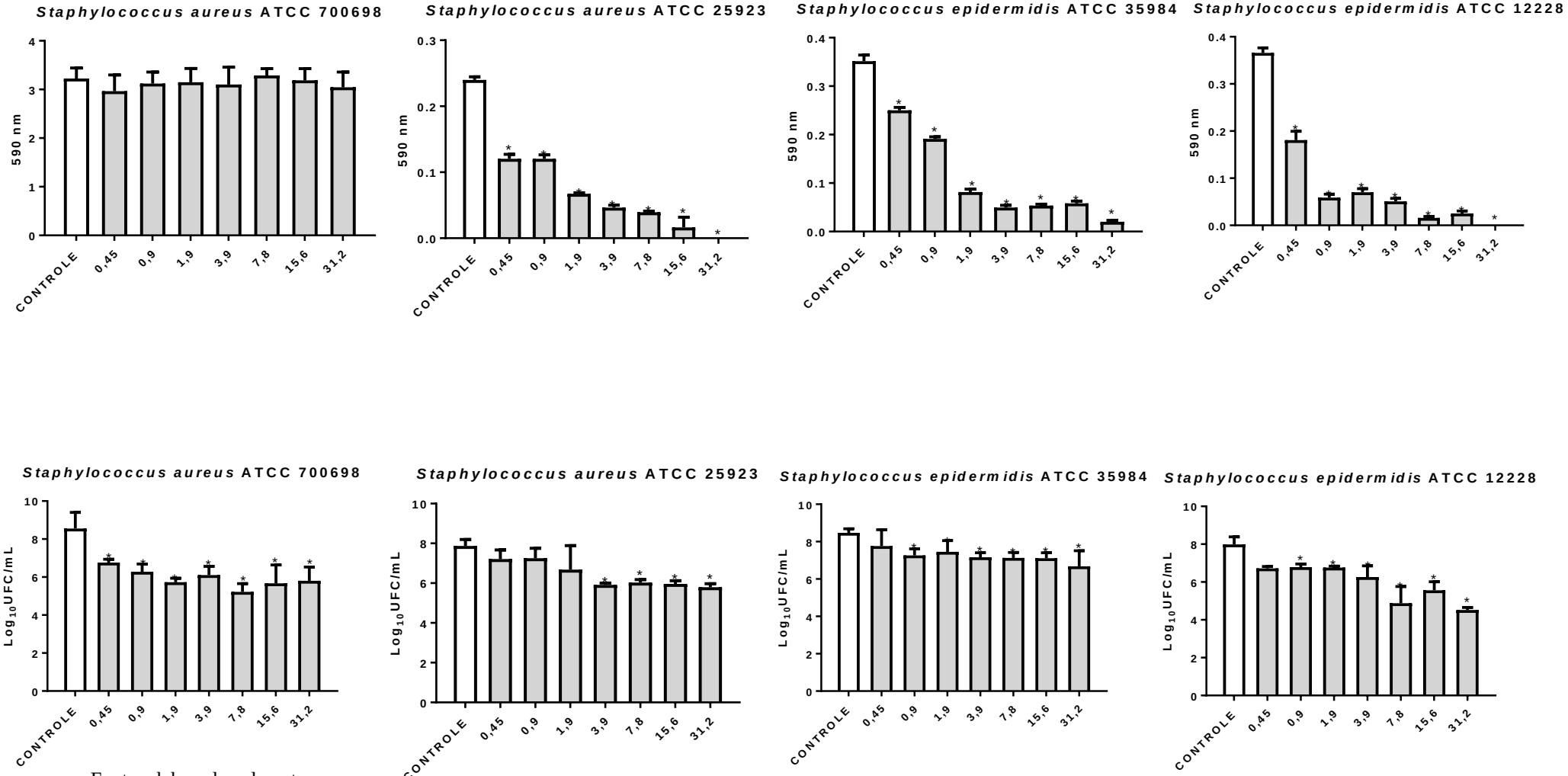
Fonte: elaborada pelo autor.

Figura 18- Efeito antimicrobiano do CLF1 em diferentes concentrações sobre a formação da biomassa e da enumeração de células viáveis dos biofilmes Gram-positivas.



Fonte: elaborada pelo autor.

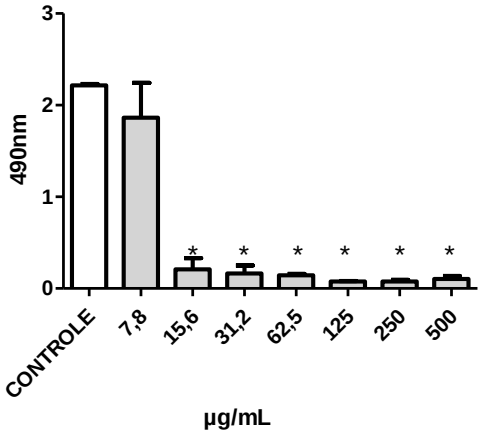
Figura 19- Efeito antimicrobiano do CLF1 em diferentes concentrações sobre a formação da biomassa e da enumeração de células viáveis dos biofilmes pré-formados de Gram-positivas.



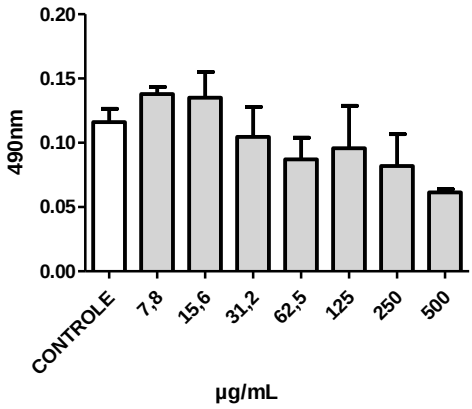
Fonte: elaborada pelo autor.

Figura 20. Determinação da atividade metabólica da inibição dos biofilmes. ***** Significativamente diferente (p <0,01) em comparação com o grupo controle.

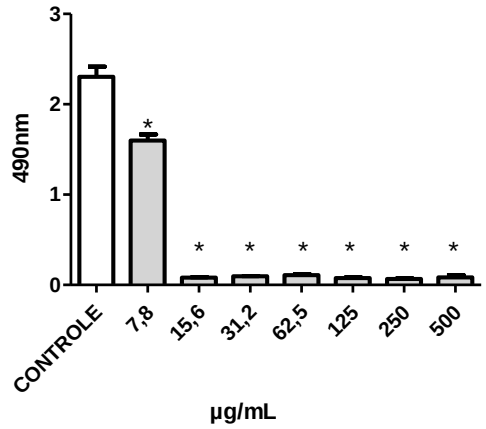
Staphylococcus aureus **ATCC 25923**



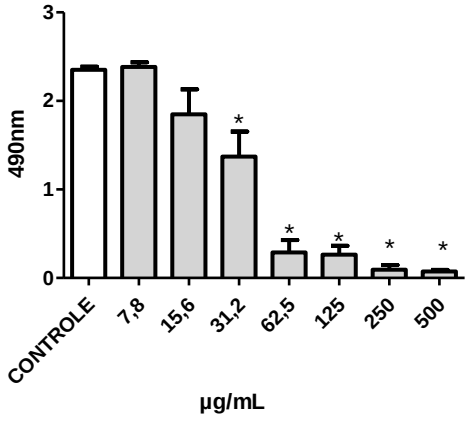
Staphylococcus epidermidis **ATCC 12228**



Staphylococcus aureus **ATCC 700698**



Staphylococcus epidermidis **ATCC 35984**



Fonte: elaborada pelo autor.

PERSPECTIVAS

Diante das evidências, os vegetais são fontes de metabólitos secundários e óleos essenciais com utilidade em tratamento de várias patologias. Nesta perspectiva, o uso de espécies do gênero *Myroxylon* na medicina popular parece apropriado, no entanto, ainda, há necessidade de mais estudos em modelos *in vitro* e *in vivo* que comprovem o uso desses vegetais como antimicrobiano, anti-inflamatório, antiparasitário, anticancerígeno, antioxidante e cicatrizante. Este presente trabalho constatou-se que a isoflavona 7-hidroxi-4', 6-dimetoxi-isoflavona isolada da casca assim como o óleo essencial da folha de *M. peruiferum* L.f, apresentaram atividades antibacteriana sobre bactérias Gram-positivas, antifúngica para fungos dermatófitos e antioxidante. Já o triterpeno 3β,6β,16β-trihidroxilupe-20(29)-eno, isolado das folhas de *C. leprosum,* têm potencial antibacteriano, antibiofilme e efeito sinérgico com ampicilina e tetraciclina, considerando-se como um possível insumo biotecnológico no tratamento de patologias associadas a biofilmes formados por *S. aureus* e *S. epidermidis*. Contudo, este estudo aponta a necessidade de novas investigações relativas aos mecanismos de ação das amostras através da microscópia eletrônica de varredura assim como a determinação da curva de morte.

REFERÊNCIAS

ABOELSOUD, N. H. Herbal medicine in ancient Egypt. **Journal of Medicinal Plants Research,** v. 4, n. 2, p. 82-86, 2010.

AGRA, M. F. *et al.* Medicinal and poisonous diversity of the flora of "Cariri Paraibano", Brazil. **J Ethnopharmacol,** v. 111, n. 2, p. 383-95, 2007.

AGUIAR, C. L. *et al*. Analysis of isoflavonoids from leguminous plant extracts by RPHPLC/DAD and electrospray ionization mass spectrometry. **International Journal of Food Sciences and Nutrition**. v. 58, p. 116-124, 2007.

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. I Morfologia, histologia de órgãos secretores e obtenção de bálsamo. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 9, p.115-133, 1971.

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. II Caracterização física do bálsamo e análise qualitativa de alguns componentes. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 10, p. 73-96, 1972a

AKISUE, G. Secreções de *Myroxylon peruiferum* L.f. III Caracterização física e química do óleo essencial do bálsamo e análise qualitativa de alguns componentes. **Revista de Farmácia e Bioquímica da Universidade de São Paulo**, v. 10, p. 151-165, 1972b.

AKTAS, Z. *et al*. Resistance to macrolide, lincosamide and streptogramin antibiotics in staphylococci isolated in Istanbul, Turkey. **J Microbiol,** v. 45, n. 4, p. 286-290. 2007.

AL-FATIMI, M. *et al*. Antioxidant, antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. **Journal of Ethnopharmacology**, v. 111, p 657-666, 2007.

ALLEN, G.M. *et al*. 50 Common native important plants in Florida's ethnobotanical history. University of Florida. **Circular 1439**, p. 1-21, 2012.

ALVES, C. Q. *et al*. Métodos para determinação de atividade antioxidante *in vitro* em substratos orgânicos. **Química Nova**, v. 33, n. 10, p. 2202-2210, 2010.

ALVES, M. J. *et al*. Phenols, flavonoids and antioxidant and cytotoxic activity of leaves, fruits, peel of fruits and seeds of *Piptadenia moniliformis* Benth (Leguminosae - Mimosoideae). **Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas**, v. 13, n. 5, p. 466-476, 2014.

AMARI; MARQUES; DAVIES. The putative enoyl-coenzyme A hydratase DspI is required for production of the *Pseudomonas aeruginosa* biofilm dispersion autoinducer *cis*-2-decenoic acid. **J. Bacteriol**.,v. 195, p. 4600-4610, 2013.

AN, S. W; DONG, Y. H; ZHANG, L. H. The Impact and molecular genetics of bacterial biofilms. United Kingdom: **Environmental Molecular Microbiology, Caister Academic Press**, p. 211-226, 2009.

ANDERL, J.N.; FRANKLIN, M. J.; STEWART, S. P. Role of Antibiotic Penetration Limitation in *Klebsiella pneumoniae* Biofilm Resistance to Ampicillin and Ciprofloxacin**.** **Antimicrobial Agents and Chemotherapy**, v. 44, n. 7, p. 1818-1824, 2000**.**

ANDERSEN, Ø. M.; MARKHAM, K. R.; Flavonoids: Chemistry, Biochemistry and Applications. **Taylor & Francis**. Nova Yorque, 2006.

ANDRADE, M. A. *et al*. Essential oils: in vitro activity against *Leishmania amazonensis*, cytotoxicity and chemical composition. **BMC Complementary and Alternative Medicine**, v.16, p. 1-8, 2016.

ANGEH, J. E. *et al*. Antimicrobial and anti-inflammatory activity of four known and one new triterpenoid from *Combretum imberbe* (Combretaceae). **J. Ethnopharmacol**. v. 1, n. 1, p. 56-60, 2007.

ASKARIAN, M.; MAHMOUDI, H.; ASSADIAN, O. Incidence of Nosocomial Infections in a Big University Affiliated Hospital in Shiraz, Iran: A Six-month Experience. **Int J Prev Med,** v. 4, n. 3, p. 366-72. 2013.

AVALOS-PERALTA, P., GARCÍA-BRAVO, B., Camacho, F. M. Sensitivity to *Myroxylon pereirae* resin (balsam of Peru). A study of 50 cases. **Contact Dermatitis**, v. 52, p. 304-306, 2005.

AWAAD *et al*. Novel flavonoids with antioxidant activity from a Chenopodiaceous plant. **Pharmaceutical Biology**, v.50, n.1, p. 99-104, 2012.

AZEVEDO, L.F.P. *et al*., Triagem fitoquímica e atividade antioxidante de *Costus spicatus* (Jacq.) S.w. **Rev. Bras. Pl. Med**., v.16, n.2, p.209-215, 2014.

BÄHRLE-RAPP, M. *Myroxylon balsamum* var. pereirae. In: Springer Lexikon Kosmetik und Körperpflege. Heidelberg: **Springer Medizin Verlag**, 2007.

BARBOSA, K. B. F. *et al*. Estresse oxidativo: conceito, implicações e fatores modulatórios. **Revista de Nutrição**, v. 23, n. 4, p. 629-643, 2010.

BARREIROS, A. L. B. S.; JORGE, M. D**.** Estresse oxidativo: relação entre geração de espécies reativas e defesa do organismo. **Quim. Nova**, v. 29, n. 1, p. 113-123, 2006.

BEECH, B. I.; SUNNER, J. A.; HIRAOKA, K. Microbe-surface interactions in biofouling and biocorrosion processes. **Int. Microbiol**., v. 8, p. 157-168, 2005.

BERNARD *et al*. Gly-cosylated flavones as selective inhibitors of topoisomerase IV. **Antimicrob. Agents Ch**., v. 41, n. 5, p. 992-998,1997.

BFG. Growing knowledge: an overview of Seed Plant diversity in Brazil. **Rodriguésia** v. 66, p. 1085-1113, 2015.

BILLINGS *et al*. The extracellular matrix component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. **PLOS Pathog**., v. 9, n.8, p.1-12, 2013.

BIRBEN, *et al*. Oxidative stress and antioxidant defense, W**orld Allergy Organ. J.** v, 5 1, p, 9-19, 2012.

BJARNSHOLT *et al*. The in vivo biofilm. **Trends in microbiology**. v. 21, p.466-474, 2013.

BLUMENTHAL, J.A. *et al*. Mental stress induced ischemia in the laboratory and ambulatory ischemia during daily life: Associatio and hemodynamic features. **Circulation,** v. 92, p. 2102-2108, 1995.

BOELENS, H.M., RIJKE, D; HARING, H.G..Studies of some balsamics in perfumery. **Perf Flavor** v. 6, p. 7-14, 1982.

BOJASE, G. *et al*. Antimicrobial flavonoids from *Bolusanthus speciosus*. **Planta medica**, v. 68, p. 615-620, 2002.

BOLES; HORSWILL. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. **PLoS Pathog**, v.4, n. 4, p. 1-13, 2008.

BORGES, J. S. **Análise comparativa do proteoma e metaboloma de raízes de dois clones de *E. grandis* x *E. camaldulensis,* sendo um tolerante e um susceptível a condições de estresse hídrico**. 2013. 199f. Dissertação (Mestrado em Ciencias)- Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, São Paulo 2013.

BOURGAUD, F. *et al*. Production of plant secondary metabolites: a historical perspective. **Plant Sci.**, v. 161, n. 5, p. 839-851, 2001.

BOWLES, E.J. Chemistry of Aromatherapeutic Oils. Australia: **Allen & Unwin**, 2003.

BRAVO, L. B. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. **Nutr. Rev.**, v. 56, n. 11, p. 317- 333, 1998.

BRESCÓ *et al*. Pathogenic Mechanisms and Host Interactions in *Staphylococcus epidermidis* Device-Related Infection. **Front Microbiol**, v. 2, p. 1-24, 2017.

BUFFET-BATAILLONA, S. *et al*. Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds-a critical review. **Int J Antimicrob Agents**, v. 39, p. 381-389, 2012.

BURT, S. Essential oils: Their antimicrobial properties and potential applications in food- A review. **International Journal of Food Microbiology**, v. 94, p. 223-253, 2004.

BUSH, k. *et al*. Tackling antibiotic resistance. **Nat Rev Microbiol**, v. 9, n. 12, p. 894-896, 2011.

CAI, Y.; SUN, M.; CORKE, H. Antioxidant activity of betalains from plants of the Amaranthaceae. J. Agric. **Food Chem**., v. 51, p. 2288-2294, 2003.

CARSON, S.N. *et al*. Using a Castor Oil-Balsam of Peru-Trypsin Ointment to Assist in Healing Skin Graft Donor Sites. **Ostomy Wound Manage**, v. 49, p. 60-64, 2003.

CARTAXO, S. L.; ALMEIDA SOUZA, M. M.; ALBUQUERQUE, U. P. Medicinal plants with bioprospecting potential used in semi-arid northeastern Brazil. **Journal of Ethnopharmacology**, v. 131, n. 2, p. 326-342, 2010.

CARVALHO T.A. *et al*. Potencial bactericida de extratos e substâncias isoladas de *Myroxylon peruiferum* (cabreúva) frente a micobactérias do trato respiratório. **In: XVI Congresso de Iniciação Científica**. UFSCar, Sorocaba, 2008.

CASTRO, R. D. Atividade antifúngica do óleo essencial de *Cinnamomum zeylanicum* Blume (canela) e de sua associação com antifúngicos sintéticos sobre espécies de *Candida*. **Tese de doutorado**. Universidade Federal da Paraíba, 2010.

CAVALCANTE, G.M. *et al*. Atividade antimicrobiana de *Artocarpus heterophyllus* Lam. (Moraceae) sobre o desenvolvimento de *Streptococcus pneumoniae* e *Escherichia coli*. **Scientia Plena**, v.9, p. 1-7, 2013.

CAVAR *et al*. Chemical composition and antioxidant and antimicrobial activity of essential oil of Artemisia annua L. from Bosnia. **Ind. Crops. Prod**, v.37, p. 479-485, 2012.

CETIN, E. S. *et al*. Distribution of genes encoding resistance to macrolides, lincosamides and streptogramins among clinical staphylococcal isolates in a Turkish university hospital. **J Microbiol Immunol Infect,** v. 43, n. 6, p. 524-529, 2010.

CHANDRA *et al*. Antimicrobial Resistance and the Alternative Resources with Special Emphasis on Plant-Based Antimicrobials-A Review. **Plants (Basel**), v. 6, n.2, p.1-11, 2017.

CHANTRAINE, J.M. *et al*. Insecticidal activity of essential oils on *Aedes aegypti* larvae. **Phytotherapy Research**, v. 12, p. 350-354, 1998.

CHAVES, E. M. F.; BARROS, R. M. F.; ARAÚJO, F. S. Flora Apícola do Carrasco no Município de Cocal, Piauí, Brasil. **Revista Brasileira de Biociências,** v. 5, p. 555- 557, 2007.

CHEN, X. *et al*. Reactive oxygen species regulate T cell immune response in the tumor microenvironment, **Oxid. Med. Cell. Longev**, v. 2016, p. 1-10, 2016.

CHENG *et al*. Contribution of coagulases towards *Staphylococcus aureus* disease and protective immunity. **PLoS pathogens**, v. 6, n. 8, p. 1-18, 2010.

CHENG, B. *et al*. Study on the plant resources of nerolidol and their uses. **Linchan Huaxue Yu Gongye**, v. 16, p. 22-28, 1996.

CHEW Y.L. *et al*. Antioxidant activity of three edible seaweeds from two areas in South East Asia. **Food Science and Technology**, v. 41, p. 1067-1072, 2008.

CHEYNIER, V. Polyphenols in foods are more complex than often thought. **Am. J. Clin. Nutr.**, v. 81, n. 1, p. 223S-229S, 2005.

CHIKARA, S, *et al*. Oxidative stress and dietary phytochemicals: Role in câncer chemoprevention and treatment. **Cancer Letters**, n. 413, p. 122-134, 2018.

CHOUHAN, S.; KANIKA, S.; GULERIA, S. Antimicrobial Activity of Some Essential Oils-Present Status and Future Perspectives. **Medicines**, v.4, n. 58, p.1-21, 2017.

COATES; MORAN; HORSBURGH. Staphylococci: colonizers and pathogens of human skin. **Future Microbiol**, v. 9, p. 75-91, 2014.

COLOMBO, A. L. *et al*. Brazilian guidelines for the management of candidiasis -a joint meeting report of three medical societies: Sociedade Brasileira de Infectologia, Sociedade Paulista de Infectologia and Sociedade Brasileira de Medicina Tropical. **The Brazilian Journal of infectious diseases**, v. 17, n. 3, p. 283-312, 2013.

COSTA, A.F. Farmacognosia. Lisboa: **Calouste Gulbenkian**, 1994.
COSTA, M.C.M.F. *et al*. Essential oils from leaves of medicinal plants of Brazilian flora: chemical composition and activity against *Candida* species. **Medicines**, v. 27, p. 1-8, 2017.

COSTA, R. O. *et al*. Uso do iodeto de potássio na dermatologia: considerações atuais de uma droga antiga. **Anais Brasileiros de Dermatologia**, v. 88, n. 3, p. 401-407, 2013.

COULON *et al*. Chemical analysis of cellular and extracellular carbohydrates of a biofilm-forming strain *Pseudomonas aeruginosa* PA14. **PLoS One**, v. 5, n. 12, 2010.

COWAN, M.M. Plant products as antimicrobial agents. **Clin Microbiol Ver**, v. 12, p. 564-582, 1999.

COWARD, L. *et al*. Genistein, dadzein, and their β-glicoside conjugates: antitumor isoflavones in soybean foods from american and asian diets. **Journal of Agriculture and Food Chemistry**. v. 41, p. 1961-1967, 1993.

CUSHNIE, T. P. T.; LAMB, A. J. Antimicrobial activity of flavonoids. **International Journal of Antimicrobial Agent**. v. 26, n. 5, p. 343–356, 2005.

CUSTÓDIO, D. L.; VEIGA-JUNIOR, V. F. True and common balsams. **Revista Brasileira de Farmacognosia** v. 22, n. 6, p. 1372–1383, 2012.

DAGLIA M. Polyphenols as antimicrobial agents. **Curr Opin Biotechnol**. v. 23, p.174-181, 2011.

DAVIES, D. Understanding biofilm resistance to antibacterial agents. **Nat. Rev. Drug Discov**, v. 2, p. 114-122, 2003.

DE CARVALHO, R.B.F. *et al.* Composição química e atividade anticolinesterásica de uma fração ativa do extrato de folhas de *Citrus limon* (L.) Burm. **Quimica Nova**, v, 36, p. 1375-1379, 2013.
DEWICK, P. M. **Medicinal natural products: a biosynthetic approach**. 2. ed. London: John Wiley & Sons, 517p, 2002.

DEWICK, P.M. The mevalonate and methylerythritol phosphate pathways: terpenoids and steroids. In: **Medicinal Natural Products: a biosynthetic approach**. 3 ed, Chichester: John Wiley e Sons, cap. 5, p. 187-306, 2009.

DHONGADE, H.J; CHANDEWAR, A.V. An *in-vitro* investigation of the antioxidant activity of *Phyllanthus amarus*. **International Journal of Biomedical and Advance Research** v, 4, p. 435-439, 2013.

DI PASQUA, R. *et al*. Changes in the proteome of *Salmonella enterica* serovar Thompson as stress adaptation to sublethal concentrations of thymol. **Proteomics**, v.10, p. 1040-1049, 2010.

DI STASI, L.C. HIRUMA-LIMA, C.A. Plantas medicinais na Amazônia e na Mata Atlântica. 2 ed. **Revista e ampliada**, São Paulo, 2002.

DIAS, D. A.; URBAN, S.; ROESSNER, U. A historical overview of natural products in drug discovery. **Metabolites**, v.2, n. 2, p. 303-336, 2012.

DIXON, R. A.; PASINETTI, G. M; Flavonoids and Isoflavonoids: From Plant Biology to Agriculture and Neuroscience. **Plant Physiology**. v. 154, p. 453-457, 2010.

DJILANI, A.; DICKO, A. **The therapeutic benefits of essential oils**. INTECH Open Access Publisher, 2012.

DONLAN, R. M.; COSTERTON, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. **Clinical Microbiology Reviews**, v. 15, p. 167-193, 2002.

DOUGLAS, L. J. *Candida* biofilms and their role in infection. **Trends Microbiol**., v. 11, n. 1, p. 30-36, 2003.

DUAN, X.J. *et al.* Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata.* **Food Chemistry**, v. 95, p. 37-43, 2006.

DUDAREVA, N; PICHERSKY, E; GERSHENZON, J. Biochemistry of plant volatiles. **Plant Physiol**, v. 135, p.1893-1902, 2004.

DUNNE, W. M. Bacterial adhesion: Seen any good biofilms lately? **Clin. Microbiol. Rev**., v. 15, n. 2, p. 155-166, 2002.

EDRIS, A. E. Pharmaceutical and Therapeutic Potentials of Essential Oils and Their Individual Volatile Constituents: A Review. **Phytoterapy Research**, v.21, p.308-323, 2007.

ELOUENNASS, M. *et al*. *In Vitro* Activities of Ertapenem and Imipenem against Clinical Extended Spectrum Beta-Lactamase-Producing Enterobacteriaceae Collected in Military Teaching Hospital Mohammed V of Rabat. **Interdiscip Perspect Infect Dis,** v. 2012, p. 6464-80, 2012.

EL-SHAER, N.S. Evaluation of some natural products and plant acids against UVB radiation *(in vitro*). **Alex J Pharm Sci**, v. 20, p. 7-11, 2006.

EVARISTO, F. F. V. *et al*. Antibacterial effect on mature biofilms of oral streptococci and antioxidant activity of 3β,6β,16β-trihydroxylup-20(29)-ene from *Combretum leprosum*. **Medicinal Chemistry Research**, v. 1, p. 1, 2017.

EVARISTO, F. F. V. *et al*. Antimicrobial Effect of the Triterpene 3β,6β,16β-Trihydroxylup-20(29)-ene on Planktonic Cells and Biofilms from Gram Positive and Gram Negative Bacteria. **Bio**. **Med. Research International**. 2014.

FACUNDO, V. A. *et al*. "Triterpenes and flavonoids from *Combretum leprosum*, " **Phytochemistry**, v. 32, n 2, p. 411-415, 1993.

FACUNDO, V. A. *et al.* Arjunolic Acid in the Ethanolic Extract of *Combretum leprosum* Root and its Use as a Potential Multi-Functional Phytomedicine and Drug for Neurodegenerative Disorders: Anti-Inflammatory and Anticholinesterasic Activities. **Journal of the Brazilian Chemical Society,** v. 16, n. 6B, p. 1309-1312, 2005.

FAUSTINO, A. R. C. **Preparação de novos Triterpenóides Semi-Sintéticos**. 2015. Dissertação (Mestrado em Química Farmacêutica Industria) - Universidade de Coimbra, Coimbra, 2015.

FAVRE-BONTÉ *et al*. Autoinducer production and *quorum-sensing* dependent phenotypes of *Pseudomonas aeruginosa* vary according to isolation site during colonization of intubated patients. **BMC Microbiol**, v.7, n. 33, 2007.

FERGUSON, L.R., PHILPOTT, M. Nutrition and mutagenesis. **Annu. Rev. Nutr**, v. 28, p. 313-329, 2008.

FERNANDES, R.S. *et al*. Validação do método de extração e quantificação de 7-hidróxi- 4',6 dimetóxi-isoflavona em culturas de células em suspensão e calos de *Dipteryx odorata.* **Eclética Química**, v.34, p.13-18, 2009.

FILIZOLA, B. C.; SAMPAIO, M. B. Boas Práticas de Manejo para o Extrativismo Sustentável de Cascas. Brasília: **Instituto; Sociedade, População e Natureza**; 2015.

FLEMMING, H. C.; WINGENDER, J. The biofilm matrix. **Nat. Rev. Microbiol**., v. 8, p. 623-633, 2010.

FLUCKIGER, F. A. HANBURY, D. Balsamum tolunatum. **Pharmacographia**, v. 15, p.177-184, 1874.

FONTANAY, S. *et al*. Ursolic, oleanolic and betulinic acids: Antibacterial spectra and selectivity indexes. **Journal of Ethnopharmacology**, v. 120, p. 272–276, 2008.

FONTENELLE, R.O.S. *et al*. Antifungal activity of essential oils of *Croton* species from the Brazilian Caatinga biome. **Journal of Applied Microbiology**, v.104, p. 1383-1390, 2008.

FONTENELLE, R.O.S. *et al*. Chemical composition, toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham. **J Antimicrob Chem**, v. 59, p. 934-940, 2007.

FORMICA, J. V.; REGELSON, W. Review of the biology of quercetin and related bioflavonoids. **Food Chem. Toxicol**, v. 33, n. 12, p. 1061-1080, dez. 1995.

FOSTER *et al*. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. **Nature reviews Microbiology**, v. 12, p.49-62, 2014.

FRANÇA, I.S.X. *et al*. Medicina popular: benefícios e malefícios das plantas medicinais. **Revista Brasileira de Enfermagem**, v. 61, p. 201-208, 2008.

FRANZ, C.; NOVAK, J. Chemistry of Essential Oils. In: BASER, K. Hüsnü Can; BUCHBAUER, Gerhard. **Handbook of essential oils:** science, technology, and applications. CRC Press, p. 39-82, 2010.

FRITSCHE, S.; STEINHART, H. Occurrence of hormonally active compounds in food: a review. **Eurpean Food Research and Technology**. v. 209, p. 153-179, 1999.

GANESAN, P; KUMAR, C.S; BHASKAR, N. Antioxidant properties of methanol extract an its solvent fractions obtained from selected Indian red seaweeds. **Beoresource Techonology, v.** 99, p. 2717-2723, 2008.

GARCIA, R.G; MALO, A.L; PALOU, E. Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against *Listeria innocua*. Jo**urnal of Food Science**, v.76, p. 95-100, 2011.

GELLATLY; HANCOCK. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. **Pathog. Dis**., v. 67, p. 159-173, 2013.

GHAFOOR; HAY; REHM. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. **Appl. Environ. Microbiol**., v. 77, p. 5238–5246, 2011.

GHAHARI *et al*. Chemical Composition, Antioxidant and Biological Activities of the Essential Oil and Extract of the Seeds of *Glycine max*(Soybean) from North Iran. **Curr Microbiol**, n. 74, p. 522-531,2017.

GIL-CHÁVEZ, J.G. *et al*. Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food Ingredients: An Overview. **Comprehensive Reviews in Food Science and Food Safety**, v. 12, n. 1, p. 5-23, 2013.

GIULIETTI, A.M. *et al*. Biodiversidade e conservação das plantas no Brasil. **Megadiversidade**, v. 1, p. 52-60, 2005.

GOBBO-NETO, L; NORBERTO, P.L. Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. **Química Nova**, v.30, p. 374-381, 2007.

GOMES, E. C. S. *et al*. Plantas da Caatinga de uso terapêutico: Levantamento Etnobotânico. **Engenharia Ambiental, Espírito Santo do Pinhal**, v.5, p.74-85, 2008.

GONÇALVES, A.L, FILHO, A.A. MENEZES, H. Estudo comparativo da atividade antimicrobiana de extratos de algumas árvores nativas. **Arquivos do Instituto Biológico**, v. 72, p. 353-358, 2005.

GONÇALVES, A.L. Estudo da atividade antimicrobiana de algumas árvores medicinais nativas com potencial de conservação/recuperação de florestas tropicais. **Tese de doutorado**, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rio Claro, 2007.

GOODMAN; GILMAN'S. **Manual of Pharmacology and Therapeutics**. Nova Iorque: McGraw Hill, 2008.

GORRINI, C., HARRIS, I.S., MAK, T.W. Modulation of oxidative stress as an anticâncer strategy, **Nat. Rev. Drug Discov**, v.12, n.12, p. 931-947, 2013.

GOSSAN, D. P. A. Antibacterial and cytotoxic triterpenoids from the roots of *Combretum racemosum*. **Fitoterapia**, v. 110, p. 89-95, 2016.

GOTTENBOS, B. *et al*. Pathogenesis and prevention of biomaterial centered infections. **Jounal of Materials Science. Materials in Medicine**, v. 13, p. 717-722, 2002.

GOTTLIEB, O.R. MAGALHAES, M.T. The chemistry of Brazilian Leguminosae. I. Isolation of 3',4',7-trimethoxyisoflavone (cabreuvin) from *Myroxylon balsamum* and *Myrocarpus fastigiatus*. **An Assoc Bras Quim**, v.18, p.89-97, 1959.

GREAY, S.J; HAMMER, K.A. Recent developments in the bioactivity of mono- and diterpenes: anticancer and antimicrobial activity. **Phytochemistry Reviews**, v.14, p.1-6, 2011.

GUGGENBERGER *et al*. Two distinct coagulase-dependent barriers protect *Staphylococcus aureus* from neutrophils in a three dimensional in vitro infection model. **PLoS pathogens**, v. 8, n. 1, p. 1-17, 2012.

GUIDO, R. C., ANDRICOPULO, A. D. OLIVA, G. Planejamento de fármacos, biotecnologia e química medicinal: aplicações em doenças infecciosas. **Estudos Avançados**, v. 24, n.7, p. 81-98, 2010.

GUIMARÃES, A.G; SERAFINI, M.R; QUINTANS-JUNIOR, L.J. Terpenes and derivatives as a new perspective for pain treatment: a patent review. E**xpert Opinion on Therapeutic Patents**, v. 24, p. 243-265, 2014.

GUIMARAES, D. O; MOMESSO, L. S. PUPO, M. T. Antibióticos: importância terapêutica e perspectivas para a descoberta e desenvolvimento de novos agentes. **Química Nova**, v. 33, n. 3, p. 667-679, 2010.

GULATI, M.; NOBILE, C. J. *Candida albicans* biofilms: development, regulation, and molecular mechanisms. **Microbes and Infection**, v.18, n.5, p.310-21, 2016.

GÜLÇIN, I. Antioxidant activity of food constituents: an overview. **Arch Toxicol**, v. 86, n. 3, p. 345-391, 2012.

HARBORNE, J.B., GOTTLIEB, O.R. MAGALHAES, M.T. Occurrence of the isoflavone afromosin in cabreuva wood. **J Org Chem**, v. 28, p. 881-882, 1963.

HARTMANN, T. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. **Phytochemistry**, v. 68, n. 22-24, p. 2831-2846, 2007.

HARTMANN, T. Global harmonization of herbal health claims **Ent. Exp. Appl.**, v. 80, p. 177-179, 1996.

HARTWELL, J.L. Plants used against cancer. A survey. **Lloydia**, v. 33, p. 97-194, 1970.

HAVSTEEN, B. H. The biochemistry and medical significance of the flavonoids, **Pharmacology & Therapeutics**, n. 96, n. 67, 2002.

HEINRICH, M. *et al*. Fundamentals of Pharmacognosy and Phytotherapy. Edinburgh: **Churchill Livingstone**, 2008.

HERRMANN, M. *et al*. Fibronectin, fibrinogen, and laminin act as mediators of adherence of clinical staphylococcal isolates to foreign material. **The Journal of Infectious Disease**, v. 158, p. 693-701, 1988.

HJORTH, N. Eczematous allergy to balsams, allied perfumes and flavouring agents, with special reference to balsam of Peru. **Acta Derm Venereol (Stockh)**, v. 41, p. 1-216, 1961.

HOIBY, N. *et al*. The clinical impact of bacterial biofilms. **International Journal of Oral Sciences**, v. 3, p. 55-65, 2011.

HORINOUCHI, C.D. *et al*. *Combretum leprosum* Mart. (Combretaceae): potential as an antiproliferative and anti-inflammatory agent. **J. Ethnopharmacol**. v.145, p. 311-319, 2013.

HUBER *et al*. *Pseudomonas aeruginosa* renews its virulence factors. **Environ. Microbiol. Rep.**, v. 8, p. 564–571, 2016.

HUMMELOVA, J. *et al*. The relationship between structure and *in vitro* antibacterial activity of selected isoflavones and their metabolites with special focus on antistaphylococcal effect of demethyltexasin. **Applied Microbiology**, v. 60, p. 242-247, 2014.

HURBER, L. S; AMAYA-RODRIGUEZ, D. B; Flavonóides e flavonas. **Alim. Nutr.** v.19, n.1, p. 97-108, 2008.

HURLOW, J. *et al*. Clinical biofilms: a challenging frontier in wound care. **Adv Wound Care**, v. 4, n. 5, p. 295-301, 2015.

IBRAHIM, R.K.; **Flavonoids, encyclopedia of life sciences**, 2001.

INENAMI, T. O. Estudo do inibidor de germinação em sementes de *Myroxylon peruiferum* L.f. (Cabriúva)- Leguminosae. **Tese de Doutorado**, Universidade Estadual de Campinas, Campinas, 1984.

JAKOBSEN; TOLKER-NIELSEN; GIVSKOV. Bacterial Biofilm Control by Perturbation of Bacterial Signaling Processes. **International Journal of Molecular Sciences**. v. 18, n. 9, p. 1-27, 2017.

JANG, D.S. et al. Potential cancer chemopreventive constituents of the seeds of *Dipteryx odorata* (tonka bean). **J. Nat. Prod**, v. 66, p. 583-587, 2003.

JANKOWSKY, L. Atividade farmacológica de extratos obtidos de a partir de resíduos madeireiros. **Tese de doutorado**, Universidade Estadual de Campinas, Campinas, 2005.

JHA, H.C; ZILLIKEN, F; BREITMAIER, E. Carbon-13 chemical shift assignments of chromones and isoflavones. **Can. J. Chern**, v. 58, p.1211-1219, 1980.

JOO; OTTO. Mechanisms of resistance to antimicrobial peptides in staphylococci. **Biochim Biophys Acta**, v. 1848, p. 3055-3061, 2015.

KAMILOGLU, S. *et al*. Investigating the antioxidant potential of Turkish herbs and spices. **Quality Assurance and Safety of Crops & Foods**, V. 6, n. 2, p. 151-158, 2014.

KARADENIZ, A. *et al*. Antioxidant activity of some Turkish medicinal plants. **Nat. Prod. Res**., v. 29, p. 2308-2312, 2015.

KATERERE, D. R. *et al*. Antimicrobial activity of pentacyclic triterpenes isolated from African Combretaceae. **Phytochemistry**, v. 63, p. 81-88, 2003.

KATSAROU, A. *et al.* Frequency of immediate reactions to the European standard series. **Contact Dermatitis**, v. 41, p. 276-279, 1999.

KATZUNG, B. **Farmacologia Básica e Clínica**. Brasil: McGraw Hill. 10ª ed, 2007.

KHAN, S.M.A; MALIK, A; AHMAD, I. Anti-candidal activity of essential oils alone and in combination with amphotericin B or fluconazole against multi-drug resistant isolates of *Candida albicans*. **Medical Mycology**, v.50, p. 33-42, 2012.

KHORSHIDIAN, N. *et al*. Potential application of essential oils as antimicrobial preservatives in cheese. **Innovative Food Science and Emerging Technologies**, v. 45, p. 62–72, 2018.

KOKARE, C. R. *et al*. Biofilm: Importance and applications. **Indian Journal of Biotechnology**. v. 8, p. 159-168, 2009.

KORSAK, D.; POPOWSKA, M.; MARKIEWICZ, Z. Analysis of the murein of *Listeria monocytogenes* EGD mutant lacking functional penicillin binding protein 5 (PBP5). **Pol. J. Microbiol**., v. 54, p. 339-342, 2005.

KROON, S. Standard photopatch testing with Waxtar1, paraaminobenzoic acid, potassium dichromate and balsam of Peru. **Contact Dermatitis**, v. 9, p. 5-9, 1983.

KUREK, A. *et al*. Modulation of antibiotic resistance in bacterial pathogens by oleanolic acid and ursolic acid. **Phytomedicine**, v.19, p. 515-519, 2012.

KUREK, A. *et al*. Oleanolic acid and ursolic acid affect peptidoglycan metabolism in *Listeria monocytogenes*. **Antonie van Leeuwenhoek**, v. 97, p. 61-68, 2010.

KWIECINSKI *et al.* Staphylokinase control of *Staphylococcus aureus* biofilm formation and detachment through host plasminogen activation**. J Infect Dis**, v. 213. p. 139-148, 2016.

LAGO, J. Mecanismos de Resistência e Selecção de Antibióticos. Lisboa: **Jornadas bioMérieux**, 2011.

LARSEN, W. G. Perfume dermatitis. **J Am Acad Dermatol**, v.12, p.1-9, 1985.

LATTANZIO, N.A. *et al*. Busca de inibidores da Transtirretina (TTR) humana em extratos e substâncias isoladas de *Myroxylon peruiferum* (Fabaceae). **In: Sociedade Brasileira de Química** (SBQ), Águas de Lindóia-SP, 2007.

LECHARTIER, B.; HARTKOORN, R. C.; COLE, S. T. *In vitro* combination studies of benzothiazinone lead compound BTZ043 against *Mycobacterium tuberculosis*. **Antimicrobial agents and chemotherapy**, v. 56, n. 11, p. 5790-5793, 2012.

LEE, J-H; LEE J-S. Chemical Composition and Antifungal Activity of Plant Essential Oils against *Malassezia furfur*. **Journal of Microbiology and Biotechnology**, v. 38, p. 315-321, 2010.

LEON, H.; H. ALAIN. Dicotiledoneas: Casuarinaceas a Meliaceas. **Fl. Cuba**, v, 2p. 1-456, 1951.

LI *et al*. Gram-positive three-component antimicrobial peptide-sensing system. **Proc. Natl. Acad. Sci. U.S.A**, v. 104, p. 9469-9474, 2007.

LI, T. *et al*. Isoflavonoid compound from tobacco rhizome and preparation and application thereof. Patent CN102351827A, filled Aug 16, 2011 issued Feb 15, 2012.

LIMA, J. R.; MANSANO, V. F. A família Leguminosae na Serra de Baturité, Ceará, uma área de Floresta Atlântica no semiárido brasileiro. **Rodriguésia-Instituto de Pesquisas Jardim Botânico do Rio de Janeiro**, v. 62, n. 3, 2011.

LIMA, T. B. *et al*. Bacterial resistance mechanism: what proteomics can elucidate. **Faseb Journal**, v. 27, n. 4, p. 1291-303, 2013.

LIN, C. W. *et al.* DPPH Free-Radical Scavenging Activity, Total Phenolic Contents and Chemical Composition Analysis of Forty-Two Kinds of Essential Oils. **Journal of Food and Drug Analysis**, v. 17, p. 386-395, 2009.

LINARES, E; BYE JR, R. A. A study of four medicinal plant complexes of Mexico and adjacente United States. Journal of ethnopharmacology, v. 19, p. 153-183, 1987.

LINDE, G. *et al*. Quimiotipos, Extracción, Composición y Aplicaciones del Aceite Esencial de *Lippia alba.* **Revista Brasileira de Plantas Medicinais**, v. 18, n. 1, p. 191-200, 2016.

LINNAEUS FILIUS, C. *Myroxylon peruiferum*. **Supplementum Plantarum** v. 34, p. 232-234, 1781.

LIRA, S. R. D. *et al*. Preliminary studies on the analgesic properties of the ethanol extract of *C. leprosum*. **Pharm Biol**., V. 40, p. 213-5, 2002.

LISTER; HORSWILL. *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal. **Front Cell Infect Microbiol**, v.4, p. 1-9, 2014.

LLARRULL, L. I. *et al*. The future of the beta-lactams. **Curr Opin Microbiol,** v. 13, n. 5, p. 551-557. 2010.

LOIOLOA, M. I. B. *et al.* Leguminosas e seu potencial de uso em comunidades rurais de São Miguel do Gostoso-RN. **Revista Caatinga**, v. 23p. 59-70, 2010.

LONGHI-BALBINOT, D.T. *et al*. Anti-inflammatory effect of triterpene 3beta, 6beta, 16beta-trihydroxylup-20(29)-ene obtained from *Combretum leprosum* Mart & Eich in mice. **J. Ethnopharmacol**. v. 142, p. 59-64. 2012.

LONGHI-BALBINOT, D.T. *et al.* Spinal antinociception evoked by the triterpene 3beta, 6beta, 16beta-trihydroxylup-20(29)-ene in mice: evidence for the involvement of the glutamatergic system via NMDA and metabotropic glutamate receptors. **Eur. J. Pharmacol.** v. 623, p. 30-36, 2009.

LOPES, R.M., OLIVEIRA, T.D., NAGEM, T.J., PINTO A.D.S. **Flavonoides. Biotecnologia Ciência & Desenvolvimento**, v. 3, n. 14, 2010.

LORENZI, H. Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil. Nova Odessa: Ed. **Plantarium**, 1992.

LORENZI, H. Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil. Nova Odessa: **Plantarun**, 1998.

LORENZI, H.E; MATOS, F.J. A. Plantas medicinais no Brasil/ Nativas e exóticas. Nova Odessa, **Instituto Plantarum**, 2002.

ORENZI, H.E; MATOS, F.J.A. Plantas Medicinais no Brasil: nativas e exóticas. Nova Odessa: **Plantarum**, 2008.

LUPETTI, A. *et al*. Molecular basis of resistance to azole antifungals. **Trends Mol Med**, v. 8, n.2, p. 76-81, 2002.

MA, Z. Z. *et al*. Three New Triterpenoids from *Peganum nigellastrum*. **Nat. Prod**., v. 63, p. 390-392, 2000.

MAALEJ, S. M. *et al*. Comparison of disc diffusion, Etest and agar dilution for susceptibility testing of colistin against Enterobacteriaceae. **Lett Appl Microbiol,** v. 53, n. 5, p. 546-51. 2011.

MACEDO, P. M. *et al*. New posology of potassium iodide for the treatment of cutaneous sporotrichosis: study of efficacy and safety in 102 patients. **Journal of the European Academy of Dermatology and Venereology**, v. 29, n. 4, p. 719-724, 2015.

MACHADO, T.B. *et al.* Brazilian phytopharmaceuticals-evaluation against hospital bacteria. **Phytother Res**, v. 19p. 519-525, 2005.

MAIOLO, E. M. Activities of fluconazole, caspofungin, anidulafungin, and amphotericin B on planktonic and biofilm *Candida* species determined by microclorimetry. **Antimicrobial Agents and Chemotherapy**. v.58, n.5, p.2709-2717, 2014.

MALAQUIAS, G. *et al*. Utilização na medicina popular, potencial terapêutico e toxicidade em nível celular das plantas *Rosmarinus officinalis* L., *Salvia officinalis* L. e *Mentha piperita* L. (Família Lamiaceae). **Revista Intertox de Toxicologia, Risco Ambiental e Sociedade**, v. 7, n. 3, p. 50-68, 2014.

MARANDUBA, A. *et al*. Isoflavonoids from *Myroxylon peruiferum.* **Phytochemistry**, v. 18, p.815-817, 1979.

MARANGONI, M. S. *et al*. Pyricularia blast on white oats -a new threat to wheat cultivation. **Tropical Plant Pathology**, v.38, n.3, 2013.

MARTENS, S.; MITHOFER, A.; Flavones and Flavone synthases. **Phytochemistry Phytochem**. V.66, n. 20, p.2399-2407, 2005.

MATHIAS, L. *et al.* A New Pentacyclic Triterpene Isolated from *Myroxylon balsamum* (syn. *Myroxylon peruiferum*). Journal of the Brazilian **Chemical Society**, v. 11p. 195-198, 2000.

MATOS NETO, F.A. Abordagem etnobotânica de plantas medicinais usadas para tratamento de lesões de pele associado, ou não, a bactérias ou fungos no município de Itapajé, Ceará, Brasil. **Trabalho de conclusão de curso**, Universidade Estadual Vale do Acaraú, Sobral, 2013.

MATOS, **Francisco José Abreu. Introdução a fitoquímica experimental**. 3. ed. Fortaleza: UFC, 148 p, 2009.

MCADOW *et al.* Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood. **PLoS pathogens**, v. 7, n. 10, p. 1-12, 2011.

MCCONOUGHEY *et al*. Biofilms in periprosthetic orthopedic infections. **Fut Microbiol**, v. 9, p. 987-1007, 2014

MELO, A. S. *et al*. Biofilm production and evaluation of antifungal susceptibility amongst clinical *Candida* spp. Isolates, including strains of the *Candida parapsilosis* complex. **Medical Mycology**. v.49, p.253-262, 2011.

MENDES, J.M. Investigação da atividade antifúngica de óleo essencial de *Eugenia coryophyllata* Thunb. sobre cepas de *Candida tropicalis*. **Dissertação**, Universidade Federal da Paraíba, 2011.

MEZZARI, A.; FUENTEFRIA, A. M. **Micologia no laboratório clínico.** Barueri-SP: Manole, 2012.

MIGUEL, M.G. Antioxidant and anti-inflammatory activities of essential oils: a short review. **Molecules**, v. 15, p. 9252-9287, 2010.

MILIAUSKAS, G.; VENSKUTONIS, P.R.; VAN BEEK, T.A. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. **Food Chem**., v. 85, p. 231-237, 2004.

MOGHADDAM; KHODI; MIRHOSSEINI. *Quorum sensing* in bacteria and a glance on *Pseudomonas aeruginosa*. **Clin. Microbial**., v.3, n. 4, p. 1-10, 2014.

Monroe, D. Looking for chinks in the armor of bacterial biofilms, **PLoS Biol**, v. 5, n. 11, p. 2458- 2461, 2007.

MONTANARI, C. A.; BOLZANI, V. S. Planejamento racional de fármacos baseado em produtos naturais. **Química Nova**, v. 24, n. 1, p. 105-111, 2001.

MOORMEIER *et al*. Temporal and stochastic control of *Staphylococcus aureus* biofilm development. **MBio**, v.5, p.1341-1314, 2014.

MOORMEIER *et al*. Use of microfluidic technology to analyze gene expression during *Staphylococcus aureus* biofilm formation reveals distinct physiological niches. **Appl Environ Microbiol**, v.79, p.3413–3424, 2013.

MOORMEIER; BAYLES. *Staphylococcus aureus* biofilm: a complex developmental organism. **Mol Microbiol**, v. 104 p.365-376, 2017.

MORADALI *et al*. Alginate polymerization and modification are linked in *Pseudomonas aeruginosa*. **MBio**, v. 6, 2015.

MORADALI; GHODS; REHM. *Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. **Frontiers in Cellular and Infection Microbiology**, v. 7, p. 1-29, 2017.

MORAIS, S. M. *et al*. Ação antioxidante de chás e condimentos de grande consumo no Brasil. **Revista Brasileira de Farmacognosia**, v. 19, n. 1B, p. 315-320, 2009.

MORAR, M.; WRIGHT, G. D. The genomic enzymology of antibiotic resistance. **Annu Rev Genet,** v. 44, p. 25-51. 2010.

MORGENSTERN, M. *et al*. Antibiotic resistance of commensal *Staphylococcus aureus* and coagulase-negative staphylococci in an international cohort of surgeons: a prospective point-prevalence study. **PLoS ONE**, v. 11, p.1-16, 2016.

MORIARITY, D. M. *et al*. Lupeol is the cytotoxic principle in the leaf extract of *Dendropanax* cf. querceti. **Planta Med**., v. 64, p. 370-372, 1998.

MOSQUITO, S. *et al*. Molecular mechanisms of antibiotic resistance in *Escherichia coli*-associated diarrhea. **Rev Peru Med Exp Salud Publica,** v. 28, n. 4, p. 648-56. 2011.

MUKNE, A.P., VISWANATHAN, V., PHADATARE, A.G. Structure pre -requisites for isoflavones as effective antibacterial agents. **Pharmacognosy Review**., v. 5, n.9. p. 13-18, 2011.

MULCAHY; ISABELLA; LEWIS. *Pseudomonas aeruginosa* biofilms in disease. **Microb. Ecol**., v. 68, p.1-12, 2014.

MUÑOZ, V. *et al*. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach Part III. Evaluation of the antimalarial activity of plants used by Alteños Indians. **Journal of Ethnopharmacology**, v. 71p. 123-131, 2000.

N'DIAYE *et al*. Skin-bacteria communication: involvement of the neurohormone Calcitonin Gene Related Peptide (CGRP) in the regulation of *Staphylococcus epidermidis* virulence. **Sci. Rep**, v. 6, p. 1-15, 2016.

NAMVAR, *et al*. Clinical characteristics of *Staphylococcus epidermidis*: a systematic review. **Hygiene and Infection Control**, v.9, n.3, 2014.

NASCIMENTO, P. G. *et al*. Antibacterial and antioxidant activities of ursolic acid and derivatives. **Molecules**, v. 19, p. 1317-1327, 2014.

NASCIMENTO-NETO, L. G. Effect of the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene isolated from the leaves of *Combretum leprosum* Mart. on cutaneous wounds in mice. **Journal of Ethnopharmacology**, v. 171, p. 116-120, 2015.

National Committee Clinical Laboratory Standards (NCCLS) M27A. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Approved Standard, M27-A3; USA: Wayne, 2008.

National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6, 6th ed. National Committee for Clinical Laboratory Standards, Wayne, Pa, 2003.

NAVES, Y.R. Presence of nerolidol in papilionaceous essential oils. **Helv Chim Acta** v. 32, p. 2181-2185, 1949.

NAZZARO, F. *et al*. Effect of essential oils on pathogenic bacteria. **Pharmaceuticals**, v. 6, n. 12, p. 1451-1474, 2013.

NELSON, D. L.; COX, M. M. **Princípios de Bioquímica de Lehninger.** 6. ed. Porto Alegre: Artmed, 2014.

NIKAIDO, H. Prevention of drug access to bacterial targets: Permeability barriers and active efflux. **Science**, v. 264, n. 5157, p. 382-388, 1994.

NISNEVITCH, M. Photosensitizers as an Alternative to Antibiotics. **Recent Pat Antiinfect Drug Discov**. 2013.

NOLVACHAI, Y.; MARRIOTT, P. J. GC for flavonoids analysis: Past, current, and prospective trends. **J Sep Sci**., v. 36, v. 1, p. 20-36, 2013.

NUNES, P.H. *et al*. Antiulcerogenic activity of *Combretum leprosum*. **Pharmazie**, v. 64, p. 58–62. 2009.

O'GARA, J. P. Into the storm: Chasing the opportunistic pathogen *Staphylococcus aureus* from skin colonisation to life-threatening infections. **Environmental Microbiology**, v. 19, n. 10, p. 3823–3833, 2017.

OHSAKI, A. *et al*.Microanalysis of a selective potent anti-*Helicobacter pylori* compound in a Brazilian medicinal plant, *Myroxylon peruiferum* and the activity of analogues. **Bioorganic & Medicinal Chemistry Letters**, v. 9, p. 1109-1112, 1999.

OLIVEIRA, A.B. *et al.* Isoflavonoids from *Myroxylon balsamum*. **Phytochemistry**, v. 17, p. 593-595, 1978.

OOTANI, Marcio Akio. **Atividade inseticida, antifúngica e herbitóxica dos óleos essenciais de *Eucalyiptus citriodora* e *Cymbopogon nardus***. 2010. 121 f. Dissertação (Mestrado em Produção Vegetal). Universidade Federal do Tocantins, Gurupi, 2010.

OPDYKE, D.L.J. Monographs on fragrance raw materials: fennel oil, bitter. **Food Cosmet Toxicol**, v. 14, p. 309-310, 1976.

OZKAN, G. *et al*. Potential use of turkish medicinal plants in the treatment of various diseases. **Molecules**, v. 21, n. 257, p. 1-32, 2016.

PAIVA, P. M. G. *et al*. Antimicrobial activity of secondary metabolites and lectins from plants. Badajoz: **Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology**, p. 396-406, 2010.

PANKEY, G., SABATH, L. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram positives bacterial infections. **Oxford Journals**, v.38, p. 864-865, 2013.

PARK, S. N; AHN, S. J; KOOK, J. K. Oleanolic acid and ursolic acid inhibit peptidoglycan biosynthesis in *Streptococcus mutans* UA159. **Brazilian Journal of Microbiology**, v. 46, p. 613-617, 2015.

PARROT, D. *et al.* Review-Lichen-Associated Bacteria as a Hot Spot of Chemodiversity: Focus on Uncialamycin, a Promising Compound for Future Medicinal Applications. **Planta Med.** v. 82, n. 13, p.1143-1152, 2016.

PARVIZI *et al*. Periprosthetic joint infection: the economic impact of methicillin-resistant infections. **J Arthroplasty**, v. 25, p. 103-107, 2010.

PAUL, J. H.; JEFFREY, W. H. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in Vibrio proteolytica. **Appl Environ Microbiol**., v. 50, p. 431-437, 1985.

PAVITHRA, D.; DOBLE, M. Biofilm formation, bacterial adhesion and host response on polymeric implants-issues and prevention. **Biomedical Materials**, v. 3, p. 1-13, 2008.

PERIASAMY *et al*. How *Staphylococcus aureus* biofilms develop their characteristic structure. **Proc Natl Acad Sci**, v. 109, n. 4, p. 1281-1286, 2012.

PETROSILLO, N.; IOANNIDOU, E; FALAGAS, M. E. Colistin monotherapy vs. combination therapy: evidence from microbiological, animal and clinical studies. **Clin Microbiol Infect,** v. 14, n. 9, p. 816-27, 2008.

PIERCE, C. G. A simple and reproducible 96 well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. **Nature Protocols**. v.3, n.9, 1494-1500, 2008.

PIERCE, C. G. *et al*. Antifungal therapy with an emphasis on biofilms. **Current Opinion in Pharmacology**, v. 13, p.1-5, 2013.

PIETROVSKI, E. F. *et al*. Antinociceptive properties of the ethanolic extract and of the triterpene 3β,6β,16β -trihidroxilup-20(29)-ene obtained from the flowers of *Combretum leprosum* in mice. **Pharmacology, Biochemistry and Behavior**, v. 83, p. 90-99, 2006.

PLAPER, A. *et al*. Characterization of quercetin binding site on DNA gyrase. **Biochem. Biophys. Res. Commun**., v. 306, n. 2, p. 530-336, 2003.

POPOVA, M. *et al.* A scientific note on the high toxicity of propolis that comes from *Myroxylon balsamum* trees. **Apidologie**, v. 33, p.87-88, 2002.

PROMDEN *et al*. Structure and antioxidant activity relationships of isoflavonoids from *Dalbergia parviflora*. **Molecules**, v. 19, p. 2226–2237, 2014.

QIU, J. *et al.* Subinhibitory concentrations of perilla oil affect the expression of secreted virulence factor genes in *Staphylococcus aureus*. **PLoS ONE**, v. 6, p. 1-8, 2011.

QUITANS-JÚNIOR, L. *et al*. Antinociceptive Action and Redox Properties of Citronellal and Essential Oil Present in Lemongrass. **J Med Foot**. v.14, n.6, p.630- 639, 2011.

RAI, R. V; JAMUNA, B. A. Nanoparticles and Their Potential Application as Antimicrobials, Science against Microbial Pathogens: Communicating Current Research and Technological Advances. In: Méndez-Vilas, A., Ed., **Formatex, Microbiology Series**, v. 1, n. 3, p. 197-209, 2011.

RAO *et al*. Synthesis and antioxidant activity of galloyltyrosine, derivatives from young leaves of *Inga laurina*. **International Journal of Pharma and Bio Sciences**, v. 2, n. 4, p. 39-44, 2011.

RASAMIRAVAKA *et al*. The formation of biofilms by *Pseudomonas aeruginosa*: A review of the natural and synthetic compounds interfering with control mechanisms. **BioMed Res. Int.**, v. 2015, 2015.

RAUT, J. S.; KARUPPAYIL, S. M. A status review on the medicinal properties of essential oils. **Industrial Crops and Products**, v. 62, p. 250-264, 2014.

REZENDE, F. M. *et al*. Vias de síntese de metabólitos secundários em plantas. **VII Botânica no Inverno**. São Paulo: Instituto de Biociências da Universidade de São Paulo, Departamento de Botânica, p. 332, 2017.

RHEE, S.G., Cell signaling. H_2O_2, a necessary evil for cell signaling, **Science**, v.312, n. 5782, p. 1882-1883, 2006.

RIBEIRO *et al*. New frontiers for anti-biofilm drug development. **Pharmacol Ther**, v. 160, p. 133-144, 2016.

RIBEIRO; MONTEIRO; FERRAZ. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. **Biomatter**, v. 2, p. 176–194, 2012.

ROBBERS, J.E; SPEEDIE, M.K; TYLER, V.E. Pharmacognosy and Pharmacobiotechnology. Baltimore: **Williams & Wilkins**, 1996.

ROCHA E SILVA, H. *et al*. Constituintes químicos das cascas do caule de *Cenostigma macrophyllum*: ocorrência de colesterol. **Química Nova**, v. 30, p. 1877-1881, 2007.

ROCHFORD, E.T.; RICHARDS, R.G.; MORIARTY, T.F. Influence of material on the development of device-associated infections. **Clinical Microbiology and Infection**, v. 18, p. 1162-1167, 2012.

RODRIGO, R.; MIRANDA, A.; VERGARA, L. Modulation of endogenous antioxidant system by wine polyphenols in human disease. Clinica chimica acta. **International journal of clinical chemistry**, v. 412, n. 5-6, p. 410-424, 2011.

ROGINSKY, V.; LISSI, E. A. Review of methods to determine chainbreaking antioxidant activity in food. **Food Chemistry,** v. 92, p. 235-254, 2005.

ROSKOY, Y.R**.** *et al*. International Legume Database and Information Service (ILDIS). Disponível em http://www.ildis.org (acesso em 10-IV-2017), 2007.

RÚA, J. *et al*. Antibacterial activity against foodborne *S. aureus* and antioxidant capacity of various pure phenolic compounds. **Foodborne Pathogen and Diseases**, v. 8, p. 149-157, 2011.

RYBTKE *et al*. *Pseudomonas aeruginosa* Biofilm infections: community structure, anti-microbial tolerance and immune response. **J. Mol. Biol**., v. 427, p. 3628-3645, 2015.

SAAD, A. *et al*. Anticandidal activity of the essential oils of *Thymus maroccanus* and *Thymus broussonetii* and their synergism with amphotericin B and fluconazol. **Phytomedicine**, v. 17, p.1057-1060, 2010.

SAAD, N. Y.; MULLER, C. D.; LOBSTEIN, A. Major bioactivities and mechanism of action of essential oils and their components. **Flavour Frag. J.**, v. 28, p. 269-279, 2013.

SAHOO, S.; SINGH, S.; NAYAK, S. Chemical composition, antioxidant and antimicrobial activity of essential oil and extract of *Alpinia malaccensis* Roscoe (Zingiberaceae). **International Journal of Pharmacy and Pharmaceutical Sciences**, v. 6, p. 183-188, 2014.

SALES, M. D. C. *et al.* Antifungal activity of plant extracts with potential to control plant pathogens in pineapple. **Asian Pacific Journal of Tropical Biomedicine**, v. 6, p. 26-31, 2016.

SANCHEZ-VIOQUE *et al*. Polyphenol composition and antioxidant and metal chelating activities of the solid residues from the essential oil industry. **Ind. Crops Prod**. v. 49, p.150-159, 2013.

SANDHAR, H.K. *et al*. A Review of Phytochemistry and Pharmacology of Flavonoids. **Internationale Pharmaceutica Sciencia**. v.1, n.1, p. 25-41, 2011.

SANDOVAL, P; CHOQUE, J; URIONA, P. Cartilla popular sobre las plantas utiles de los Alteños de Mizque-Cochabamba. **Fonama-Cibe-Umsa**, Cochabamba, Bolivia, 1996.

SANTI *et al*. Chemical Composition, Antioxidant and Antimicrobial Activity of the Oil and Plant Extract *Myrocarpus frondosus* Allemão. American Journal of Plant Sciences, v.8, p.1560-1571,2017.

SANTOS CADE, M; TORRES, K.R; LEONART, R. Plantas medicinais/herbarium, flora et scientia. São Paulo. **Ícone**, 1988.

SANTOS, R. I. Metabolismo básico e origem dos metabólitos secundários. In: SIMÕES, C. M. O. et al. (Org.). **Farmacognosia: da planta ao medicamento**. 5. ed. rev. ampl. Florianópolis: Ed. da UFSC; Porto Alegre: Ed. da UFRGS, p. 403-434, 2004.

SARTORI, A.L.B. *et al*. A revision of the genus *Myroxylon* (Leguminosae: Papilionoideae). **Kew Bulletin**, v. 70, p. 1-12, 2015.

SARTORI, A.L.B. *Myroxylon* in Lista de Espécies da Flora do Brasil. **Jardim Botânico do Rio de Janeiro**. Available in: Acesso em: 16 nov. 2017.

SARTORI, A.L.B. **Revisão taxonômica e estudos morfológicos, *Myrocarpus Allemão, Myroxylon* L.f. e *Myrospermum* Jacq**. 2000.162f.Tese (doutorado em Biologia Vegetal). Instituto de Biologia. Universidade Estadual de Campinas, Campinas, São Paulo. 2000.

SCHNUCH, A. *et al*. Contact allergy to fragrances: frequencies of sensitization from 1996 to 2002. Results of the IVDK. **Contact Dermatitis**, v. 50, p. 65-76, 2004.

SEMWAL *et al*. From arrow poison to herbal medicine-The ethnobotanical, phytochemical and pharmacological significance of *Cissampelos* (Menispermaceae). **J Ethnopharmacol**, v.155, n. 2, p. 1011-1028, 2014.

SENA, L.A., CHANDEL, N.S. Physiological roles of mitochondrial reactive oxygen species, **Mol. Cell**, v. 48, n. 2, p.158-167, 2012.

SEO, S-M.; PARK, H-M;PARK, IL-K. Larvicidal Activity of Ajowan (*Trachyspermum ammi*) and Peru Balsam (*Myroxylon pereira*) Oils and Blends of Their Constituents against Mosquito, Aedes aegypti, Acute Toxicity on Water Flea, *Daphnia magna*, and Aqueous Residue. **Journal of Agricultural and Food Chemistry**, v. 60, p. 5909-5914, 2012.

SHAHIDI, F.; AMBIGAIPALAN, P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects-A review. **Journal of Functional Foods**, v. 18, p. 820-897, 2015.

SHARMA *et al*. *Pseudomonas aeruginosa* biofilm: potential therapeutic targets. **Biologicals**, v.42, p. 1-7, 2014.

SILVA, F.M; PAULA, J.E; ESPINDOLA, L.S. Evaluation of the antifungal potential of Brazilian cerrado medicinal plants. **Mycoses**, v. 52, p.511-517, 2009.

SILVA, M. I. G. *et al*. Bioactivity and potential therapeutic benefits of some medicinal plants from the Caatinga (semi-arid) vegetation of Northeast Brazil: a review of the literature. **Revista Brasileira de Farmacognosia**, v. 22, n. 1, p. 193-207, 2012.

SILVA, M. J. D. *et al*. Ethanolic extract of *Mimosa caesalpiniifolia* leaves: Chemical characterization and cytotoxic effect on human breast cancer MCF-7 cell line. **South African Journal of Botany**, v. 93, p. 64-69, 2014.

SILVA, M. J. *et al*. Avaliação da atividade antioxidante e antimicrobiana dos extratos e frações orgânicas de *Mimosa caesalpiniiflolia* Benth. (Mimosaceae). R**ev Ciên Farm Básica Apl**, v.33, p. 267-274, 2012.

SILVA, S. *et al. Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. **FEMS Microbiology Reviews**, v. 36, n. 2 p. 288-305, 2012.

SILVA, S. M. F. Q. *et al*. Atividade *in vitro* de extratos brutos de duas espécies vegetais do cerrado sobre leveduras do gênero *Candida*. **Revista Ciência & Saúde Coletiva**, v. 17, n. 6, p. 1649-1656, 2012.

SILVA-JÚNIOR, C. A. *et al*. *Myroxylum peruiferum*: antioxidant activity and phenolic content. **Current Topics in Phytochemistry**, v.12, p.21-25, 2015.

SIMAS, N.K**.** *et al.* Produtos naturais para o controle da transmissão da dengue- Atividade larvicida de *Myroxylon balsamum* (óleo vermelho) e de terpenóides e fenilpropanóides. **Quim Nova**, v.27, p.46-49, 2004.

SIMOES, M.; BENNETT, R. N.; ROSA, E. A. S. Understanding antimicrobial activities of phytochemicals against multidrug resistantbacteria and biofilms. **Natural Product**, v.26, p.746-757, 2009.

SIMOES, M.; SIMOES, L. C.; VIEIRA, M. J. A review of current and emergent biofilm control strategies. **Lwt-Food Science and Technology,** v. 43, n. 4, p. 573 583, 2010.

SONG *et al*. Clinical and economic impact of methicillin-resistant *Staphylococcus aureus* colonization or infection on neonates in intensive care units. **Infect Control Hosp Epidemiol**, v.31, p.177-182, 2010.

SOTO, S.M. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. **Virulence**, v. 4, n. 3, p. 223-229, 2013.

SOUZA, R. K. D.; MENDONÇA, A. C. A. M.; SILVA, M. A. P. Ethnobotanical, phytochemical and pharmacological aspects Rubiaceae species in Brazil. **Revista Cubana de Plantas Medicinales**, v. 18, n. 1, p. 140-156, 2013.

SOUZA, V. C; LORENZI, H. Botânica Sistemática: guia ilustrado para identificação das famílias de fanerógamas nativas e exóticas no Brasil, baseado em APGII. 2 ed. Nova Odessa, SP: **Instituto Plantarum**, 2008.

STOODLEY, P. *et al*. Biofilm material properties as related to shear-induced deformation and detachment phenomena. **J. Ind. Microbiol. Biotechnol**, v. 29, n. 6, p. 361-367, 2002.

STREMPEL *et al*. Human host defense peptide LL-37 stimulates virulence fator production and adaptive resistance in *Pseudomonas aeruginosa*. **PLoS ONE 8**, v. 8, n. 12, p. 1-12, 2013

SUNTRES, Z. E., COCCIMIGLIO, J., ALIPOUR, M. The bioactivity and toxicological actions of carvacrol. **Critical Reviews in Food Science and Nutrition**, v. 55, n.3, p. 304 318, 2015.

TA, C. A. K; ARNASON, J. T. Mini Review of Phytochemicals and Plant Taxa with Activity as Microbial Biofilm and Quorum Sensing Inhibitors. **Molecules,** v. 21, n.1, p. 21- 29, 2015.

TAIZ, L.; ZEIGER, E. **Fisiologia Vegetal**, 3. ed. Porto Alegre: Artmed, 2004.

TAIZ, L.; ZEIGER, E. **Fisiologia vegetal**. 4.ed. Porto Alegre: Artmed, 2009. 848p.

TALIBI, I. *et al.* Antifungal activity of some Moroccan plants against *Geotrichum candidum*, causal agent of postharvest citrus sour rot. **Crop Protection**, v.35, p.41-46, 2012.

TANAKA, S. *et al.* Immediate contact reactions to fragrance mix constituents and *Myroxylon pereirae* resin. **Contact dermatites**, v.51, p. 20-21, 2004.

TAPWAL, A. *et al*. In Vitro antifungal potency of plant extracts against five phytopathogens. **Brazilian Archives of Biology and Technology**, v.54, n.6, p.1093-1098, 2011.

TAYLOR, L. Herbal secrets of the rainforest: the healing power of over 50 medicinal plants you should know about. **Rocklin: Prima Healh**, 1998.

TECHATHUVANAN, C. *et al*. Efficacy of comercial natural antimicrobials alone and in combinations against pathogenic and spoilage microorganisms. **Journal of Food Protection**, v. 77, n. 2, p. 269–275, 2014.

TEHRANI, F., SADEGHI, E. Effect of mint essential oil on growth of *Listeria monocytogenes* during the ripening and storage of Iranian white brined cheese. **Journal of Applied Environmental and Biological Sciences**, v. 5, p. 150-154, 2015.

TEMPONE, A.G. *et al*. Brazilian flora extracts as source of novel antileishmanial and antifungal compounds. **Memórias do Instituto Oswaldo Cruz**, v. 103, p.443-49,2008.

THAMMAVONGSA *et al*. Staphylococcal manipulation of host immune responses. **Nature reviews Microbiology**, v.13, p.529-543, 2015.

THOMER *et al.* Antibodies against a secreted product of *Staphylococcus aureus* trigger phagocytic killing. **The Journal of experimental medicine**, v. 213, p. 293-301, 2016.

TIELEN *et al*. Alginate acetylation influences initial surface colonization by mucoid *Pseudomonas aeruginosa*. **Microbiol. Res**., v.160, p. 165-176, 2005.

TOLKER-NIELSEN, T. *Pseudomonas aeruginosa* biofilm infections: From molecular biofilm biology to new treatment possibilities. **APMIS Suppl**, v. 122, p. 1-51, 2014.

TOLMACHEVA, A. A.; ROGOZHIN, E. A.; DERYABIN, D. G. Antibacterial and quórum sensing regulatory activities of some traditional Eastern-European medicinal plants. **Acta Pharmaceutica,** v. 64, n. 2, p. 173-186, 2014.

TOMAINO *et al*. Influence of heating on antioxidant activity and the chemical composition of some spice essential oils. **Food Chem**, v. 89, p. 549-554, 2005.

TOMAZONI, E. Z. *et al*. Atividade antifúngica do óleo essencial de *Cinnamomum zeylanicum* Ness sobre fungos fitopatogênicos do tomateiro (*Lycopersicon esculentum* Mill.). In: VIII CONGRESSO BRASILEIRO DE AGROECOLOGIA, 2013, Porto Alegre. **Anais eletrônicos**. Porto Alegre: Universidade de Caxias do Sul: 2013. Disponível em: <www.abaagroecologia.org.br/revistas/index.php/cad/article/download/13846/9464>. Acesso em 18 nov. 2017.

TRENTIN, D. S. *et al*. Potential of medicinal plants from the Brazilian semi-arid region (Caatinga) against *Staphylococcus epidermidis* planktonic and biofilm lifestyles. **Journal of Ethnopharmacology**, v.137, p.327-335, 2011.

TRENTIN, D. S. *et al*. Tannins possessing bacteriostatic effect impair *Pseudomonas aeruginosa* adhesion and biofilm formation. **PLoS One**, v. 8, p. 1-13, 2013.

TSAO, R.; YANG, R.; YOUNG, J. C. Antioxidant isoflavones in osage orange, maclura pomifera (Raf.) schneid. **Journal Agricultural and Food Chemistry**. v. 51, p. 6445-6451, 2003.

TSUCHIYA, H.; IINUMA, M. Reduction of membrane fluidity by antibacterial sophoraflavanone G isolated from *Sophora exigua*. **Phytomedicine. Stuttgart**. v. 7, n. 2, p. 161-165, 2000.

VAARA, M. Agents that increase the permeability of the outer membrane. **Microbiological Reviews**, v. 56, n.3, p. 395-411, 1992.

VALGIMIGLI, L. PRATT, D. A. Antioxidants in Chemistry and Biology. **Encyclopedia of Radicals in Chemistry, Biology and Materials**, 2012.

VASCONCELOS *et al*. Antibacterial and antioxidant activities of aerriobtusone A isolated from *Lonchocarpus obtusus*. **Biomed res Int**, v. 2014, p. 1-9, 2014.

VIAU. C. M. *et al*. The natural triterpene 3β,6β,16β-trihydroxy-lup-20 (29)-ene obtained from the flowers of *Combretum leprosum* induces apoptosis in MCF-7 breast cancer cells. **BMC Complementary and Alternative Medicine**, v. 14, n. 280, p. 1-12, 2014.

VILLARES, A. *et al*. Content and Profile of Isoflavones in Soy-Based Foods as a Function of the Production Process. **Food Bioprocess Technology**. v. 4, p. 27-38, 2011.

VISHNUVATHAN, V.J; LAKSHMI, K.S; SRIVIDYA, A.R. Medicinal uses of formononetin - a review. **The Journal of Ethnobiology and Traditional Medicine**, v. 126, p.1197-1209, 2016.

VUUREN; HOLL, Antimicrobial natural product research: A review from a South African perspective for the years 2009-2016. **Journal of Ethnopharmacology**, n. 208, p. 236–252, 2017.

WADA, S-I.; IIDA, A.; TANAKA, R. Screening of Triterpenoids Isolated from *Phyllanthus flexuosus* for DNA Topoisomerase Inhibitory Activity. **Nat. Prod**,. v. 64, p. 1545-1547, 2001.

WAHID, A. *et al*. Heat tolerance in plants: An overview. **Environ. Exp. Bot.**, v. 61, n. 3, p. 199-233, 2007.

WAHLBERG, I. *et al.* Constituents of commercial Tolu balsam. **Acta Chem Scand**, v. 25, p. 3285-3295, 1971.

WALKER, T. D. The medicines trade in the Portuguese Atlantic World: acquisition and dissemination of healing knowledge form Brazil (c. 1580-1800). In: **Social History of Medicine Advanced Access**. Oxford: Oxford University Press, 2013.

WANG, Q., WANG, H., XIE, M. Antibacterial mechanism of soybean isoflavone on S*taphylococcus aureus*. **Arch Microbiol**., v.192, p. 893-898, 2010.

WANG, T; JÓNSDÓTTIR, R; ÓLAFSDÓTTIR, G. Total phenolic compounds, radical scavenging and metal chelation of extracts from *Icelandic seaweeds*. **Food Chemistry**, v. 116, p. 240-248, 2009.

WANG. *et al*. Antibacterial and Synergistic Activity of Pentacyclic Triterpenoids Isolated from *Alstonia scholaris*. **Molecules**, v. 21, n.139, p. 1-11, 2016.

WANNER, J. *et al*. Chemical composition and antibacterial activity of selected essential oils and some of their main compounds. **Nat Prod Commun**, v.5, p.1359-1364, 2010.

WATSON, L; DALLWITZ, M.J. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Disponível em http://delta-intkey.com (acesso em 10-V-2017), 2009.

WEI; MA. Biofilm matrix and its regulation in *Pseudomonas aeruginosa*. **Int. J. Mol. Sci**., v. 14, n. 10, 2013.

WHITE, R.L.; BURGESS, D.S.; MANDURU, M.; BOSSO, J.A. Comparasion of three different in vitro methods of detecting synergy: Time-kill, *checkerboard* and E-test. **Antimicrobial agents and chemotherapy.** v. 40, p.1914-1918, 1996.

WILLDENOW, K. Species plantarum, 4 ed. Impensis G. C. **Nauk, Berolin**,1799.

WRIGHT, G. D. Bacterial resistance to antibiotics: enzymatic degradation and modification. **Adv Drug Deliv Rev,** v. 57, n. 10, p. 1451-1470. 2005.

YARWOOD *et al*. *Quorum sensing* in *Staphylococcus aureus* biofilms. **J Bacteriol**, v.186, p. 1838-1850, 2004.

YIN, H.; XU, L.; PORTER, N.A. Free Radical Lipid Peroxidation: Mechanisms and Analysis. **Chem. Rev**., v. 111, n. 10, p. 5944–5972, 2011.

ZAPOTOCZNA *et al.* An essential role for coagulase in *Staphylococcus aureus* biofilm development reveals new therapeutic possibilities for device-related infections. **J Infect Dis**, v.212, p. 1883-1893, 2015.

ZHENG, W.; WANG, S.Y. Antioxidant activity and phenolic compounds in selected herbs. **J. Agric. Food Chem.** v. 49, p. 5165-5170, 2001.

ZHU, M. Application of 7-hydroxy-4'-methoxy-isoflavone in preparation of antifungal drugs. Patent CN103565794A, filled Nov 08, 2013 issued Feb 12, 2014.

ZORE, G. *et al*. Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. **Phytomed**, v.18 p.1181–1190, 2011.

ZWENGER, S.; BASU, C. Plant terpenoids: applications and future otentials. **Biotechnol. Mol. Biol**., v. 3, p.1-7, 2008.

ANEXO I e II

ESPECTROS DO COMPOSTO ISOLADO DE *Myroxylon peruiferum* L.f

Figura 1. Espectro de RMN[1] H (piridina, 500 MHz) da 7-hidroxi-6,4'-dimetoxi-isoflavona

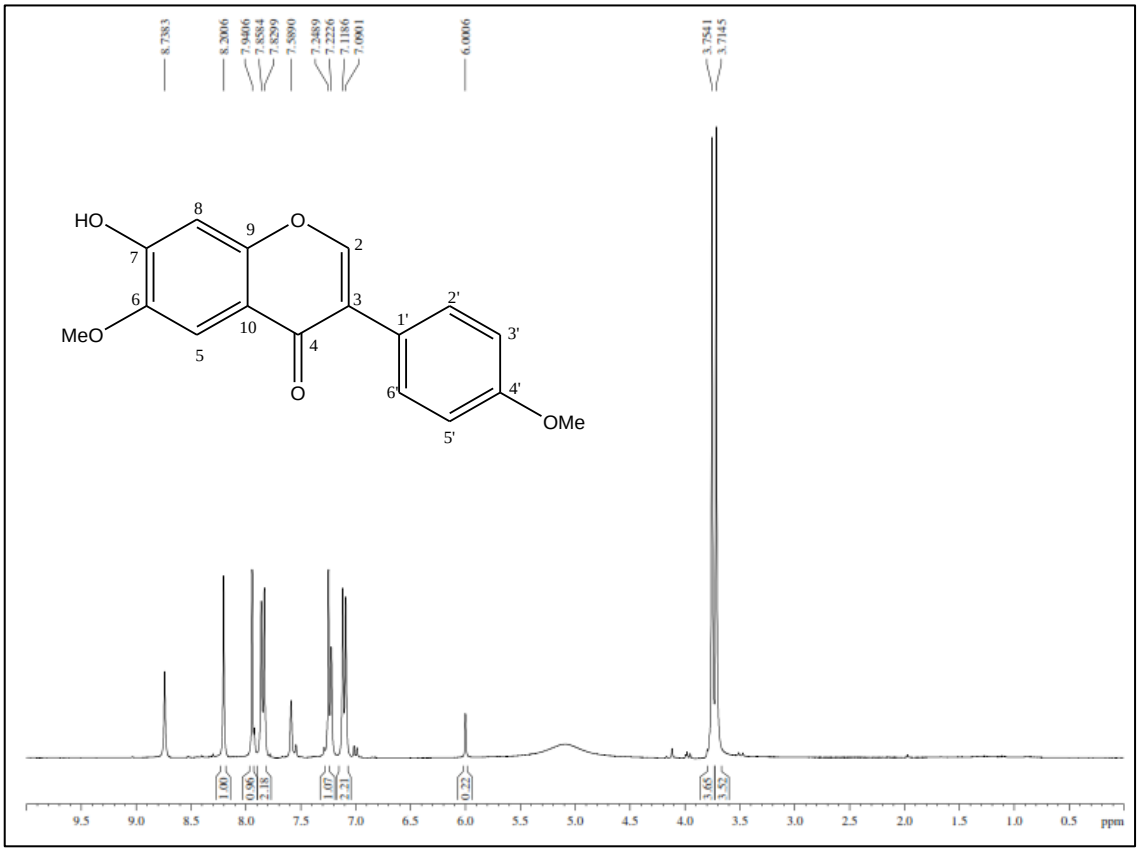


Figura 2. Expansão do espectro de RMN[1] H (piridina, 500 MHz) da 7-hidroxi-6,4'-dimetoxi-isoflavona

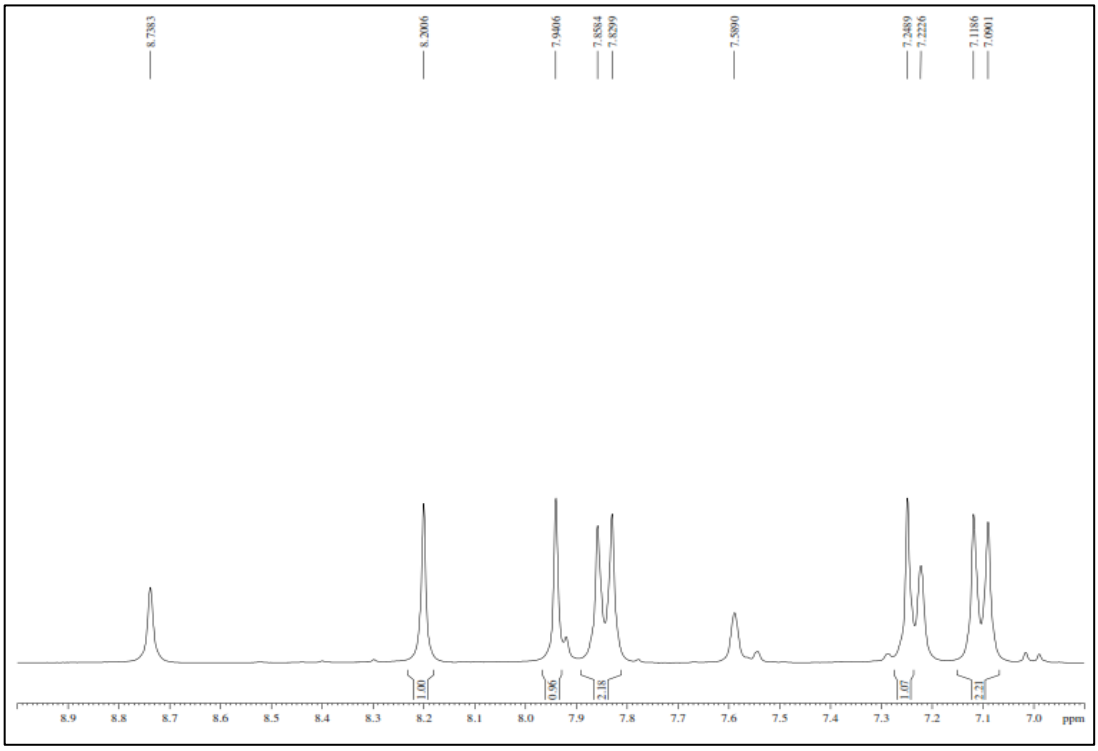


Figura 3. Espectro de RMN^{13} C (piridina, 125 MHz) da 7-hidroxi-6,4'-dimetoxi-isoflavona

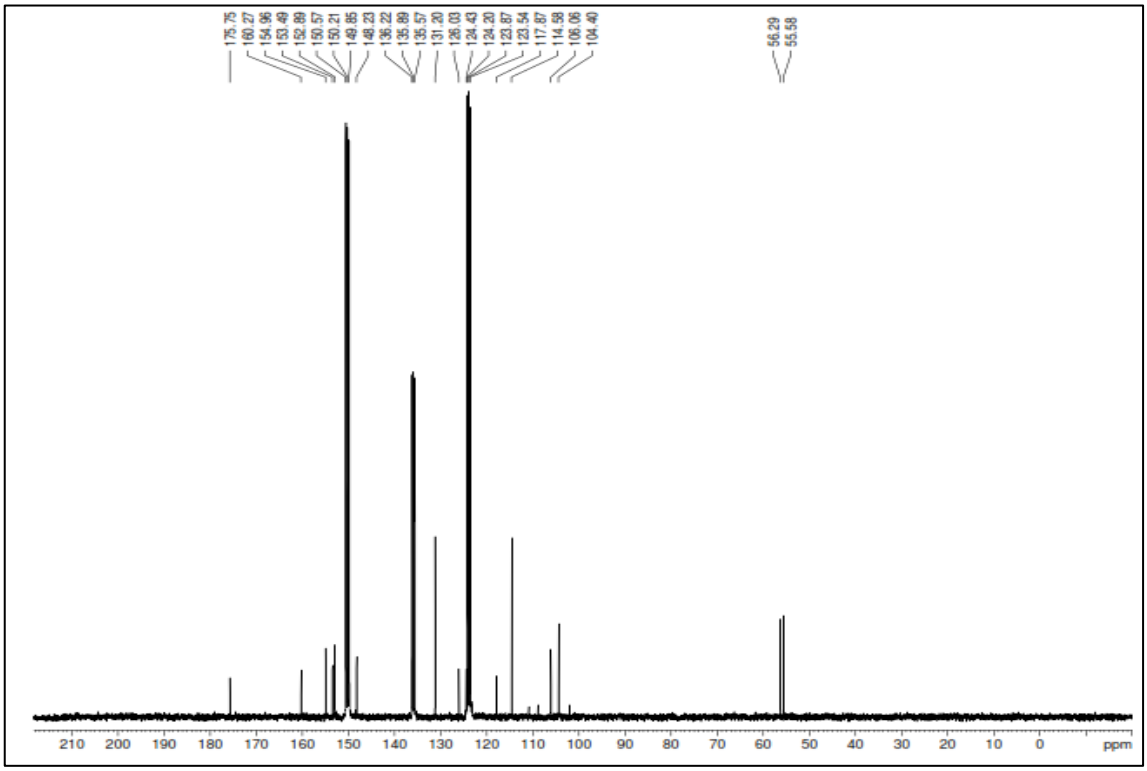


Figura 4- Espectro de RMN^{13}C-DEPT 135° (piridina, 500 MHz) da 7-hidroxi-6,4'-dimetoxi-isoflavona

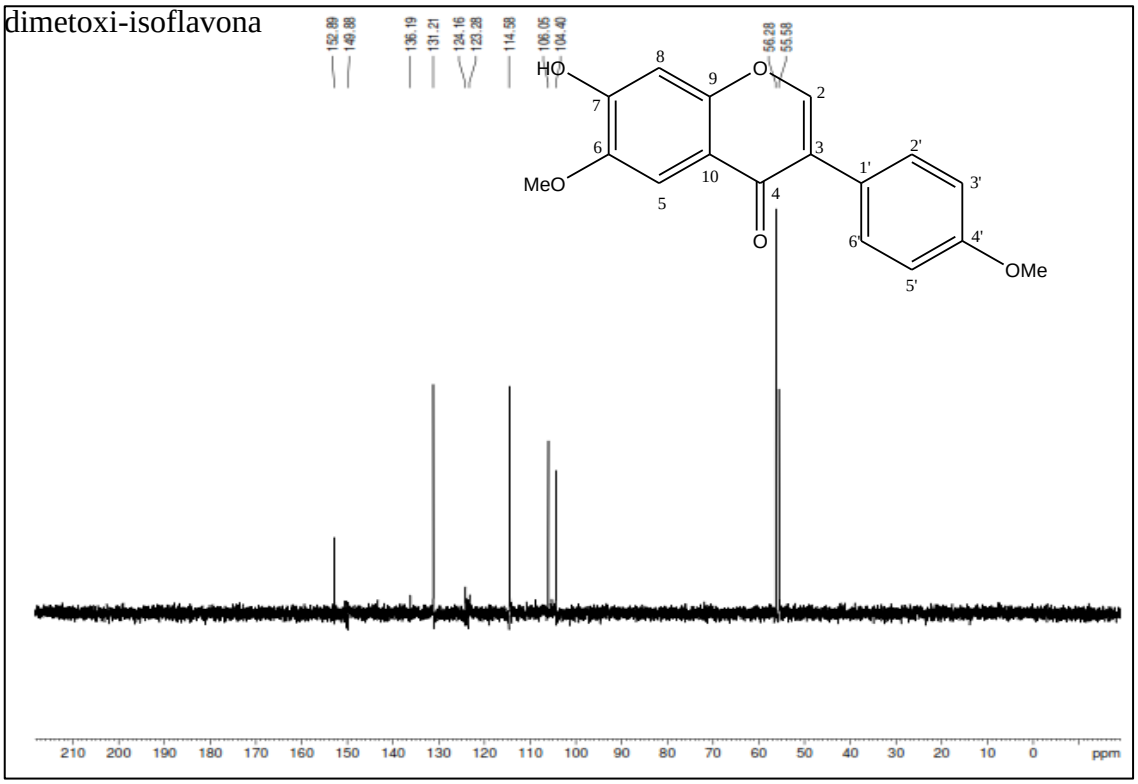


Figura 5- Mapa de correlação heteronuclear-HSQC (piridina, 500 MHz) da 7-hidroxi-6,4’-dimetoxi-isoflavona

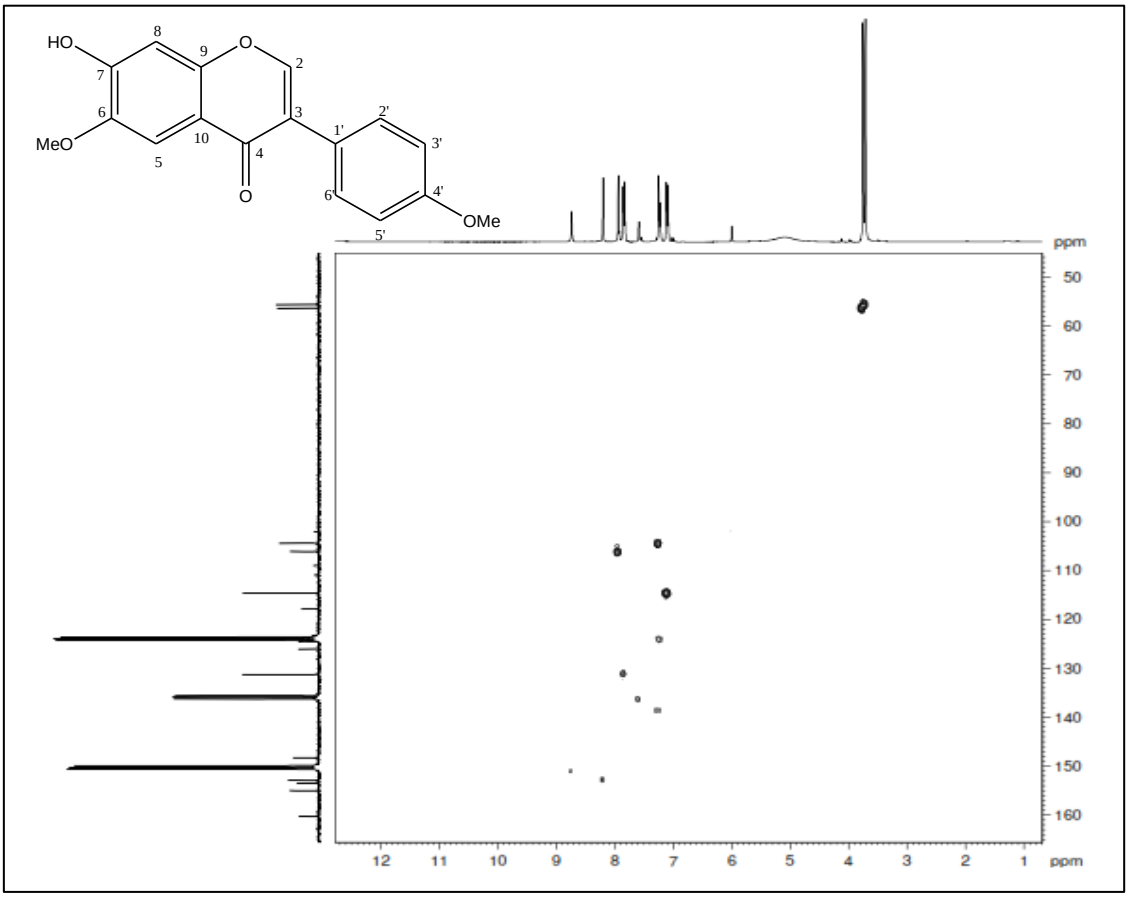


Figura 6 - Mapa de correlação heteronuclear-HSQC expansão (piridina, 500 MHz) da 7-hidroxi-6,4’-dimetoxi-isoflavona

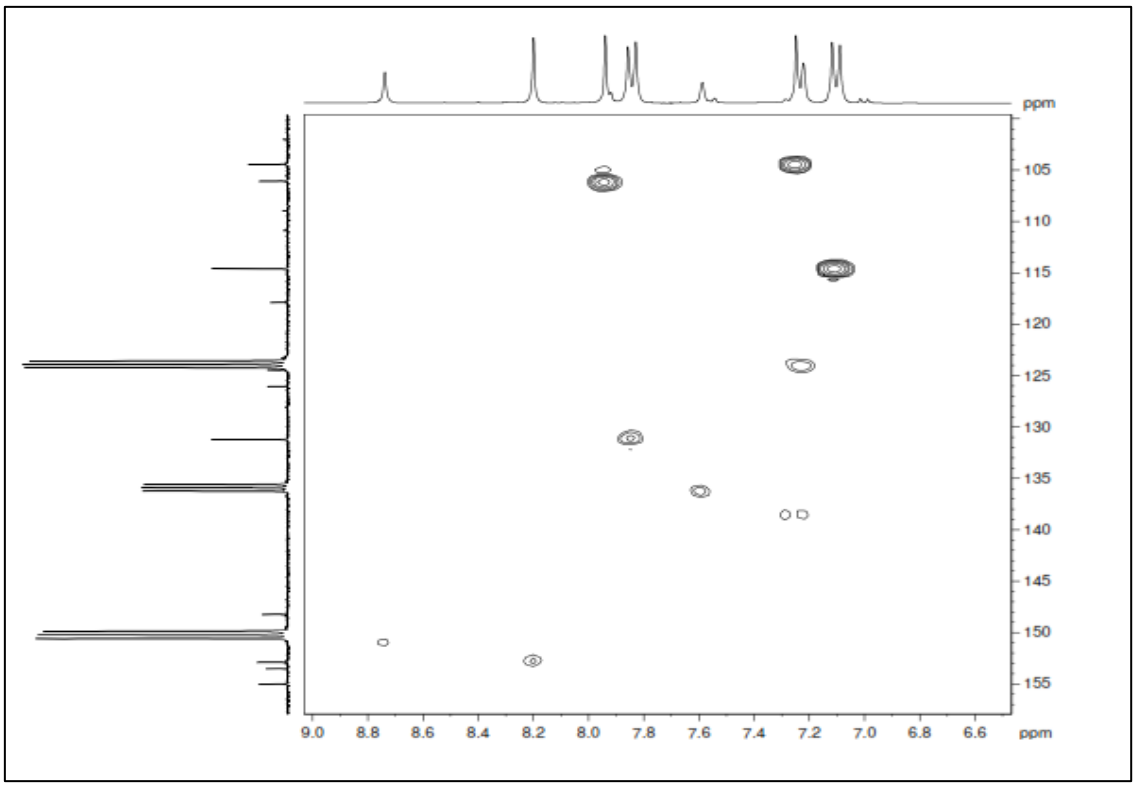


Figura 7- Mapa de correlação heteronuclear - HMBC (piridina, 500 MHz) da 7-hidroxi-6,4’-dimetoxi-isoflavona

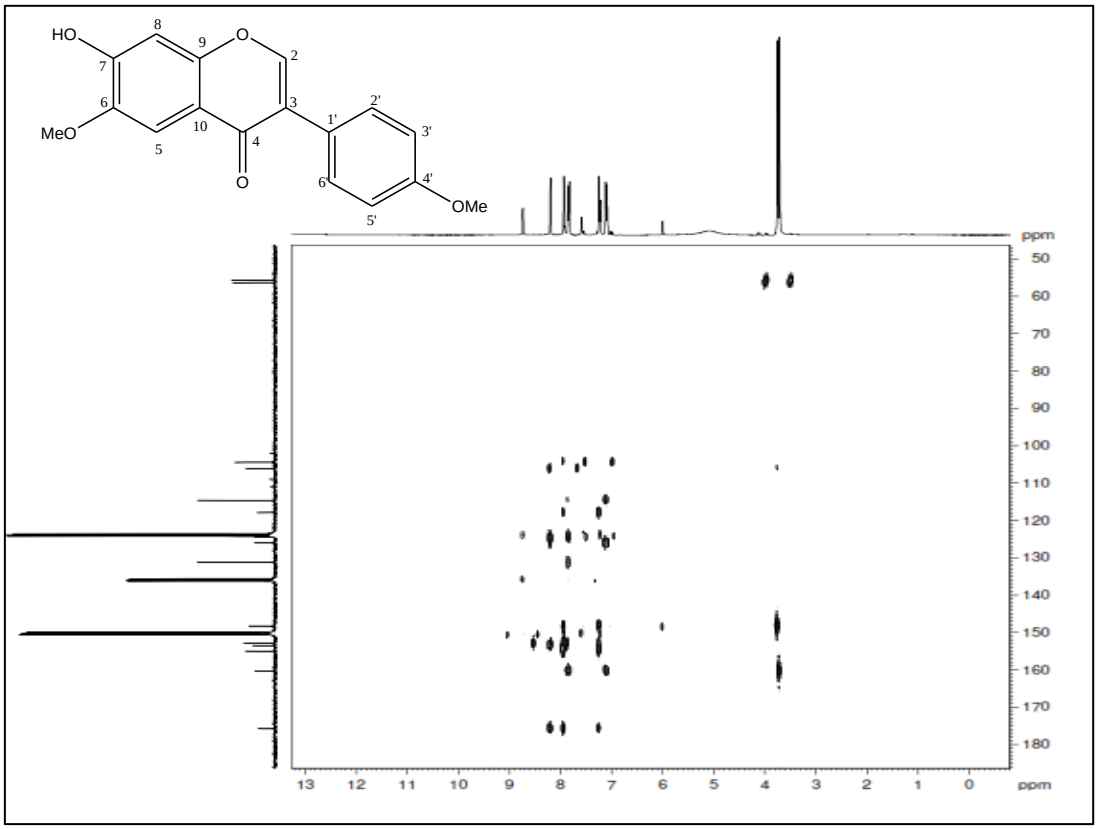
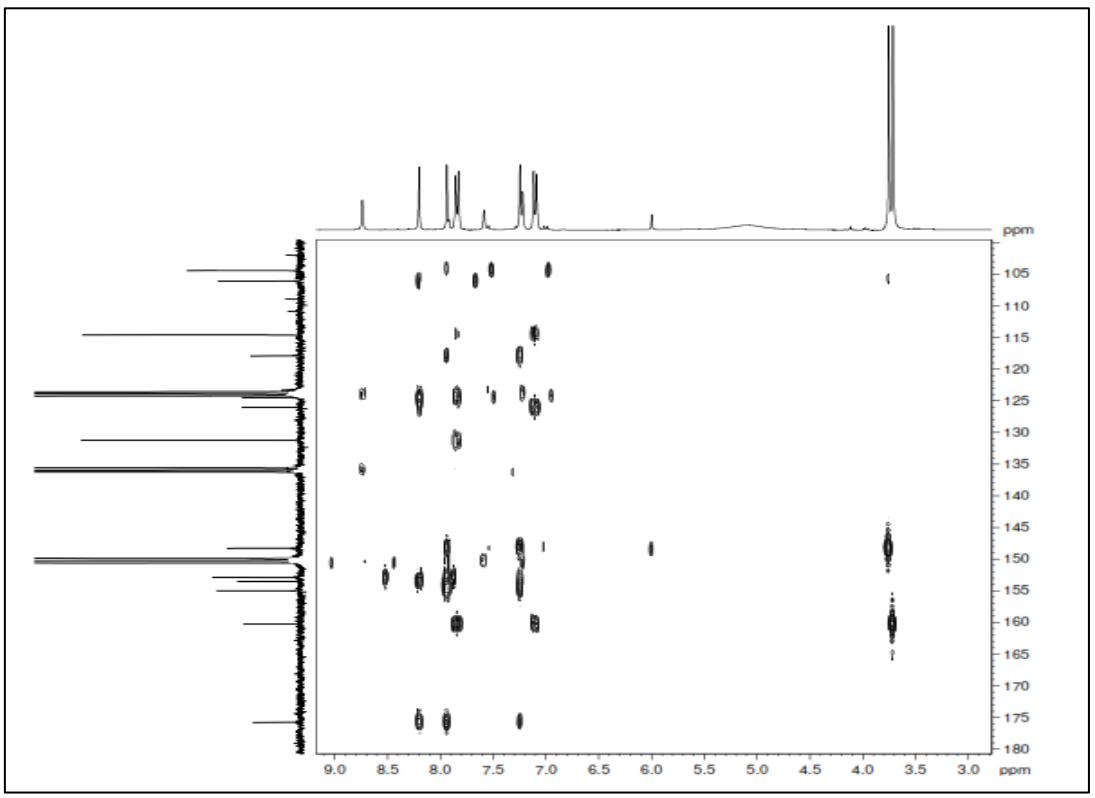


Figura 8 - Mapa de correlação heteronuclear – HMBC expansão (piridina, 500 MHz) da 7-hidroxi-6,4’-dimetoxi-isoflavona



ESPECTROS DO ÓLEO ESSENCIAL DE *Myroxylon peruiferum* L.f

Figura 1: Espectro de massa do tolueno

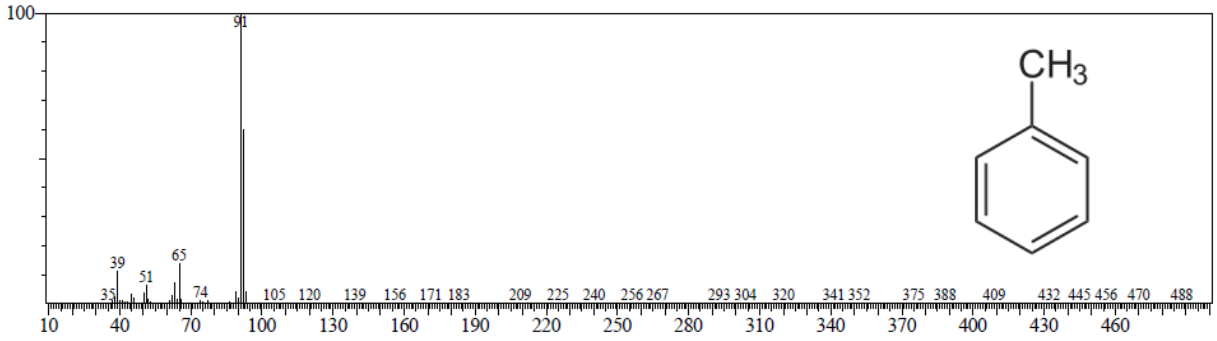


Figura 2: Espectro de massa do 2E-hexenol

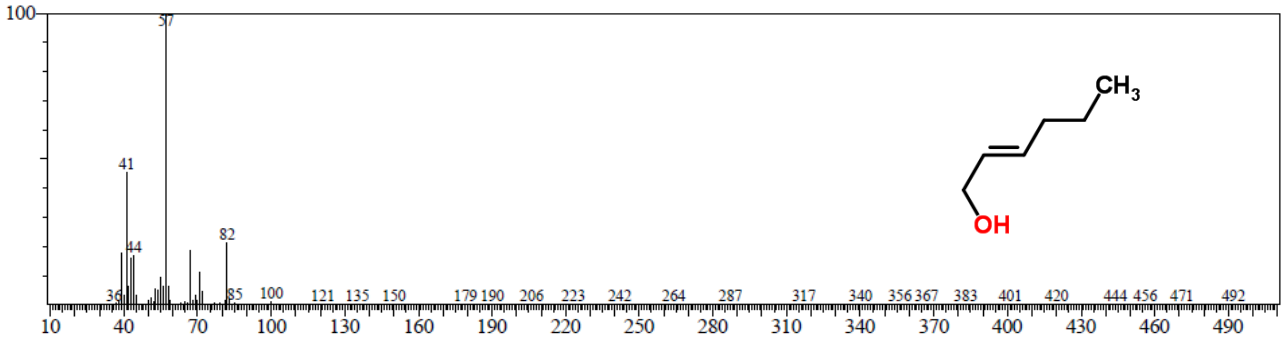


Figura 3: Espectro de massa do 1-Hexanol

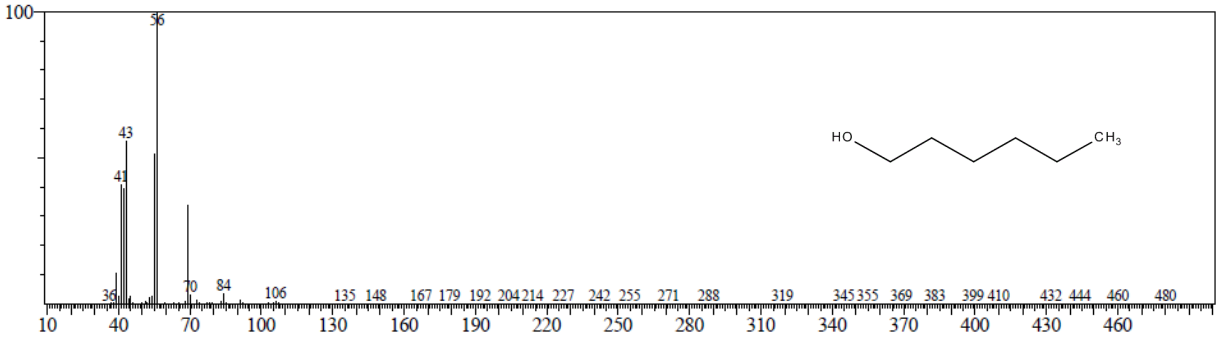


Figura 4: Espectro de massa do α-tujeno

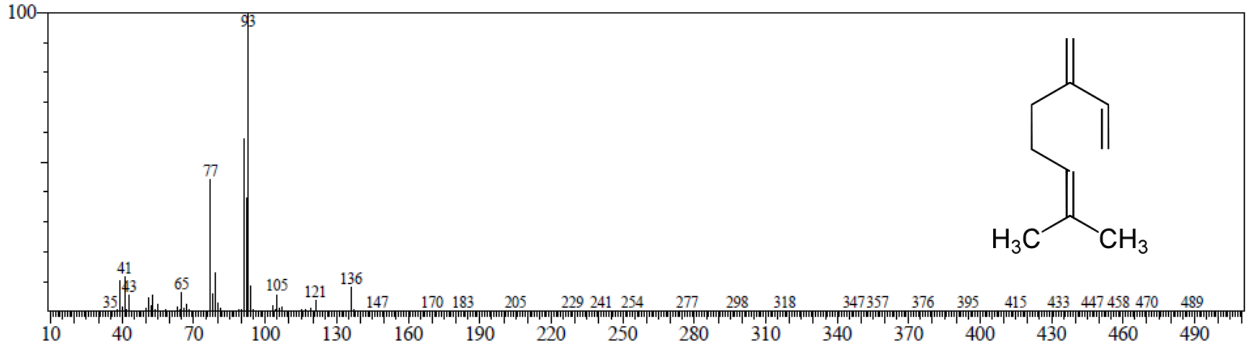


Figura 5: Espectro de massa do α-pineno

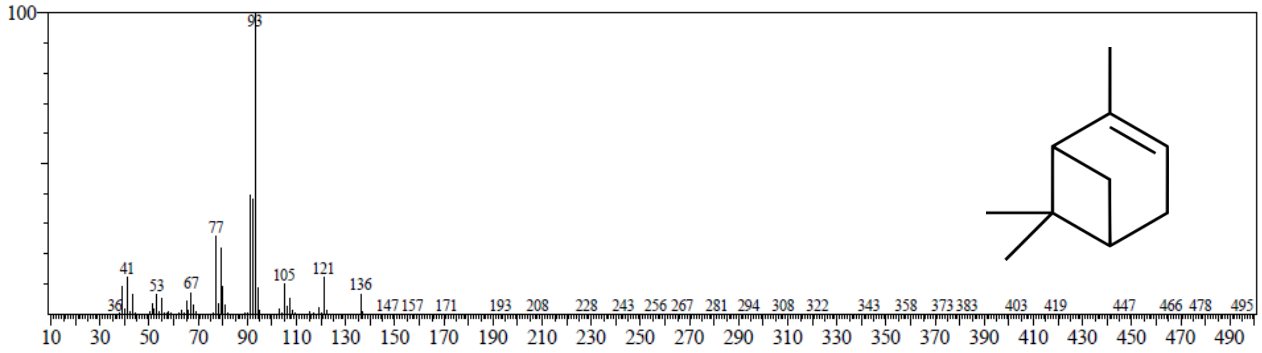


Figura 6: Espectro de massa do β-felandreno

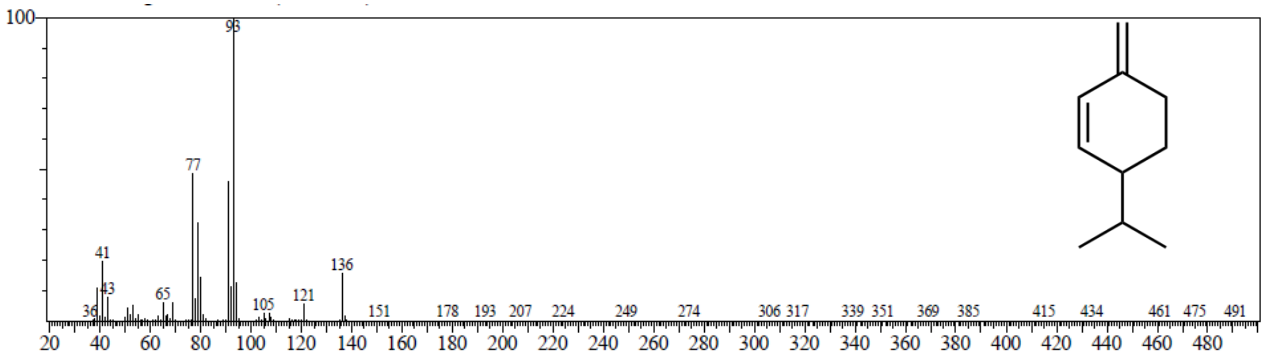


Figura 7: Espectro de massa do β-pineno

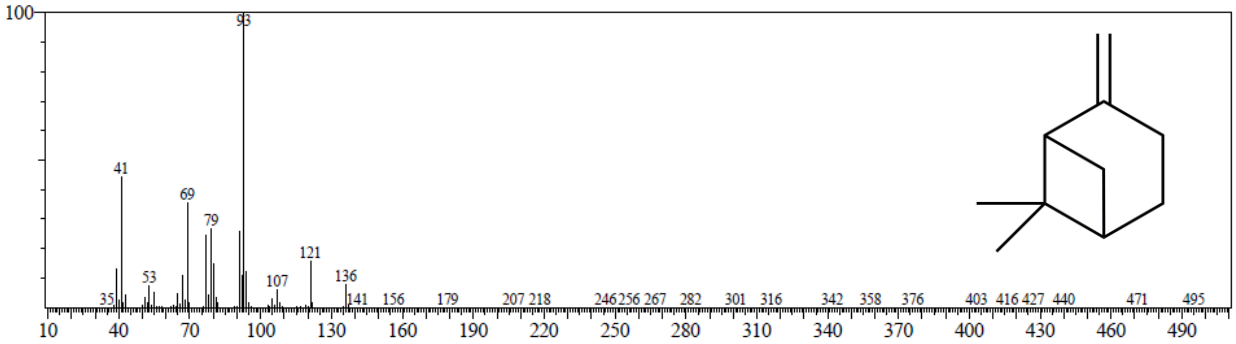


Figura 8: Espectro de massa do Mirceno

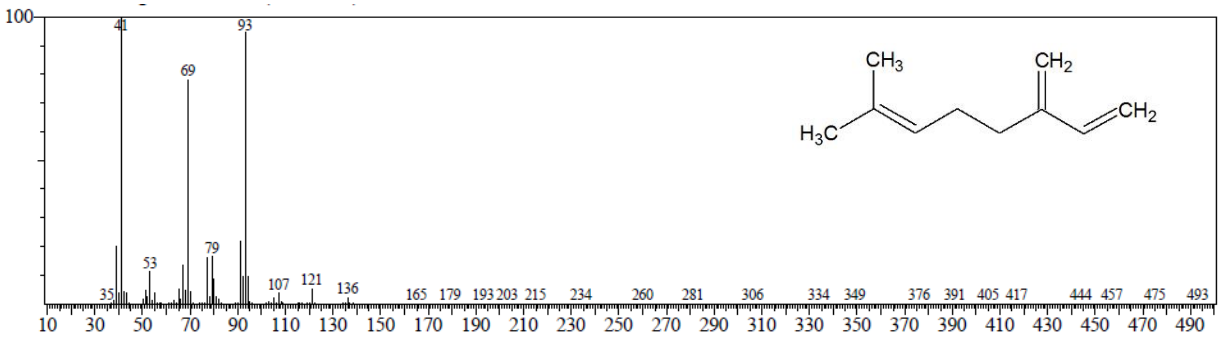


Figura 9: Espectro de massa do δ-2-careno

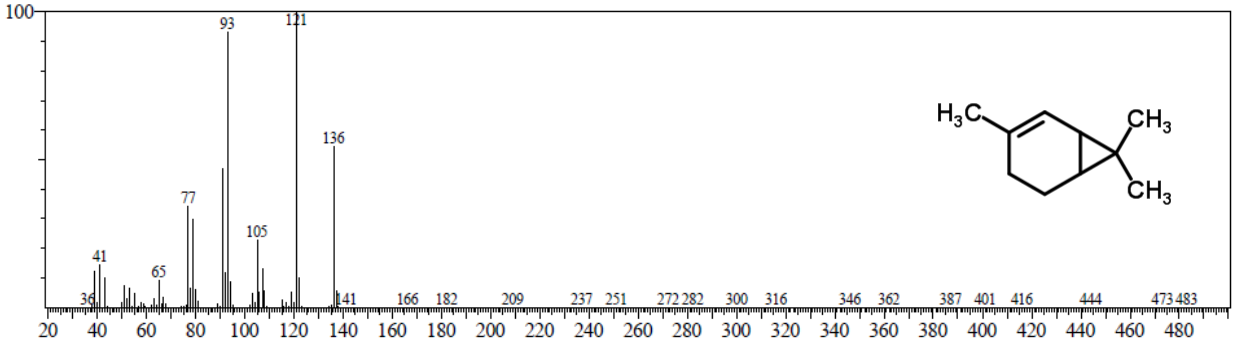


Figura 10: Espectro de massa do limoneno

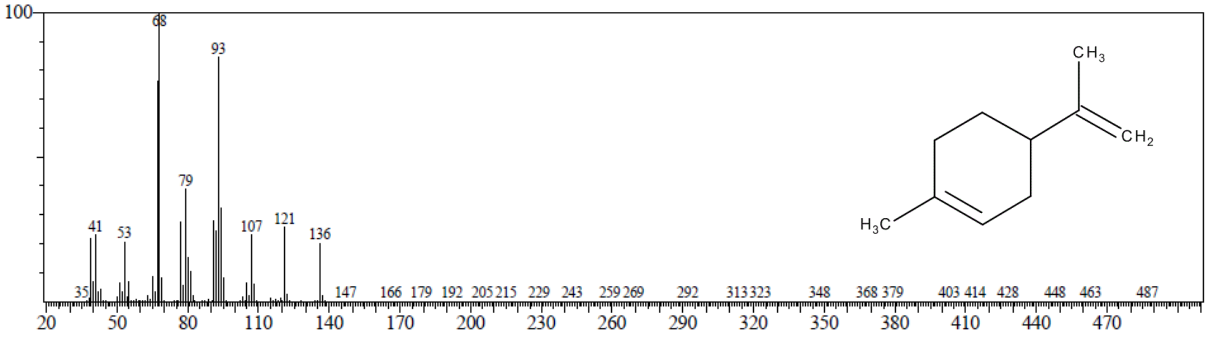


Figura 11: Espectro de massa do γ--terpineno

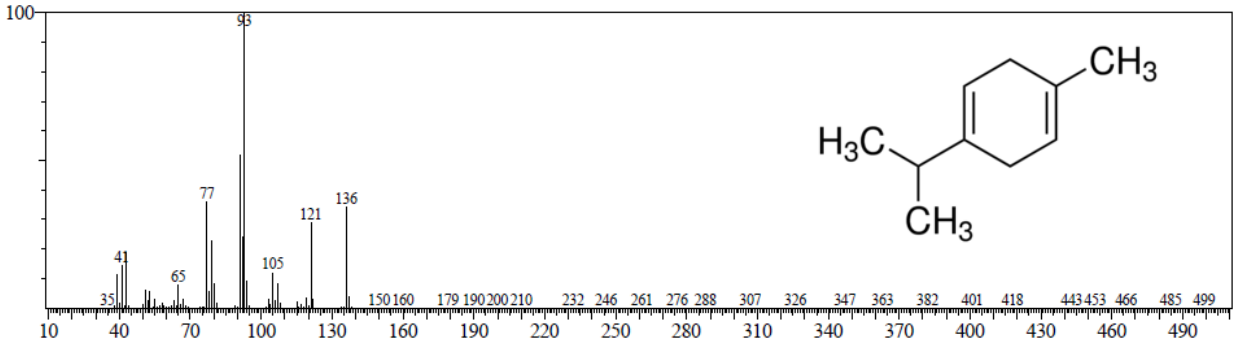


Figura 12: Espectro de massa do terpinoleno

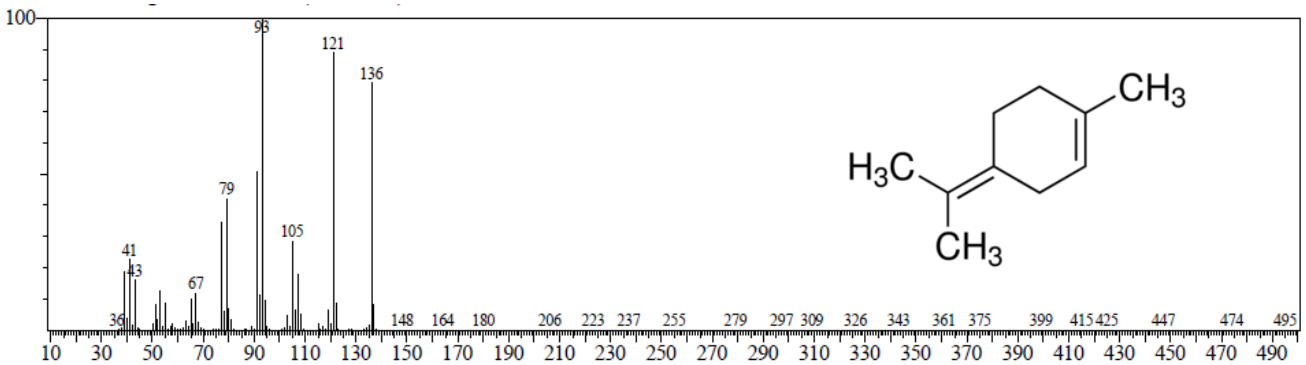


Figura 13: Espectro de massa do 4-terpineol

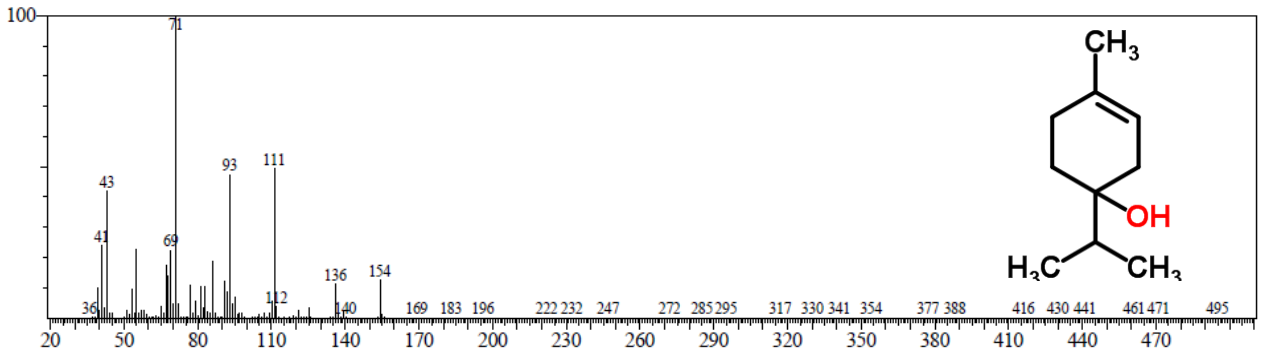


Figura 14: Espectro de massa do Nerol

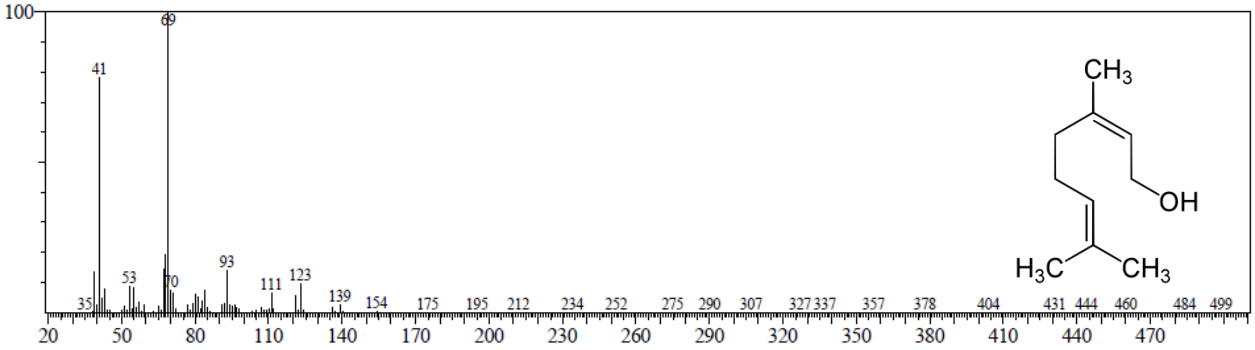


Figura 15: Espectro de massa do α-copaeno

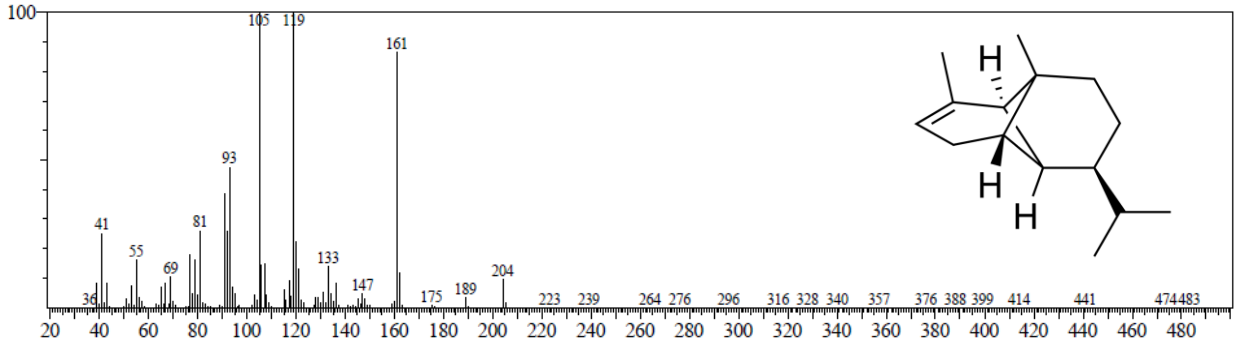


Figura 16: Espectro de massa do E- cariofileno

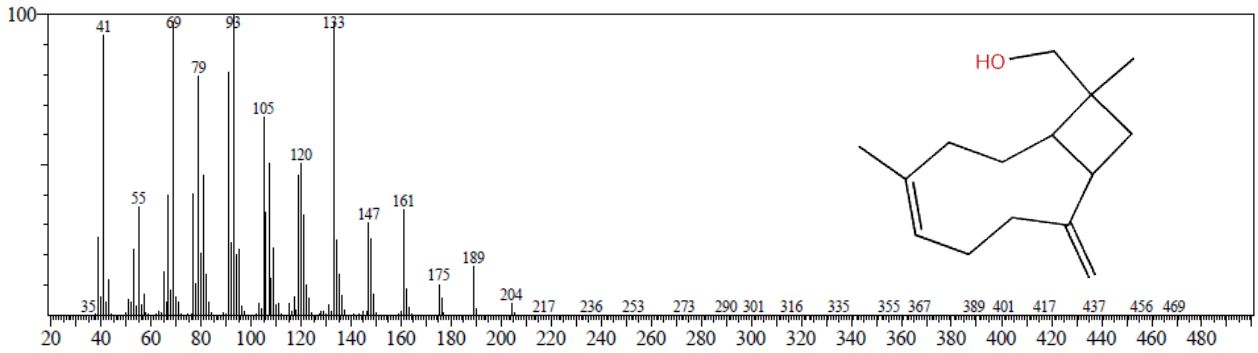


Figura 17: Espectro de massa do γ-elemeno

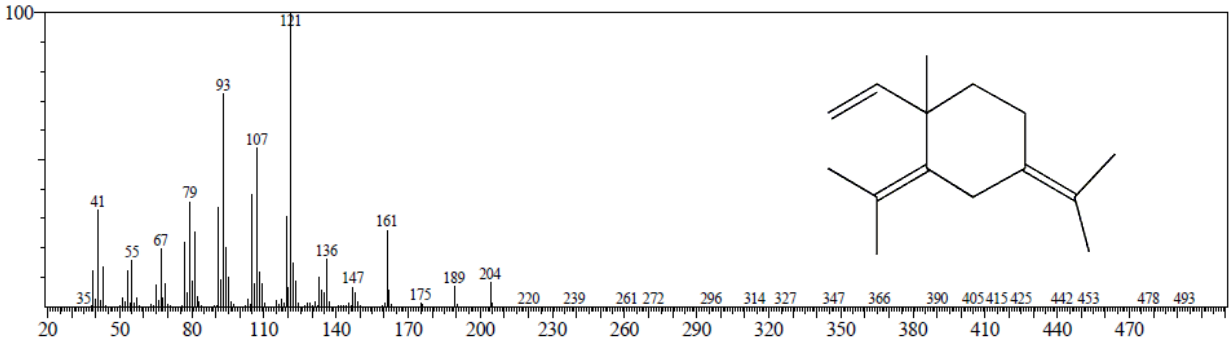


Figura 18: Espectro de massa do Germacreno D

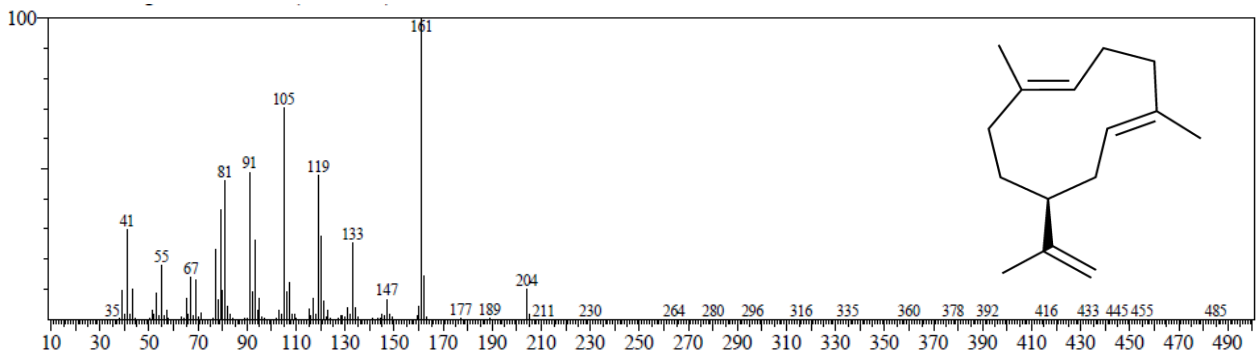


Figura 19: Espectro de massa do δ-cadineno

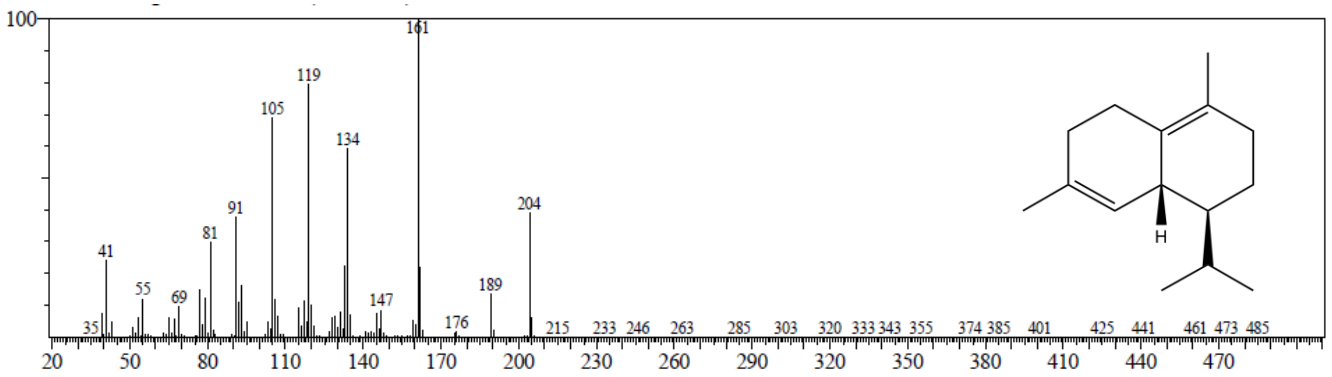


Figura 20: Espectro de massa do α-humuleno

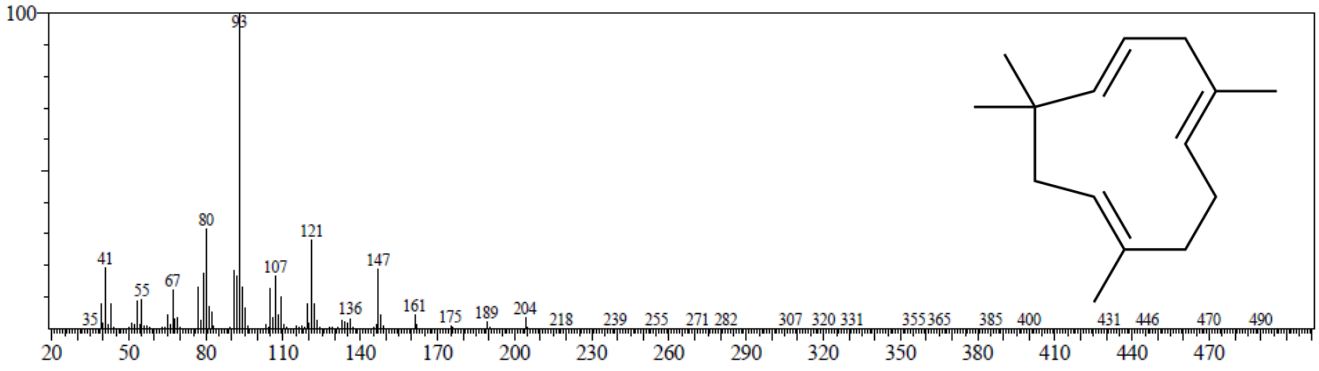


Figura 21: Espectro de massa do Germacreno B

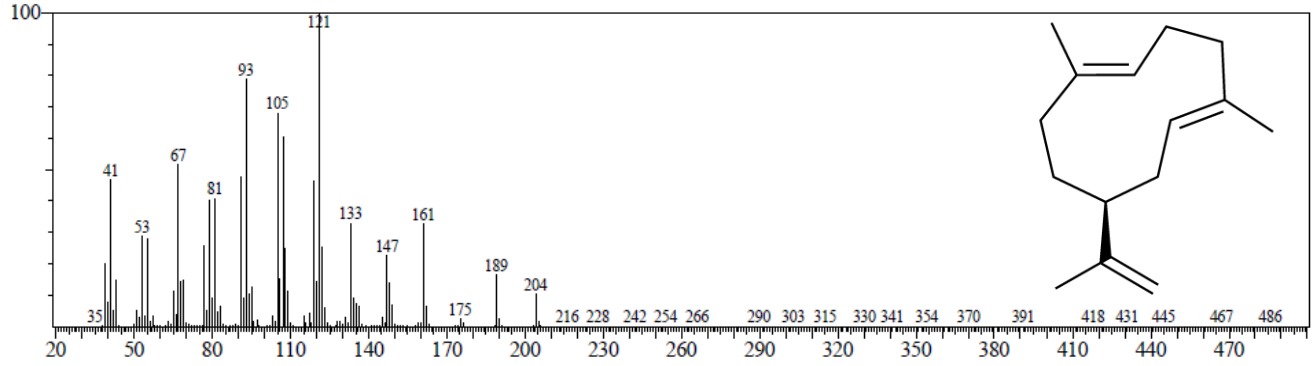


Figura 22: Espectro de massa do Espatulenol

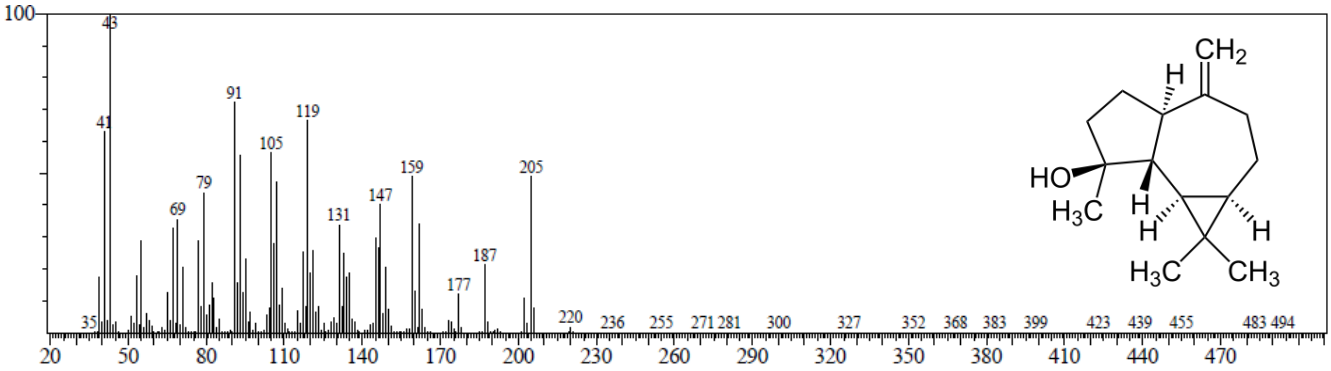


Figura 23: Espectro de massa de Óxido de cariofileno

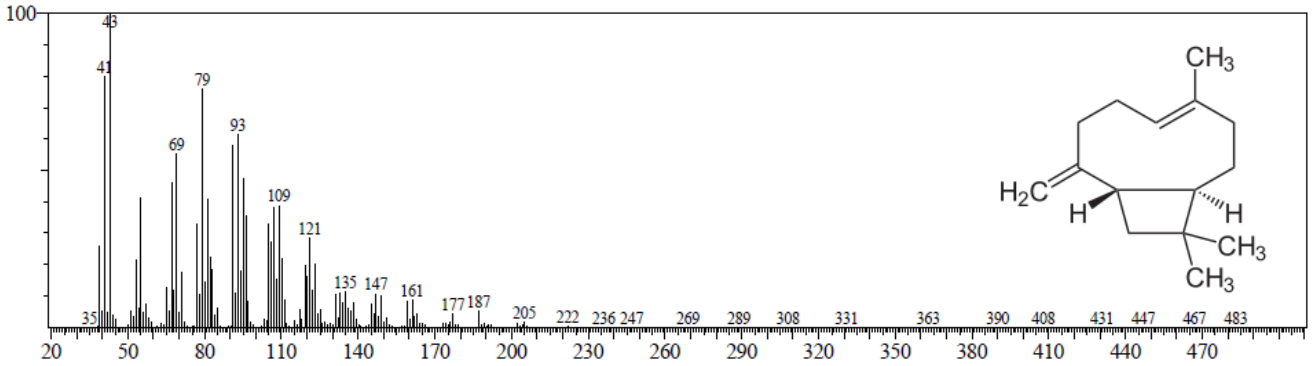


Figura 24: Espectro de massa do Ledol

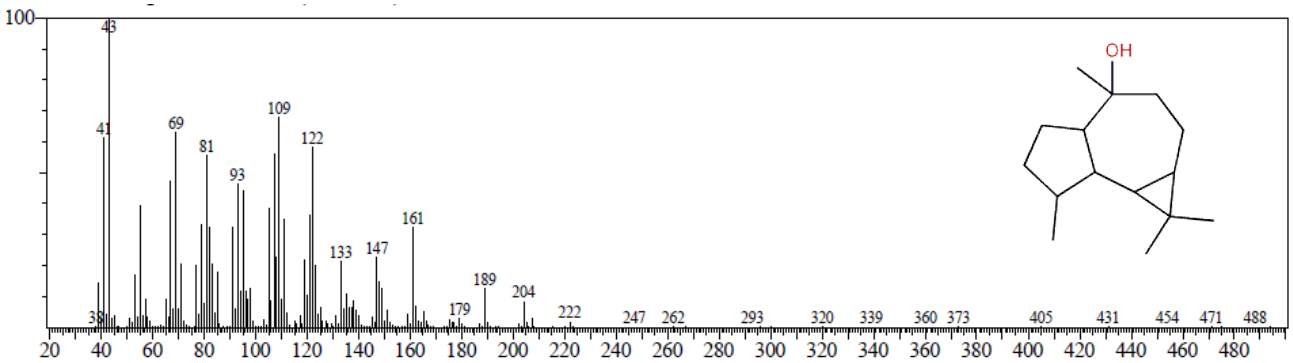
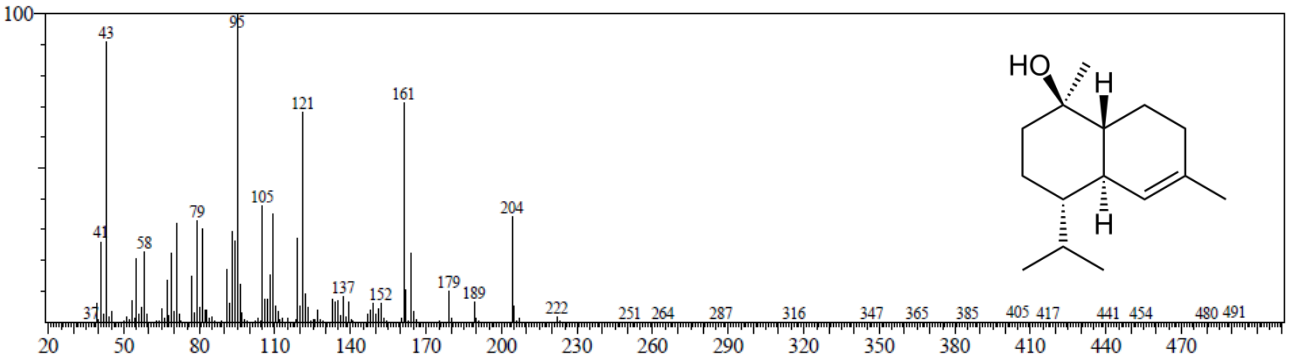


Figura 25: Espectro de massa do α-cadinol



ESPECTROS DO COMPOSTO ISOLADO DE *Combretum leprosum*

Figura 1 - Espectro de absorção na região do infravermelho de CLF1

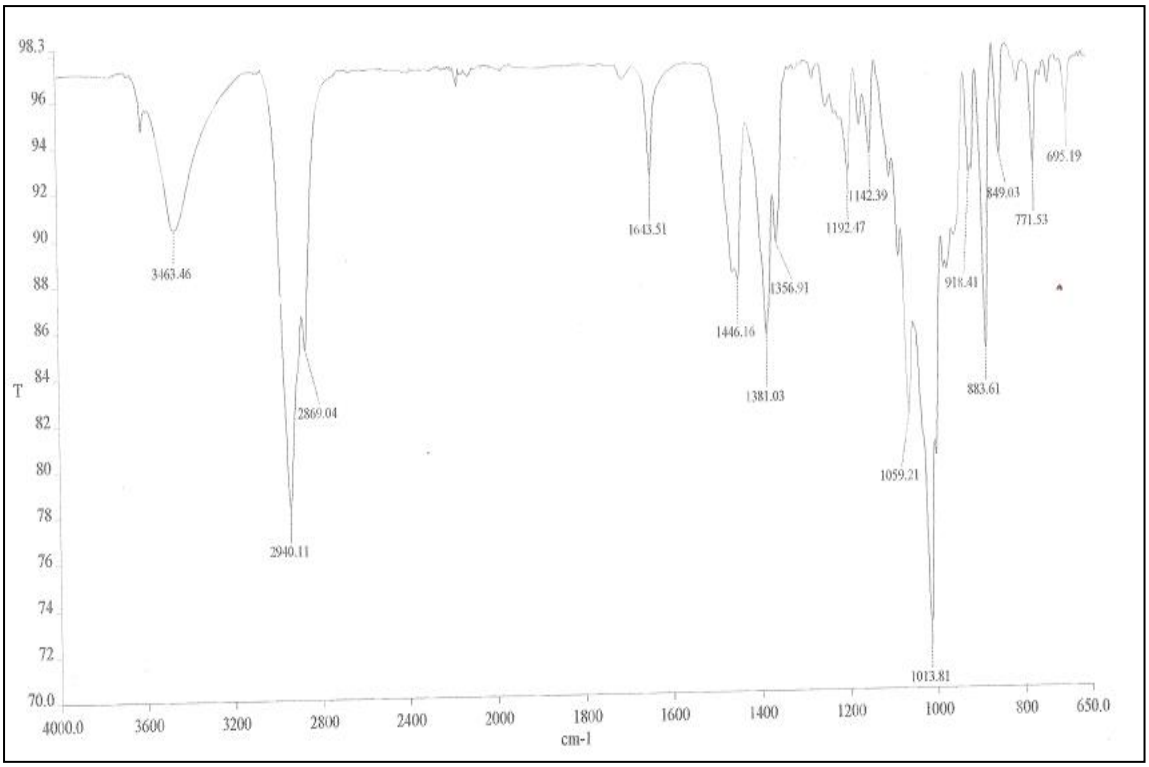


Figura 2 - Espectro de RMN ^{1}H (500 MHz, $CDCl_3$) de CLF1

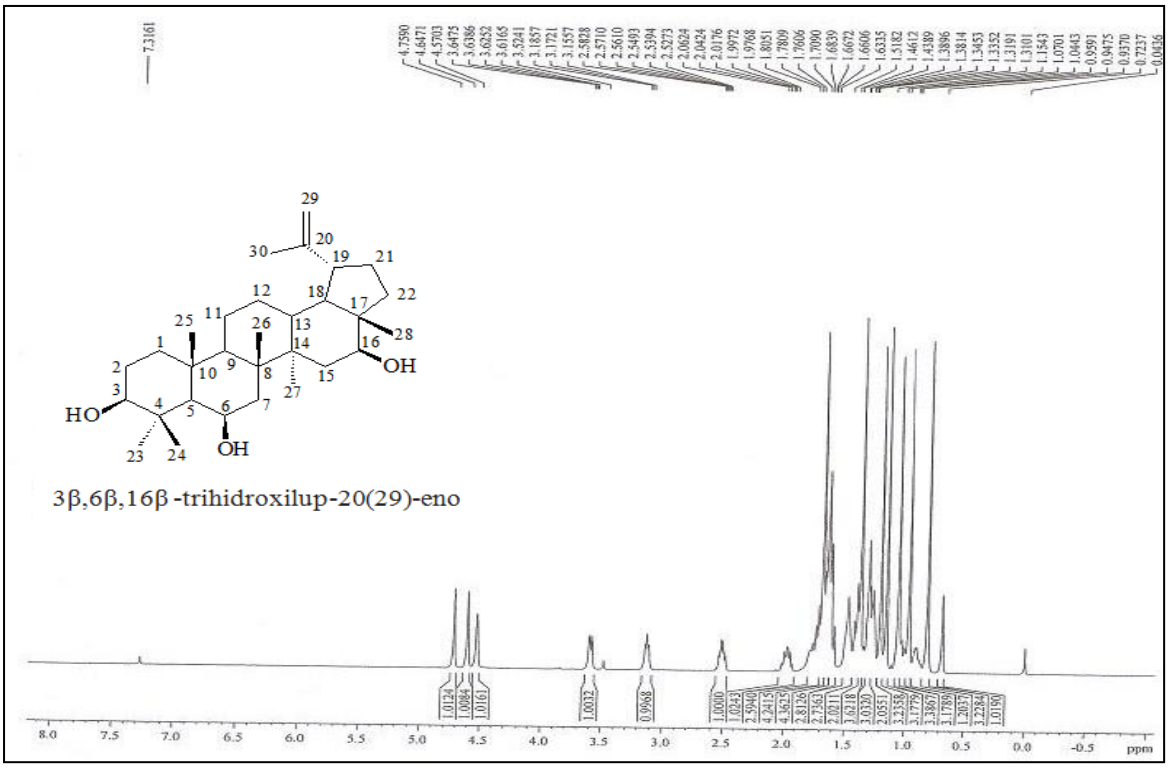


Figura 3 - Espectro de RMN ^{13}C (125 MHz, $CDCl_3$) de CLF1

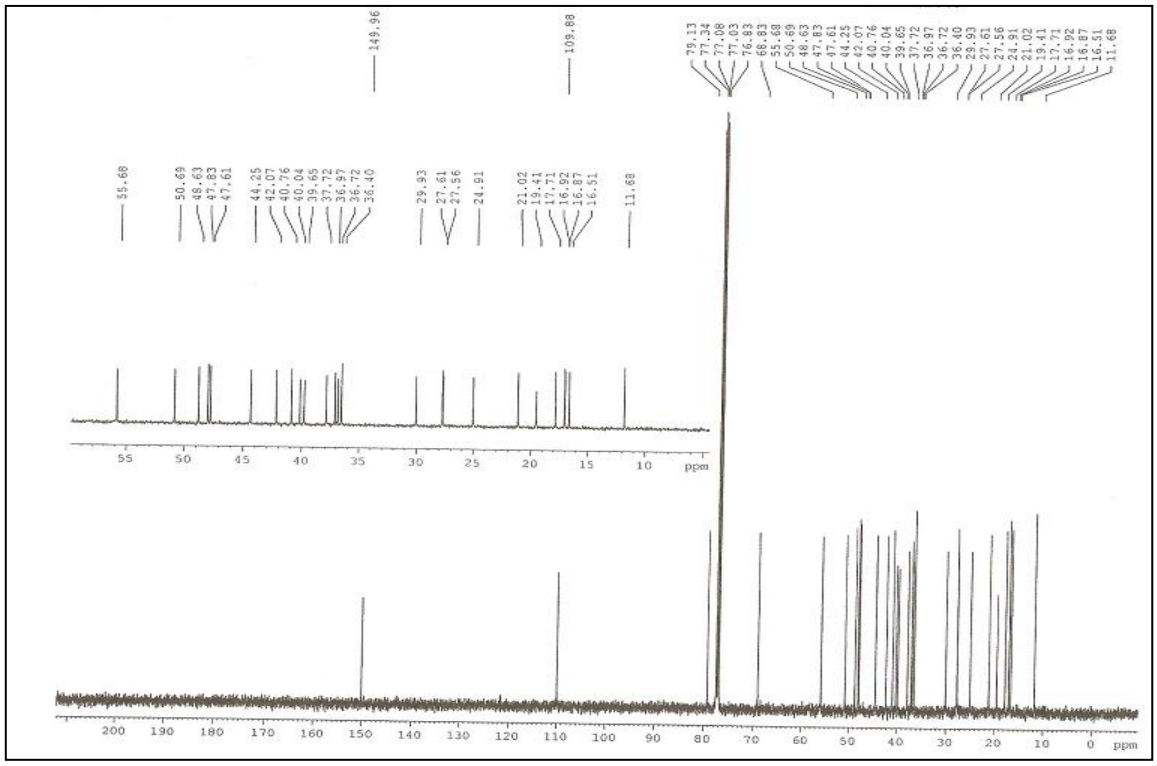


Figura 4. Espectro de RMN DEPT 135° (125 MHz, $CDCl_3$) de CLF1

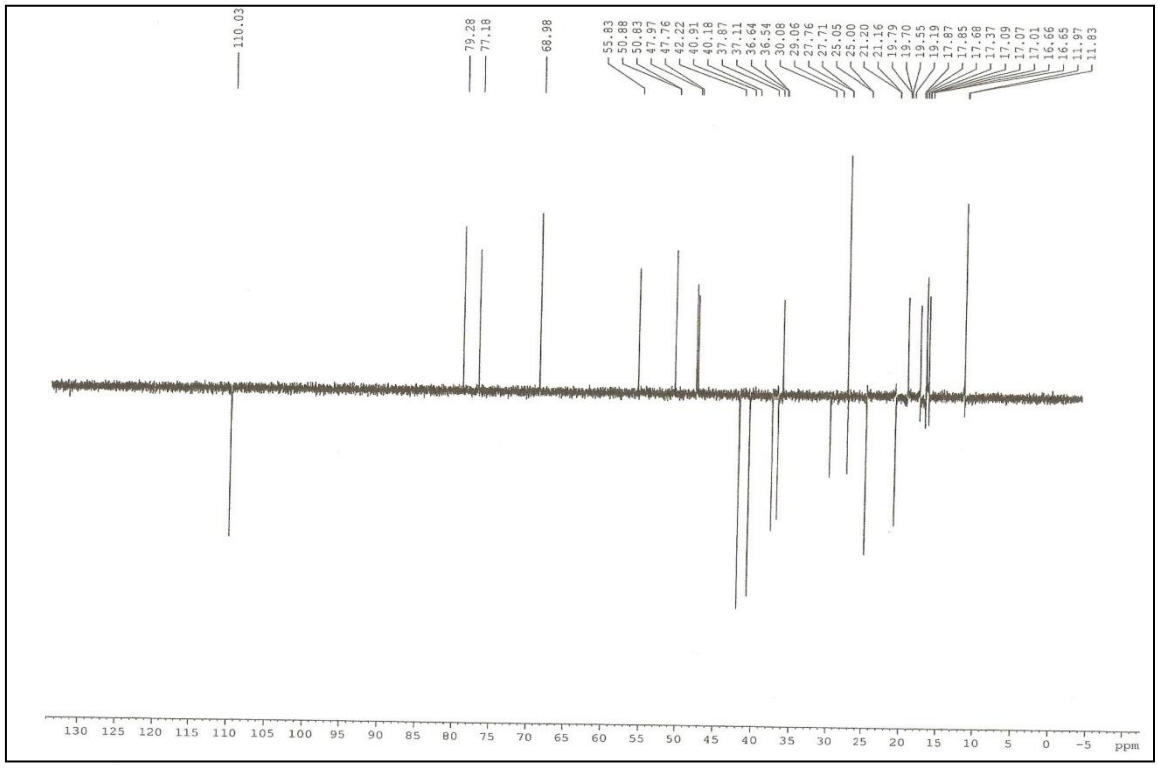
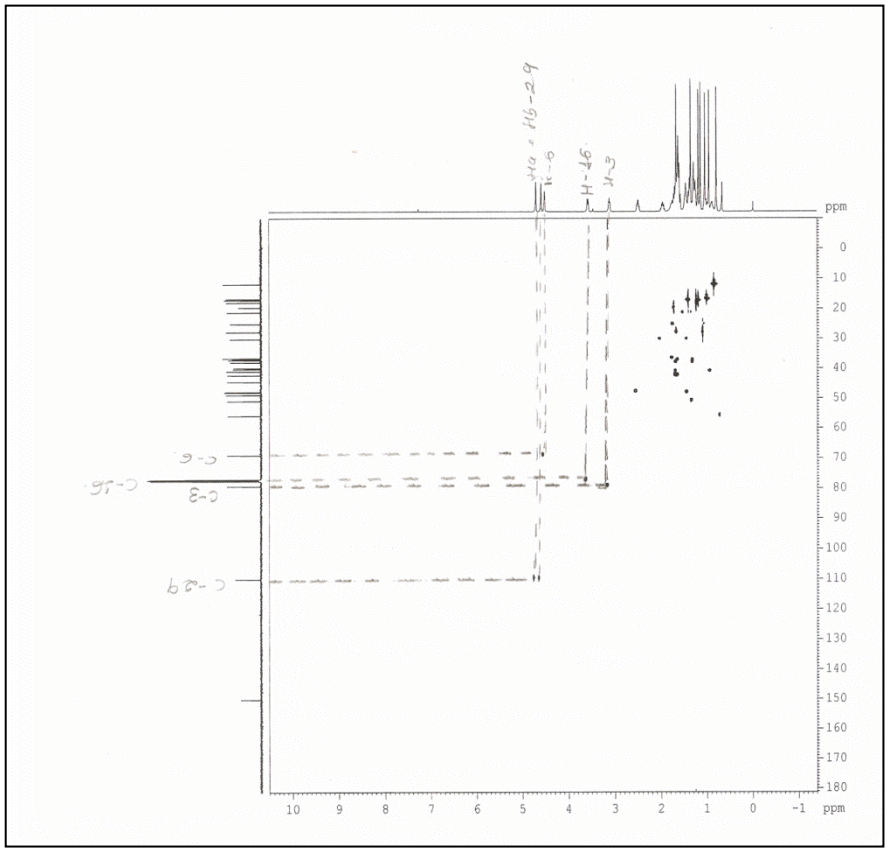


Figura 5 - Mapa de contorno HSQC (125 MHz, $CDCl_3$) de CLF1



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Full Length Research Paper

Cytotoxicity, antifungal and antioxidant activities of the essential oil from *Eupatorium ballotifolium* Kunth (Asteraceae)

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This study aimed to characterize chemically and evaluate the cytotoxicity and antioxidant, antifungal and modulatory activities of the essential oil of *Eupatorium ballotifolium*, collected in the mountainous region of Meruoca in the state of Ceará. The antioxidant activity was investigated by the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and β-carotene/linoleic-acid oxidation model system. The lethality bioassay was performed using *Artemia salina* (brine shrimp). The evaluation of the antifungal activity *in vitro* was performed by broth microdilution using strains of dermatophyte fungi and yeasts. The modulatory activity assays were performed by the checkerboard technique using ketoconazole as standard. Chromatographic analysis associated with mass spectrometry showed the main constituents of *E. ballotifolium* essential oil were β-caryophyllene (23.59%), thymol methyl ether (12.28%), germacrene D (6.56%) and bicyclogermacrene (6.47%). The brine shrimp lethality assay demonstrated potential biological activity. The essential oil showed better antioxidant action by the β-carotene/linoleic acid assay, with IC50 value of 19.47 µg/mL for essential oil, 11.32 µg/mL for thymol and 22.83 µg/mL for carvacrol, used as test standards. The broth microdilution test demonstrated that the essential oil inhibited fungal growth of all *Trichophyton rubrum* strains. In the modulation activity assay against strains of *Trichophyton rubrum,* there was synergism of essential oil on the strains of dermatophyte fungi when combined with ketoconazole.

Key words: Compositae, *Lourteigia ballotaefolia*, *Trichophyton rubrum*, antioxidant activity, antimicrobial activity.

INTRODUCTION

The Asteraceae family contains about 10% of the world's flora, and includes around 24,000 described species, grouped in 1,600 to 1,700 genera distributed in 17 tribes and 3 subfamilies (Funk et al., 2009; Petacci et al., 2012). It is the largest family of the eudicotyledons, and together with Calyceraceae, Campanulaceae, Menyanthaceae and Goodeniaceae, it forms a clade, the Asterales order (Pozner et al., 2012).

The genus *Eupatorium* L. (tribe Eupatorieae, subtribe Eupatoriinae) is significant in the Asteraceae family, comprising around 1,200 species. It is widely distributed, especially in Europe, Asia, North America and South America (Albuquerque et al., 2010). Brazil has around 250 native species with distribution in all geographical regions (Souza, 2007). *Eupatorium* species have been used to treat many diseases in folk medicine in various places in the world (Albuquerque et al., 2010).

Eupatorium ballotifolium Kunth (syn. *Lourteigia ballotaefolia* (Kunth) R.M. King & H.Rob.) is a perennial herbaceous species whose vertical growth ranges to 40 to 80 cm. It is an aromatic plant with few branches and many flowers, with pubescence and pink color with purple tones. Its leaves typically have purplish edges that are rich in oleíferas glands. The plant spreads through seeds (Silveira and Pessoa, 2005). It is popularly known in Brazil by many names, such as "maria-preta", "maria-preta-verdadeira" and "picão-roxo" (Cardoso et al., 2013).

Previous studies have demonstrated that the essential oil of the aerial parts of *E. ballotifolium* have anticholinesterase activity (Albuquerque et al., 2004), and two flavonoids were isolated from the aerial parts of the plant, nepetin and quercetin-3-O-glucoside, both showed antimitotic activity (Militão et al., 2004).

The therapeutic potential of many plants used in folk medicine has not been scientifically proved (Desoti et al., 2011). Hence, there is a need for chemical studies of natural products used in folk medicine to complement the studies to develop synthetic organic chemicals (Suffredini et al., 2006). Research of plants with antioxidant activity contributes to the development of new therapeutic strategies for inflammation, aging and chronic degenerative diseases (Fabri et al., 2011). Likewise, plants with potential antimicrobial activity can be a therapeutic alternative against multiresistant microorganisms to antimicrobial drugs (Bekele et al., 2015).

This study describes the chemical composition of the essential oil of *Eupatorium ballotifolium* (EOEB), and reports the antioxidant and antifungal activities against dermatophytes and yeasts, as well as the cytotoxicity, based on modulatory activity assays with ketoconazole.

MATERIALS AND METHODS

Plant material

The aerial parts of Eupatorium ballotifolium were collected in the flowering period in Brazil, Ceará, municipality of Alcântaras, in the Meruoca mountain region, in June, 2014 in a semideciduous forest environment located around 800 m above sea level. This region is located in the middle reaches of the Acaraú River, about 250 km from Fortaleza, the state capital. A voucher specimen (No. 3105) was deposited in Francisco José de Abreu Matos Herbarium (HUVA) and authenticated by Dr. Elnatan Bezerra de Souza of the Center for Agricultural Sciences and Biological Sciences, Vale do Acaraú State University.

Isolation of essential oil

Fresh aerial parts of *E. ballotifolium* (680 g) were subjected to hydrodistillation for 2 h in a modified Clevenger-type apparatus. The oil was dried over anhydrous Na_2SO_4 (~1 g), filtered and preserved in a sealed vial at 4°C prior to further analysis, with a yield of 0.1% (w/w).

Analysis of essential oil

The essential oil was analyzed using a Hewlett-Packard 5971 GC/MS instrument under the following conditions: dimethylpolysiloxane DB-5 fused silica capillary column (30 m × 0.25 mm i.d., 0.1 µm film thickness); carrier gas: helium (1 mL/min); injector temperature: 250°C; detector temperature 200°C; column temperature: 35 to 180°C at 4°C/min, then 180 to 250°C at 10°C/min; and mass spectra: electronic impact 70 eV. The identity of the components was achieved from their GC retention times relative to known compounds, calculated by linear interpolation relative to retention times of a series of n-alkanes and by comparison of their mass spectra with those present in the computer data bank (NIST) and published spectra (Adams, 2012).

In vitro antifungal assay

Fungal strains

The strains were obtained from the fungal collection of the Specialized Medical Mycology Center (CEMM), Federal University of Ceará, the URM Culture Collection of the Department of Mycology, Federal University of Pernambuco, and Hospital Santa Casa de Misericórdia de Sobral. In all these collections, the strains were maintained in saline (0.9% NaCl) at 28°C. At the time of the analysis, an aliquot of each suspension was taken and inoculated onto potato dextrose agar (Difco, Detroit, MI, USA), and then incubated at 28°C for 2 to 10 days.

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A total of four strains of *Trichophyton rubrum*, two strains of *Candida albicans*, one strain of *Candida parapsilosis* and one strain of *Candida tropicalis* were included in this study. *Candida* spp. strains were clinical isolates obtained from Hospital Santa Casa de Misericórdia de Sobral.

Preparation of inocula

For the broth microdilution method, standardized inocula (2.5 to 5 x 10^3 CFU mL^{-1} for *Candida* spp. and 5.0 x 10^4 CFU mL^{-1} for *T. rubrum*) were prepared by turbidimetry. Stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *T. rubrum*, respectively, grown on potato dextrose agar at 28°C. Sterile saline solution (0.9%) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat and from the blastoconidia for *T. rubrum* and *Candida* spp., respectively. The suspensions were diluted to 1:2000 for *Candida* spp. and 1:500 for *T. rubrum*, both with RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with l-glutamine without sodium bicarbonate (Sigma Chemical Co., St Louis, MO, USA), and then buffered to pH 7.0 with 0.165 M MOPS (Sigma Chemical Co.), to obtain inocula of 2.5 to 5 x 10^3 CFU mL^{-1} and 5.0 x 10^4 CFU mL^{-1}, respectively.

Broth microdilution method

The minimum inhibitory concentration (MIC) for *Candida* spp. was determined by the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute (CLSI M27-A3, 2008). The broth microdilution assay for *T. rubrum* was performed as previously described (Sousa et al., 2009) based on the M38-A document (CLSI M38-A2, 2008).

The minimum fungicidal concentrations (MFC) for both *Candida* spp. and *T. rubrum* were determined according to the study of Fontenelle et al (2008). The EOEB was prepared in 100% mineral oil. Amphotericin B (AMB) and ketoconazole (Sigma, Chemical Co., USA) were prepared in distilled water. For the susceptibility analysis, the essential oil samples were tested in concentrations ranging from 0.002 to 2.5 mg/mL.

The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for the EOEB. The microplates were incubated at 37°C and read visually after 2 days for *Candida* spp. and 5 days for *T. rubrum*. The assay for the essential oil was run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 100% inhibition of visible fungal growth. The results were read visually as recommended by CLSI. The MFC was determined by subculturing 100 µL of solution from wells without turbidity on potato dextrose, at 28°C. The MFCs were determined as the lowest concentration resulting in no growth on the subculture after 2 days for *Candida* spp. and 5 days for *T. rubrum* (Fontenelle et al., 2008).

Microdilution checkerboard assay

Assays were performed on all strains of *T. rubrum* according to the checkerboard technique (Johnson, 2004; Pyun and Shin, 2006), to determine the combined effect of the essential oil with the standard antifungal drugs. The interaction of the drugs was ascertained by calculating the fractional inhibitory concentration index (FICI). The FICI is calculated by adding the fractional inhibitory concentration (FIC) for each of the tested compounds, being defined as the addition of the MIC values of each drug in the combination divided by the MIC of the drug alone.

FIC^A = MIC of agent A in combination/MIC of agent A alone

FIC^B = MIC of agent B in combination /MIC of agent B alone

$FICI = FIC^A + FIC^B$

In the equations, A represents the EOEB and B the antifungal, ketoconazole. The turbidity of the fungal suspensions was adjusted to 0.5 McFarland standard (10^5 UFC/mL). In the solutions, the tested products were used at concentrations of their respective MICs. Initially, 50 µL of RPMI 1640 medium was added to all 96 wells of the microdilution plate. Then 50 µL of essential oil was added in the first column, in which serial dilutions were made in the plate until the 8th column, with the essential oil concentrations ranging from 5 to 0.03 mg/mL. In the vertical lines, 50 µL of standard antifungal ketoconazole was placed in concentrations ranging from 16 to 0.125 µg/mL.

Finally, 100 µL of inoculum was added to all wells. RPMI 1640 medium with inoculum was used as a negative control, while the antifungals and essential oil separately were used as positive controls at the respective MIC values. The microplates were incubated at 37°C and read visually after five days for dermatophytes. Assays were performed in triplicate. The FICI was interpreted as indicating a synergistic effect at values ≤ 0.5, an indifferent effect at values > 0.5 or ≤ 4.0, and an antagonistic effect at values > 4.0 (Odds, 2003; Johnson, 2004).

Determination of DPPH radical scavenging activity

To evaluate the antioxidant activity of the essential oil, the study used the free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, according to Fenglin et al. (2004), with modifications. The activity was investigated by the ability to scavenge the DPPH radical through variation of absorbance obtained for a stoichiometric color loss of the radical solution in the presence of antioxidant substances present in the essential oil sample.

In a test tube, 3.9 mL of a methanol solution of free radical DPPH 6.5 x 10^{-5}M was combined with 0.1 mL of the methanol solution of essential oil in the concentrations to be tested. After 60 min, the absorbance was determined with an UV-VIS spectrophotometer at a wavelength of 515 nm. Assays were performed in triplicate. The inhibition of free radical DPPH was calculated in percent using the following equation:

IP% = Abs(DPPH) - Abs(sample) / Abs(DPPH) x 100

Where IP% is the inhibition percentage; Abs (DPPH) is the absorbance of the DPPH solution and Abs (sample) is the absorbance of the solution containing the essential oil at a particular concentration. IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated by the regression equation of the concentration of the essential oil, and percentage inhibition of free radical formation/percentage inhibition DPPH was calculated (Bajpai et al., 2009).

Determination of antioxidant activity using β-carotene/linoleic acid assay

The evaluation of oxidation inhibition by β-carotene/linoleic-acid oxidation system was performed by means of a spectrophotometric assay based on the discoloration of β-carotene due to oxidation induced by oxidative degradation products of linoleic acid (Silva et al., 1999; Alves et al., 2010). The antioxidant activity was determined by measuring the ability of the volatile organic compounds to inhibit the conjugated diene hydroperoxide formation from linoleic acid and β-carotene coupled oxidation in an emulsified aqueous system, which loses its orange color when reacting with the radicals (Alves et al., 2010; Lopes-Lutz et al., 2008).

The β-carotene (Sigma, St. Louis, MO) was dissolved in 5 ml of chloroform (0.3 mg/mL), followed by adding 20 µL of linoleic acid (Sigma, St. Louis, MO) and 200 µL of Tween 40 (Sigma, St. Louis, MO). Chloroform was completely evaporated using a vacuum evaporator. After removal of $CHCl_3$, 100 ml of distilled water saturated with oxygen under constant agitation was added to form an emulsion. The solution was adjusted in the spectrophotometer to a wavelength of 470 nm. The final emulsion had absorbance between 0.6 and 0.7 nm. Then 5 ml aliquots of the emulsion were placed in test tubes followed by 100 µl of dilutions of previously prepared methanol solutions of the essential oil, at concentrations of 500 to 25 mg/ml. Sample readings were taken 2 min after contact of the methanol solutions having varied concentrations with the emulsion. Then the samples were put in a water bath at 50°C for 120 min, and a second reading was performed. The negative control consisted of 5 ml of emulsion alone (Andrade et al., 2012). The percentage inhibition was calculated from the data with the formula:

$$IP\% = [(Abs_{sample(0)} - Abs_{sample(120)}) / (Abs_{system(0)} - Abs_{system(120)})] \times 100$$

$$\% \text{ Protection} = 100 - IP\%$$

Where IP% is the inhibition percentage; $Abs_{sample(0)}$ is the absorbance of the essential oil at t=0 min; $Abs_{sample(120)}$ is the absorbance of the essential oil at t=120 min; $Abs_{system(0)}$ is the absorbance of the system at t=0 min and $Abs_{system(120)}$ is the absorbance of the system at t=120 min. Thymol and carvacrol, oxygenated monoterpenes present in many essential oils, were used as positive controls. Samples were read against a blank containing the emulsion minus beta-carotene. Each assay was repeated three times and the IC_{50} values (concentration sufficient to obtain 50% of a maximum effect estimate in 100%) were calculated by the regression equation of the concentration of the essential oil, and percentage of protection (Lopes-Lutz et al., 2008; Andrade et al., 2012).

Brine shrimp lethality bioassay

The lethality assay against *Artemia salina* Leach (Crustacea, Artemiidae) was performed according to the method proposed by Meyer et al. (1982) with adaptations. The eggs of *A. salina* were incubated at room temperature (between 22 to 29°C) in artificial brine consisting of 23 g/L of sea salt and 0.7 g/L of sodium bicarbonate in distilled water for a period of 48 h in a tank fitted with a dark compartment and another clear one.

Using a light source, the nauplii were attracted to the light, collected with a Pasteur pipette and transferred to a beaker with saline water. Extraction solutions were prepared with the solvents methanol, DMSO and saline water in concentrations of 10,000 to 1 µg/mL. The positive control was prepared with potassium dichromate ($K_2Cr_2O_7$) and saline solution, and the negative control with saline solution and DMSO. Then 10 larvae were added to test tubes containing 5 ml of each tested solution and negative and positive control solutions (Costa et al., 2009).

Assays were performed in triplicate and the number of dead larvae was counted after contact for 24 h with the solutions. For counting the number of nauplii, the study considered those that remained immobile for more than 10 s after gentle agitation of the tubes (Lhullier et al., 2006).

Hemolysis assay

Fresh blood (10 ml) was collected in EDTA tubes and centrifuged at 1000 g for 10 min at 4°C. After plasma removal, the pellet containing the red blood cells (RBCs) was washed five times with PBS and then re-suspended in PBS to obtain an 8% (v/v) suspension. Then 100 µL of this suspension was added to different microcentrifuge tubes with 100 µL of 2-fold serial dilutions of essential oil, ranging from 0.005 to 2.5 mg/ml. Final concentrations were 4% (v/v) of erythrocyte suspension, and the essential oil concentration range was 0.1 to 100 µM. The resulting suspensions were incubated with agitation for 60 min at 37°C. After incubation, the samples were centrifuged for 2 min at 1000 g. The supernatants were transferred to 96-well plates and the hemoglobin release was measured by absorbance at 540 nm, using the Biotek Synergy HT multiplate reader. Triton X-100 at 1% and 4% (v/v) RBCs in PBS with no essential oil (untreated) were used as positive and negative controls, respectively. Percentage hemolysis was determined as [(Abs_{540nm} sample-treated – Abs_{540nm} untreated)/ (Abs_{540nm}1% Triton X-100 – Abs_{540nm} untreated)] x 100, and experiments were carried out in triplicate (Ahmad et al., 2010).

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). One-way ANOVA with the Tukey test was performed followed by multiple comparisons testing where appropriate. LC_{50} values were obtained using statistical package for social sciences (SPSS) 14.0 for Windows (SPSS Inc. Chicago, USA). Significance of difference was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

The essential oil yield was 0.1% (w/w) dry weight. The chemical compositions of the volatile constituents of the EOEB and the respective percentages are reported in Table 1. The essential oil contained 25 components, accounting for 93.84%, among them mono- and sesquiterpenes, with a prevalence of sesquiterpenes. The main constituents were β-caryophyllene (23.59%), thymol methyl ether (12.28%), germacrene D (6.56%) and bicyclogermacrene (6.47%). Among these main constituents, only one monoterpene was found, thymol methyl ether. Sesquiterpene hydrocarbons predominate in the chemical composition.

A previous study of the chemical composition of the aerial parts of EOEB identified 27 components, representing 91.2% of the total content (Albuquerque et al. 2001). In this study, the EOEB showed a high content of monoterpenes, limonene (15.3%), (E)-β-ocimene (10.5%), followed by β-caryophyllene sesquiterpene (7.5%) and myrcene monoterpene (7.3%) (Albuquerque et al., 2001). Variations in the chemical composition of essential oils, secondary metabolism products of the plant, occur due to changes in abiotic factors, as well in the geographic location of the plant, season of the year and even time of day when the plants are sampled (Dudareva et al., 2004; Gobbo-Neto; Lopes, 2007). In other Eupatorium species, the chemical analysis showed a corresponding composition. In *E. capillifolium* (Lam.) Small. ex Porter & Britton, the chemical composition of the essential oil of the aerial parts contained thymol methyl ether (36.3%) as the main constituent (Tabanca et al., 2010).

Table 1. Chemical composition of the aerial parts of the essential oil from *E. ballotifolium* (EOEB).

Compound[a]	RI Lit[b]	RI[c]	EOEB
Sabinene	975	975	1.88
β-Myrcene	990	992	0.51
α-Phellandrene	1002	1004	1.32
p-Cymene	1024	1025	3.41
Limonene	1029	1023	0.88
(Z)-β-Ocimene	1037	1039	1.40
(E)-β-Ocimene	1050	1050	5.05
Terpinen-4-ol	1177	1186	2.13
Thymol methyl ether	1245	1235	12.28
δ-Elemene	1338	1350	1.87
α-Copaene	1376	1373	2.29
β-Elemene	1390	1389	5.47
β-Caryophyllene	1419	1427	23.59
α-Humulene	1454	1450	1.58
epi-Caryophyllene	1466	1471	0.76
Germacrene D	1481	1490	6.56
Bicyclogermacrene	1500	1499	6.47
Germacrene A	1509	1513	1.08
δ-Cadinene	1523	1529	5.54
Germacrene B	1561	1560	0.97
Caryophyllene oxide	1583	1583	1.38
Globulol	1590	1591	2.85
1,10-de-*epi*-Cubenol	1619	1621	0.73
epi-α-Muurolol	1642	1634	2.40
α-Cadinol	1654	1644	1.44
Total identified	-	-	93.84

[a] Order of elution on DB-5 capillary column. [b] RI_{lit} refers to the retention index taken from Adams, 2012. [c] RI_{ca} refers to the retention index experimentally calculated using C_7–C_{26} n-alkanes.

Regarding the *in vitro* antifungal activity of the EOEB, the results are summarized in Table 2. The essential oil showed no significant activity when tested against strains of *Candida* spp (*C. albicans* LABMIC 0201, *C. albicans* LABMIC 0202, *C. parapsilosis* LABMIC 0301 and *C. tropicalis* LABMIC 0401). However, the results showed MIC values ranging from 2.5 to 1.25 mg/ml for the strains of dermatophytes (*T. rubrum* LABMIC 0101, *T. rubrum* LABMIC 0102, *T. rubrum* CEMM 05-1-08 and *T. rubrum* CEMM 05-1-034). Ketoconazole was used as positive control.

There are no reports in the literature of antimicrobial activities of the *E. ballotifolium* species, so this study is groundbreaking in investigating the antifungal activity against dermatophytes, which are medically important fungi responsible for superficial skin infections, with *T. rubrum* being the species most often found to cause dermatophytosis (Cafarchia et al., 2013).

Previous studies with some of the major constituents in the composition of EOEB report antifungal activity for β-caryophyllene (Tampieri et al., 2005; Skaltsa et al., 2003; Bougatsos et al., 2004) and bicyclogermacrene (Silva et al., 2007), both sesquiterpene hydrocarbons. These results indicate that the antifungal activity found in our study may represent a combined effect of these main EOEB constituents. For the *Eupatorium* genus, numerous studies have been conducted of the antimicrobial activity, mainly involving extracts and essential oils. The data are still incipient, although *Eupatorium* has been found to be one of the largest genera of the family Asteraceae (Garcia-Sanchez et al., 2011; Roque and Bautista, 2008).

In testing the modulatory activity, the study used the strains of *T. rubrum* LABMIC 0101 and *T. rubrum* LABMIC 0102. The results (Table 3) demonstrate that the combination of ketoconazole with EOEB reduced the MICs for both strains of *T. rubrum* (Table 3). Mutual synergistic potentiation of antifungal activity of EOEB and ketoconazole occurred, with a significant reduction in the MIC of ketoconazole of 1.0 to 0.125 µg/mL on the strain *T. rubrum* LABMIC 0102. The most significant reduction occurred in the tests with strain LABMIC 0102, whose fractional inhibitory concentration index (FICI) was low

Table 2. Minimum inhibitory concentration of essential oils from *Eupatorium ballotifolium* against *Candida* spp and *Trichophyton rubrum.*

Strains	Essential oil of *E. ballotifolium*		Drug (µg/mL)	
	MIC (mg/mL)	MFC (mg/mL)	Amphotericin B	ketoconazole
C. albicans LABMIC 0201	NI	NI	2.0	–
C. albicans LABMIC 0202	NI	NI	1.0	–
C. parapsilosis LABMIC 0301	NI	NI	4.0	–
C. tropicalis LABMIC 0401	NI	NI	2.0	–
T. rubrum LABMIC 0101	1.25	–	–	1.0
T. rubrum LABMIC 0102	2.5	–	–	1.0
T. rubrum CEMM 05-1-08	2.5	–	–	1.0
T. rubrum CEMM 05-1-034	2.5	–	–	1.0
Geometric mean of *T. rubrum*	2.69	–	–	–

LABMIC, Microbiology Laboratory; CEMM, Specialized Centre of Medical Mycology.

Table 3. MIC of the ketoconazole in the presence and absence of essential oil from *Eupatorium ballotifolium* against *Trichophyton rubrum.*

Plant essential oil/Drug	*T. rubrum* LABMIC 0102			*T. rubrum* LABMIC 0101		
	MIC (µg/ml) alone	MIC (µg/ml) combined	FIC indexa	MIC (µg/ml) alone	MIC (µg/mL) Combined	FIC index
E. ballotifolium	5000	39	0.1	2500	39	1.0
ketoconazole	1.0	0.125		1.0	1.0	

[a]FIC index, index fractional inhibitory concentration (FICI).

Table 4. Antioxidant activity of the essential oil from *E. ballotifolium* and the thymol and carvacrol, tested standard.

Methods	β-carotene / linoleic acid	DPPH
Compound	IC_{50} (µg/mL)	IC_{50} (µg/mL)
E. ballotifolium	19.47	222.17
Thymol	11.32	21.71
Carvacrol	22.83	25.5

IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%.

(0.1) compared with strain LABMIC 0101, whose FICI value was 1.0. So, for LABMIC 0102 the modulatory activity was synergistic and for LABMIC 0101 the activity was indifferent.

The mechanisms of inhibiting microbial growth may be related to the hydrophobic nature of the constituents of the essential oil. Such compounds can act on the plasma membrane, causing it to become more permeable to antifungal agents, affecting the mitochondrial respiratory chain and cellular energy production. Thus, this mechanism can occur due to combined action of the antifungals with natural products at subinhibitory concentrations (Nogueira et al., 2014; Tintino et al., 2014). This is the first report of modulatory activity of a standard antifungal drug combined with EOEB.

The results presented here corroborate with other studies involving essential oils of *Thymus vulgaris* L. and *Cinnamomum cassia* L. (Pekmezovic et al., 2015), *Ocimum sanctum* L. (Amber et al., 2010), *Myrtus communis* L. (Mahboubi and Bidgoli, 2010), *Melaleuca alternifolia* (Maiden & Betche) Cheel and *Lavandula angustifolia* Mill. (Cassella et al., 2002). These studies demonstrate the ability of essential oils used to modify the antibiotic activity of drugs through the checkerboard technique. IC_{50} values of the EOEB obtained from the antioxidant assays investigated by the free radical DPPH (1.1-diphenyl-2-picrylhydrazyl) scavenging assay and β-carotene/linoleic-acid oxidation model system are shown in Table 4.

In the DPPH scavenging assay, the IC_{50} value was 222.17 µg/mL. This result indicates that the essential oil exhibited weak antioxidant capacity when compared to the positive controls: thymol, whose IC_{50} was 21.71 µg/mL and carvacrol, with IC_{50} of 25.5 µg/mL. In the test

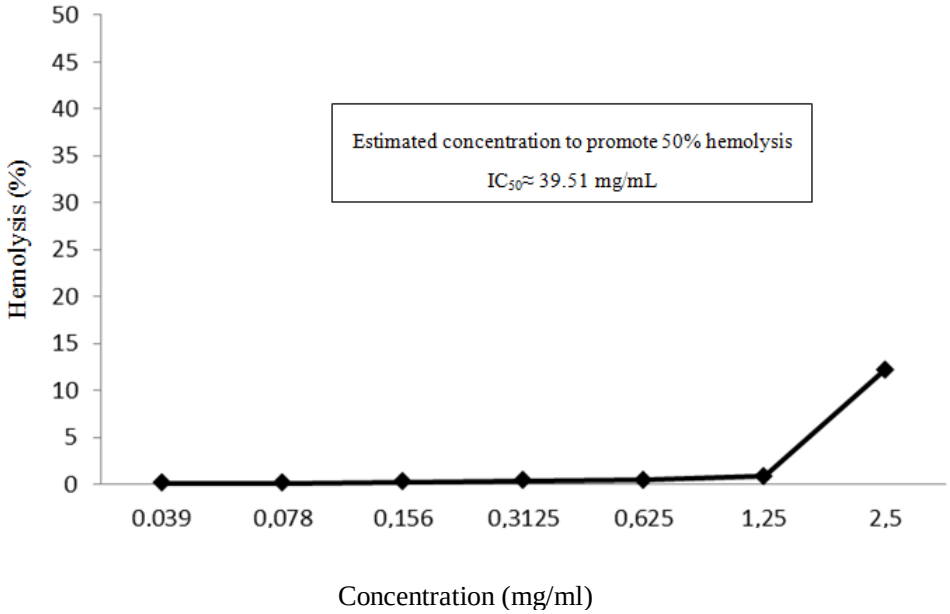

Figure 1. Hemolytic activity of the essential oil from *Eupatorium ballotifolium* (EOEB).

with β-carotene/linoleic-acid, the IC_{50} of the essential oil was 19.47 µg/mL, an excellent antioxidant action, while the IC_{50} of thymol was 11.32 µg/mL and carvacrol was 22.83 µg/mL.

The discrepant results between the two tests can be explained by the purpose of each method. The DPPH scavenging assay is based on the ability of the tested substance to sequester DPPH free radicals, which are reduced to the hydrazine compound and are routinely used to evaluate the antioxidant activity in plant extracts and pure substances such as terpenoids and flavonoids (David et al., 2007; Alves et al., 2010). The β-carotene/linoleic-acid assay is employed specifically to investigate the ability of a sample to minimize the complete oxidation of linoleic acid and β-carotene in an aqueous-lipid system which loses its orange color when it reacts with radicals produced by oxidative degradation of fatty acids (Alves et al., 2010). This method is used to investigate the antioxidant capacity of lipophilic substances, such as essential oils (Kulisic et al., 2004). Another property that makes it a useful method to test essential oils is that it does not require high temperatures, which allows determination of the antioxidant activity of thermo-sensitive substances such as essential oils (Silva et al., 1999).

Sesquiterpenes were the constituents identified in greatest quantities in the study essential oil, and the literature indicates antioxidant potential of plant-derived sesquiterpenes (Xu et al., 2008; Sghaier et al., 2011). β-caryophyllene, the main constituent found in the study (23.59%), was previously shown to have antioxidant activity and protective effect on liver fibrosis and the ability to inhibit activation of hepatic stellate cells (Calleja et al., 2013). The isolated action of β-caryophyllene or the synergism between the main constituents of the essential oil can be also be related to the antioxidant action (Candan et al., 2003). These results of antioxidant activity of the EOEB, detected by two different methods, corroborate previous studies that have indicated antioxidant potential of the genus *Eupatorium*, with activity for many species, such as *E. adenophorum* (Ahluwalia et al., 2014), *E. odoratum* (Raman et al., 2012; Chakraborty et al., 2010), *E. polystachyum* DC. (Souza et al., 2007) and *E. triplinerve* Vahl (Melo et al., 2013).

In the brine shrimp lethality bioassay, mortality rates to the EOEB varied between 0 and 100%, and the concentration required to kill 50% of the larvae (LC_{50}) was 28.89 µg/mL. LC_{50} values less than 1000 µg/mL indicate a possible spectrum of biological activities of some of the constituents, combined and/or isolated (Meyer et al., 1982). The anticholinesterase activity has been investigated of the EOEB from leaves and bark (Albuquerque et al., 2010). Lethality bioassays with A. salina have also been used in prospecting studies to screen plants with possible pharmacological activity, in Brazil (Quignard et al., 2004), India (Krishnaraju et al., 2006) and Nicaragua (Coe et al., 2010).

The tests to determine the in vitro hemolytic activity of EOEB showed that at concentrations of 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 mg/mL respectively the percentage of hemolysis ranged from 0.1 to 12.16% (Figure 1). Based on these results, the study can extrapolate hemolysis values and estimate the IC50 of 39.51 mg/mL for the essential oil. Regarding hemolytic

activity, the statistical analysis showed a significant difference at the level p = 0.05 when compared to the positive control Triton X-100 with the EOEB concentrations used.

Methods to measure the hemolytic activity *in vitro* enable determining the cytotoxic profile of the studied substance, and consist of checking for potential damage caused by the substances present in essential oils to the membranes of erythrocytes, which when undergoing lysis release hemoglobin (Miyazaki et al., 2013). At the essential oils' MIC values, the study observed hemolysis percentages between 0.8 and 12.2%. Comparison of the IC_{50} value of hemolytic activity with the MIC indicated that the oil concentration responsible for the fungistatic activity is lower than the concentration required to damage red blood cells by rupture of their membrane. However, according to the test of hemolytic activity, the EOEB showed low cytotoxic effect at the concentrations that inhibited microbial growth.

Conclusion

Chemical analysis of the EOEB identified 25 components, accounting for 93.84% of the substances, with predominance of sesquiterpene hydrocarbons. The brine shrimp lethality bioassay (*A. salina*) used as for screening allowed establishing a correlation with other potential biological activities, and the hemolytic activity assay showed that the essential oil has low cytotoxicity.

The essential oil showed *in vitro* antifungal activity against the dermatophyte *T. rubrum*, and when tested in combination with ketoconazole, the EOEB interacted synergistically, increasing its antifungal action. Antioxidant activity was evidenced by the DPPH scavenging assay, with IC_{50} of 222.17 µg/mL, while the IC_{50} value was 19.47 µg/mL in the test with β-carotene/linoleic-acid. The antioxidant potential can be related to the high content of sesquiterpenes and especially β-caryophyllene, germacrene D and bicyclogermacrene, the main constituents found in this study.

It is necessary to investigate the mechanism of action in the fungal cells and perform tests to identify the components responsible for the biological activities. It is also important to carry out tests with isolated constituents of EOEB against strains of dermatophyte fungi, modulatory activity assays with other antifungal drugs used in antifungal therapy and to investigate new toxicological aspects of the essential oil.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Adams RP (2012). Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, Allured Publishing Corporation, Illinois.

Ahluwalia V, Sisodia R, Walia S, Sati OP, Kumar J, Kundu A (2014). Chemical analysis of essential oils of *Eupatorium adenophorum* and their antimicrobial, antioxidant and phytotoxic properties. J. Pest Sci. 87(2):341-349.

Ahmad A, Khan A, Manzoor N, Khan LA (2010). Evolution of ergosterol biosynthesis inhibitors as fungicidal against *Candida*. Microb. Pathog. 48(1):35-41.

Albuquerque MRJR, Santos HS, Souza EB, Silva RM, Pessoa ODL, Braz-Filho R, Costa SMO (2010). Composição química volátil e não-volátil de *Eupatorium ballotifolium* Kunth, Asteraceae. Rev. Bras. Farmacogn. 20(4):615-620.

Albuquerque MRJR, Souza EB, Lins MUDS, Nogueira NAP, Lemos TLG, Silveira ER, Pessoa ODL (2004). Composition and antimicrobial activity of the essential oil from aerial parts of *Baccharis trinervis* (Lam.) Pers. Arkivoc 6:59-65.

Albuquerque MRJR, Souza EB, Mesquita EF, Nunes EP, Cunha AN, Silveira ER (2001). Volatile Constituents from Leaves of *Vernonia chalybaea* Mart. and *Eupatorium ballotaefolium* HBK. J. Essent. Oil Res. 13(5):376-377.

Alves CQ, David JM, David JP, Bahia MV, Aguiar RM (2010). Métodos para determinação de atividade antioxidante *in vitro* em substratos orgânicos. Quim. Nova 33(10);2202-2210.

Amber K, Aijaz A, Immaculata X, Luqman KA, Nikhat M (2010). Anticandidal effect of *Ocimum sanctum* essential oil and its synergy with fluconazole and ketoconazole. Phytomedicine 17(12):921-925.

Andrade MA, Cardoso MDG, Batista LR, Mallet ACT, Machado SMF (2012). Óleos essenciais de *Cymbopogon nardus*, *Cinnamomum zeylanicum* e *Zingiber officinale*: Composição, atividades antioxidante e antibacteriana. Rev. Ciênc. Agron. 43(2):399-408.

Bajpai VK, Al-Reza SM, Choi UK, Lee JH, Kang SC, (2009). Chemical composition, antibacterial and antioxidant activities of leaf essential oil and extracts of *Metasequioa glyptostroboides* Miki ex Hu. Food Chem. Toxicol. 47(8):1876-1883.

Bekele A, Abera R, Mebratu T, Dessie W, Getu A, Getnet B (2015). Antimicrobial activity of *Thymus schimperi* Ronninger (Lamiaceae) against standard and clinical isolates of human pathogenic bacteria. J. Med. Plants Res. 9(11):379-384.

Bougatsos C, Ngassapa O, Runyoro DK, Chinou IB (2004). Chemical composition and *in vitro* antimicrobial activity of the essential oils of two *Helichrysum* species from Tanzania. Z. Naturforsch. C Biol. Sci. 59(5-6):368-372.

Cafarchia C, Iatta R, Latrofa MS, Gräser Y, Otranto D (2013). Molecular epidemiology, phylogeny and evolution of dermatophytes. Infect. Genet. Evol. 20:336-351.

Calleja MA, Vieites JM, Montero-Meterdez T, Torres MI, Faus MJ, Gil A, Suárez A (2013). The antioxidant effect of β-caryophyllene protects rat liver from carbon tetrachloride-induced fibrosis by inhibiting hepatic stellate cell activation. Br. J. Nutr. 109(03):394-401.

Candan F, Unlu M, Tepe B, Daferera D, Polissiou M, Sökmen A, Akpulat HA (2003). Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae). J. Ethnopharmacol. 87(2):215-220.

Cardoso AD, Viana AES, Barbosa RP, Teixeira PRG, Júnior NSC, Fogaça JJNL (2013). Levantamento fitossociológico de plantas daninhas na cultura da mandioca em Vitória da Conquista, Bahia. Biosci. J. 29(5):1130-1140.

Cassella S, Cassella JP, Smith I (2002). Synergistic antifungal activity of tea tree (*Melaleuca alternifolia*) and lavender (*Lavandula*

angustifolia)essential oils against dermatophyte infection. Int. J. Aromather. 12(1):2-15.
Chakraborty AK, Roy HA, Bastia S (2010). Evaluation of antioxidant activity of the leaves of *Eupatorium odoratum* LINN. Int. J. Pharm. Pharm. Sci. 2:77-79.
Clinical and Laboratory Standards Institute (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts (Approved Standard. Document M27. CLSI). Third ed. Vol. M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
Clinical and Laboratory Standards Institute (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi (Approved Standard. Document M38. CLSI). Second ed. Vol. M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
Coe FG, Parikh DM, Johnson CA (2010). Alkaloid presence and brine shrimp (*Artemia salina*) bioassay of medicinal species of eastern Nicaragua. Pharm. Biol. 48(4):439-445.
Costa ES, Dolabela MF, Póvoa MM, Oliveira DJ, Müller AH (2009). Estudos farmacognósticos, fitoquímicos, atividade antiplasmódica e toxicidade em *Artemia salina* de extrato etanólico de folhas de *Montrichardia linifera* (Arruda) Schott, Araceae. Rev. Bras. Farmacogn. 19:834-838.
David JP, Meira M, David JM, Brandão HN, Branco A, Agra MF, Barbosa MRV, Queiroz LP, Giulietti AM (2007). Radical scavenging, antioxidant and cytotoxic activity of Brazilian Caatinga plants. Fitoterapia 78(3):215-218.
Desoti VC, Maldaner CL, Carletto MS, Heinz AA, Coelho MS, Piati D, Tiuman TS (2011). Triagem fitoquímica e avaliação das atividades antimicrobiana e citotóxica de plantas medicinais nativas da região oeste do estado do Paraná. Arq. Ciênc. Saúde Unipar 15(1):3-13.
Dudareva N, Pichersky E, Gershenzon J (2004). Biochemistry of plant volatiles. Plant Physiol. 135(4):1893-1902.
Fabri RL, Nogueira MS, Dutra LB, Bouzada MLM, Scio E (2011). Potencial antioxidante e antimicrobiano de espécies da família Asteraceae. Rev. Bras. Plantas Med. 13:183-89.
Fenglin H, Ruili L, Bao H, Liang M (2004). Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medicinal plants. Fitoterapia 75(1):14-23.
Fontenelle ROS, Morais SM, Brito EHS, Brilhante RSN, Cordeiro RA, Nascimento NRF, Kerntopf MR, Sidrim JJC, Rocha MFG (2008). Antifungal activity of essential oils of *Croton* species from the Brazilian Caatinga biome. J. Appl. Microbiol. 104(5):1383-1390.
Funk VA, Susanna A, Stuessy TF, Robinson H (2009). Classification of Compositae. In Funk VA, Susanna A, Stuessy TF, Bayer RJ (eds.). Systematics, evolution, and biogeography of Compositae. International Association for Plant Taxonomy: Vienna, Austria. pp. 171-192.
Garcia-Sanchez E, Lopez CBR, Torres RENR, Pacheco MMM (2011). A revision de *Eupatorium* (Compositae: Eupatorieae) from Michoacan. Phyton 80(2):139-146.
Gobbo-Neto L, Norberto PL (2007). Plantas medicinais: Fatores de influência no conteúdo de metabólitos secundários. Quim. Nova 30(2):374.
Johnson MD, MacDougall C, Ostrosky-Zeichner L, Perfect JR, Rex JH (2004). Combination antifungal therapy. Antimicrob. Agents Chemother. 48(3):693-715.
Krishnaraju AV, Rao TV, Sundararaju, D, Vanisree, M, Tsay, HS, Subbaraju, GV (2006). Biological screening of medicinal plants collected from Eastern Ghats of India using *Artemia salina* (brine shrimp test). Int. J. Appl. Sci. Eng. 4(2):115-125.
Kulisic T, Radonic A, Katalinic V, Milos M (2004). Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 85(4):633-640.
Lhullier C, Horta PA, Falkenberg M (2006). Avaliação de extratos de macroalgas bênticas do litoral catarinense utilizando o teste de letalidade para *Artemia salina*. Rev. Bras. Farmacogn. 16:158-163.
Lopes-Lutz D, Alviano DS, Alviano CS, Kolodziejczyk PP (2008). Screening of chemical composition, antimicrobial and antioxidant activities of *Artemisia* essential oils. Phytochemistry 69(8):1732-1738.
Mahboubi M, Bidgoli FG (2010). *In vitro* synergistic efficacy of combination of amphotericin B with *Myrtus communis* essential oil against clinical isolates of *Candida albicans*. Phytomedicine 17(10):771-774.
Melo AS, Monteiro MC, Silva JB, Oliveira FR, Vieira JLF, Andrade MA, Baetas AC, Sakai JT, Ferreira FA, Sousa PJC, Maia CFS (2013). Antinociceptive, neurobehavioral and antioxidant effects of *Eupatorium triplinerve* Vahl on rats. J. Ethnopharmacol. 147(2):293-301.
Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DJ, McLaughlin JL (1982). Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med. 45:31-34.
Militão GCG, Albuquerque MRJR, Pessoa ODL, Pessoa C, Moraes MEA, Moraes MO, Costa-Lotufo LV, (2004). Cytotoxic activity of nepetin, a flavonoid from *Eupatorium ballotaefolium* HBK. Die Pharmazie. Int. J. Pharm. Sci. 59(12):965-966.
Miyazaki CMS, Hirota BCK, Lima CP, Verdam MCS, Paula CS, Chaves SC, Pavan PMN, Miguel MD, Miguel OG (2013). Coumarin isolation and comparative study of biological activities of *Pterocaulon alopecuroides* DC and *Pterocaulon lorentzii* Malme. Int. J. Phytomed. 5(3):298-301.
Nogueira LFB, Morais EC, Brito MAD, Santos BS, Vale DL, Lucena BFF, Figueredo FG, Guedes GMM, Tintino SR, Souza CES, Nogueira RBSS, Matias EFF, Morais-Braga MFB, Cunha EVL, Lima MA, Coutinho HD (2014). Evaluation of antibacterial, antifungal and modulatory activity of methanol and ethanol extracts of *Padina sanctae-crucis*. Afr. Health Sci. 14(2):372-376.
Odds FC (2003). Synergy, antagonism, and what the chequerboard puts between them. J. Antimicrob. Chemother. 52(1):1-1.
Pekmezovic M, Rajkovic K, Barac A, Senerović L, Arsenijevic VA (2015). Development of kinetic model for testing antifungal effect of *Thymus vulgaris* L. and *Cinnamomum cassia* L. essential oils on *Aspergillus flavus* spores and application for optimization of synergistic effect. Biochem. Eng. J. 99:131-137.
Petacci F, Tavares WS, Freitas SS, Teles AM, Serrão JE, Zanuncio JC (2012). Phytochemistry and quantification of polyphenols in extracts of the Asteraceae weeds from Diamantina, Minas Gerais State, Brazil. Planta Daninha 30(1):9-15.
Pozner R, Zanotti C, Johnson LA (2012). Evolutionary origin of the *Asteraceae capitulum*: insights from Calyceraceae. Am. J. Bot. 99(1):1-13.
Pyun MS, Shin S (2006). Antifungal effects of the volatile oils from Allium plants against *Trichophyton* species and synergism of the oils with ketoconazole. Phytomedicine 13(6):394-400.
Quignard ELJ, Nunomura SM, Pohlit AM, Alecrim AM, Pinto ACS, Portela CN, Oliveira LCP, Don LC, Silva LFR, Henrique MC, Santos M, Pinto PS, Silva SG (2004). Median lethal concentrations of Amazonian plant extracts in the brine shrimp assay. Pharm. Biol. 42(3):253-257.
Raman BV, La S, Saradhi MP, Rao BN, Khrisna ANV, Sudhakar M, Radhakrishnan T (2012). Antibacterial, antioxidant activity and GC-MS analysis of *Eupatorium odoratum*. Asian J. Pharm. Clin. Res. 5(2):99-106.
Roque N, Bautista H (2008). Asteraceae: Caracterização e morfologia floral. Salvador: EDUFBA. 69 p.
Sghaier MB, Skandrani I, Nasr N, Franca M-GD, Chekir-Ghedira L, Ghedira K (2011). Flavonoids and sesquiterpenes from *Tecurium ramosissimum* promote antiproliferation of human cancer cells and enhance antioxidant activity: A structure–activity relationship study. Environ. Toxicol. Pharmacol. 32(3):336-348.
Silva FA, Borges MFM, Ferreira MA (1999). Métodos para avaliação do grau de oxidação lipídica e da capacidade antioxidante. Quim. Nova 22(1):94-103.
Silva LD, Oniki GH, Agripino DG, Moreno PR, Young MCM, Mayworm MAS, Ladeira AM (2007). Biciclogermacreno, resveratrol e atividade antifúngica em extratos de folhas de *Cissus verticillata* (L.) Nicolson & Jarvis (Vitaceae). Rev. Bras. Farmacogn. 17:361-367.
Silveira RS, Pessoa ODL (2005). Constituintes micromoleculares de plantas do nordeste com potencial farmacológico. Fortaleza: Expressão Gráfica.
Skaltsa HD, Demetzos C, Lazari D, Sokovic M (2003). Essential oil analysis and antimicrobial activity of eight *Stachys* species from Greece. Phytochemistry 64(3):743-752.
Sousa JC, Berto RF, Gois EA, Fontenele-Cardi NC, Honorio-Junior JE, Konno K, Richardson M, Rocha MFG, Camargo AACM, Pimenta DC, Cardi BA, Carvalho KM (2009). Leptoglycin: A new Glycine/Leucine-

rich antimicrobial peptide isolated from the skin secretion of the South American frog *Leptodactylus pentadactylus* (Leptodactylidae). Toxicon 54(1):23-32.
Souza TJ, Apel MA, Bordignon S, Matzenbacher NI, Zuanazzi JAS, Henriques AT (2007). Composição química e atividade antioxidante do óleo volátil de *Eupatorium polystachyum* DC. Rev. Bras. Farmacogn. 17:368-372.
Suffredini IB, Paciencia ML, Nepomuceno DC, Younes RN, Varella AD (2006). Antibacterial and cytotoxic activity of Brazilian plant extracts-Clusiaceae. Mem. Inst. Oswaldo Cruz 101(3):287-290.
Tabanca N, Bernier UR, Tsikolia M, Becnel JJ, Sampson B, Werle C, Demirci B, Baser KHC, Blythe EK, Pounders C, Wedge DE (2010). *Eupatorium capillifolium* essential oil: Chemical composition, antifungal activity, and insecticidal activity. Nat. Prod. Commun. 5:1409-1415.
Tampieri MP, Galuppi R, Macchioni F, Carelle MS, Falcioni L, Cioni PL, Morelli I (2005). The inhibition of *Candida albicans* by selected essential oils and their major components. Mycopathologia 159(3):339-345.
Tintino SR, Souza CE, Guedes GM, Costa JI, Duarte FM, Chaves MCO, Silva VA, Pessoa HL, Lima MA, Garcia CA, Coutinho HD (2014). Modulatory antimicrobial activity of *Piper arboreum* extracts. Acta Bot. Croat. 73(1):303-311.
Xu YJ, Sun SB, Sun LM, Qiu DF, Liu XJ, Jiang ZB, Yuan CS (2008). Quinic acid esters and sesquiterpenes from *Sonchus arvensis*. Food Chem. 111(1):92-97.

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Isolation, biochemical characterization and antibiofilm effect of a lectin from the marine sponge *Aplysina lactuca*

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ABSTRACT

A new lectin was isolated from the marine sponge *Aplysina lactuca* (ALL) by combining ammonium sulfate precipitation and affinity chromatography on guar gum matrix. ALL showed affinity for the disaccharides α-lactose, β-lactose and lactulose (K_a = 12.5, 31.9 and 145.5 M^{-1}, respectively), as well as the glycoprotein porcine stomach mucin. Its hemagglutinating activity was stable in neutral acid pH values and temperatures below 60 °C. ALL is a dimeric protein formed by two covalently linked polypeptide chains. The average molecular mass, as determined by Electrospray Ionization Mass Spectrometry (ESI–MS), was 31,810 ±2 Da. ESI–MS data also indicated the presence of three cysteines involved in one intrachain and one interchain disulfide bond. The partial amino acid sequence of ALL was determined by tandem mass spectrometry. Eight tryptic peptides presented similarity with lectin I isolated from *Axinella polypoides*. Its secondary structure is predominantly β-sheet, as indicated by circular dichroism (CD) spectroscopy. ALL agglutinated gram-positive and gram-negative bacterial cells, and it were able to significantly reduce the biomass of the bacterial biofilm tested at dose- dependent effect.

1. Introduction

Lectins, which are found in all living organisms, are sugar-binding proteins that differ from immunoglobulins and glycoenzymes because they are not produced as an adaptive immune response, and they contain no site for enzymatic modification of their ligands. However, lectins are important proteins of both vertebrate and invertebrate innate immune systems, and they interact with carbohydrates in a specific and reversible manner [1–3].

Several lectins from different sources have been isolated and characterized. Among the exploited invertebrates, lectins from the phylum Porifera has revealed promising pharmacological and biotechnological application potential [4]. Currently, about 150 sponges have been screened for hemagglutinating activity [4–8], and the isolation of about 40 resulted [4].

Sponge lectins form a heterogeneous group of proteins, which present a wide variety of biochemical characteristics (*i.e.*, molecular size, glycosylation, Ca^{2+} binding site and isoelectric point) and biological activities, including antimicrobial and cytotoxic activities, modulation of inflammatory response, and neuromodulatory activity [4,9,10,11–13].

Despite their biotechnological potential, only a few lectins from sponges have thus far been structurally characterized. Among those that have had their primary structure determined are representatives of some structural lectin families, including C-type lectins, tachylectins, galectins [14–16], and "orphan" lectins that do not belong to any specific family [17,18].

Irrespective of their structural features, most sponge lectins converge in one primordial characteristic: specificity. Several galactophilic lectins were isolated from sponges [4,7,10,19,20]. The genus *Aplysina*, for instance, presents galactose-binding lectins isolated from *A. archeri* and *A. lacunosa* [8].

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Aplysina lactuca is a recently described endemic species from the northeastern Brazilian coast. Specimens can be found on the upper part of rocks or coral heads, in depths varying from 5 to 22 m *A. lactuca* is a yellowish-brown sponge of very soft and flexible consistency that presents a lamellar form, which is reminiscent of the root buttresses of large tropical canopy trees [21].

In this work, we report the purification and biochemical characterization of a new lectin isolated from the marine sponge *A. lactuca* (ALL). We then describe the effect of the lectin against bacterial biofilm formation.

2. Methods

2.1. Materials

Guar gum, protein mix, α-cyano-4-hydroxycinnamic acid (CHCA), sugars and glycoprotein were purchased from Sigma (St. Louis, MO, USA). Trypsin was purchased from Promega (Madison, WI, USA). Acetonitrile (ACN), formic acid (FA), trifluoracetic acid (TFA), vinyl-pyridine (VP), iodoacetamide (IAA), β-mercaptoethanol (β-ME) and dithiothreitol (DTT), acrylamide, bis-acrylamide, ammonium persulfate and sodium dodecyl sulfate were HPLC/Spectro grade. Sodium phosphate, ammonium sulfate, Tris hydrochloride, EDTA, glycine, sodium chloride, sodium acetate violet crystal were the purest grade commercially available (for analysis grade).

2.2. Animal collection

Sponge specimens were collected on the intertidal zone of Pacheco Beach (3°41′14″S, 38°37′58″W), Caucaia, Brazil. The sponges were transported in seawater to the laboratory and stored at −20 °C until use. The species was identified, and a voucher was deposited (ID: UFPEPOR 1929) at the Zoology Department of the Universidade Federal de Pernambuco, PE, Brazil. Collections were authorized and certified by responsible environmental institutions (SISBIO ID: 33913-8).

2.3. Lectin purification

The frozen sponges were cut into small pieces, ground in a mortar and homogenized in Tris-HCl 50 mM, pH 8.0, containing NaCl 150 mM (TBS) at 1:10 (w/v). Smashed sponges were filtered through nylon tissue, and the extract was centrifuged at 7000*g* for 30 min at 4 °C. The supernatant (crude extract) was stored at −20 °C until use.

Crude extract was submitted to precipitation with 70% ammonium sulfate saturation. After 4 h at 4 °C, precipitated proteins were pelleted by centrifugation and solubilized in a small volume of TBS (F 0–70). Approximately 45 mL of F 0–70 were applied on cross-linked guar gum prepared according to Appukuttan et al. [22]. The column (1.0 × 8.0 cm) was previously equilibrated with TBS, unbound proteins were washed with equilibrium buffer and retained proteins were eluted with glycine buffer (50 mM, pH 2.6, containing NaCl 150 mM). Fractions of 2 mL were manually collected at a flow rate of 1 mL min^{-1} and monitored by absorbance measurement using an Ultrospec 2100 spectrophotometer (GE Healthcare). Retained fractions (ALL) were pooled, dialyzed against distilled water, and freeze-dried.

2.4. Hemagglutinating activity and inhibition assays

Hemagglutinating activity (HA) and inhibition assays were performed following pre-established methods [23] using human (A, B and O) and rabbit erythrocytes, both treated with proteases and untreated.

The following sugars and glycoproteins were used in the inhibition assay: D-xylose, D-ribose, L-fucose, L-arabinose, L-rhamnose, D-galactose, D-mannose, D-glucose, D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-galacturonic acid, D-fructose, D-sucrose, D-melibiose, α-D-lactose, β-D-lactose, D-lactulose, D-maltose, D-raffinose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-β-D-thiogalactose, phenyl-β-D-galactopyranoside, 4-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-β-D-galactopyranoside, 2-nitrophenyl-β-D-galactopyranoside, *O*-nitrophenyl-β-D-galactopyranoside and type 2 porcine stomach mucin (PSM).

The effects of pH, temperature, EDTA and divalent cations on hemagglutinating activity were evaluated as described by Sampaio et al. [23].

2.5. Molecular mass and sugar content

Molecular mass of ALL under denaturing condition was estimated by SDS–PAGE [24] in the presence and absence of β-ME. LMW-SDS Marker kit (GE Healthcare, UK) was used as standard.

ALL relative molecular mass was estimated by size exclusion chromatography in the BioSuite column coupled to an Acquity UPLC system (Waters Corp, MA, USA). Chromatography was conducted in TBS at a flow rate 0.5 mL min^{-1}. The column was previously calibrated with protein mix containing BSA (66 kDa), ovalbumin (45 kDa), *carbonic anhydrase* (29 kDa), ribonuclease (14 kDa) and aprotinin (6.5 kDa).

Average molecular mass was determined by Electrospray Ionization-Mass Spectrometry (ESI–MS). ALL (10 pmol μl^{-1}) was dissolved in a solution of ACN 50% containing 0.2% FA. An aliquot of 100 μL of this solution was centrifuged at 8000 × *g* for 5 min and then infused into a nanoelectrospray source coupled to a Synapt HDMS ESI-Q-ToF mass spectrometer (Waters Corp. MA, USA) using a Hamilton syringe. The instrument was calibrated with [Glu1]-Fibrinopeptide B fragments. Mass spectra were acquired by scanning at m/z ranging from 1500 to 3500, at 2 scans s^{-1}. The mass spectrometer was operated in positive mode, using a source temperature of 363 K and capillary voltage at 3.2 kV. Data collection and processing were controlled by Mass Lynx 4.1 software (Waters).

Additionally, the average molecular mass of ALL was determined by MALDI-ToF on an Autoflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), using matrix solution (10 mg mL^{-1} of CHCA on acetonitrile, water, TFA, 50, 47, 3% v/v). The spectra were acquired in linear positive mode and processed with Flex Analysis 3.4 software (Bruker Daltonics, Germany).

Neutral carbohydrate content in ALL was evaluated in accordance with Dubois et al. [25], using lactose as the standard.

2.6. Quantification of sulfhydryl groups

To quantify free cysteines, ALL (1 mg mL^{-1}) was incubated with 55 mM IAA in the dark for 45 min at room temperature and subjected to desalting by gel filtration chromatography on a Zeba™ Spin Desalting column, 7 K MWCO (Thermo Scientific, MA, USA).

To quantify total cysteines, ALL was reduced with DTT 10 mM at 56 °C for 1 h and alkylated with 55 mM IAA in the dark for 45 min at room temperature. Then, carboxamidomethylated (CAM)-ALL was desalted in Zeba™ column. Alternatively, ALL was treated with β-ME 5 mM for 45 min at 56 °C and then with *VP* 20 mM for 1 h in dark at room temperature. After that, pyridylethylated (PE)-ALL was desalted.

All samples were freeze-dried, solubilized in ACN 50% containing FA 0.2%, and subjected to ESI–MS analyses as described above.

2.7. MS/MS

SDS-PAGE was performed as described above. Protein bands were excised and treated with DTT and IAA according to pre-established methods [26]. Digestion with trypsin was carried out as described by Carneiro et al. [18]. Peptides were extracted from gel according Shevchenko et al. [26].

Tryptic peptides were separated on a reverse phase C-18 (0.075 × 100 mm) nanocolumn coupled to a nanoAcquity system. The eluates were analyzed in a hybrid mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp. MA, USA). The instrument parameters were adjusted as described by Carneiro et al. [18].

Collision induced dissociation (CID) spectra were manually interpreted, and sequenced peptides were searched online against NCBI and Uniprot databanks.

2.8. Circular dichroism measurement

Circular Dichroism (CD) spectroscopic measurement was performed on a Jasco J-815 spectropolarimeter (Jasco International Co., Tokyo, Japan) connected to a peltier with controlled temperature. ALL (0.2 mg mL^{-1} in 20 mM phosphate buffer, pH 7.0, containing 150 mM NaCl) was placed in a rectangular quartz cuvette with 0.5 mm path length. Spectra were acquired at a scan speed of 50 nm min^{-1} with a bandwidth of 1 nm. The acquisitions were performed at 190–250 nm (far UV). The analyses of secondary structure prediction were performed by DICHROWEB web server [27].

The thermodynamics parameters of ALL folding and unfolding in the presence and absence of lactulose, α-lactose and β-lactose were calculated by monitoring the *changes in ellipticity* at 205 nm as a function of temperature [28]. The lectin (8 μM) was submitted to temperatures ranging from 70 °C to 105 °C with a ramp rate of 3 °C min^{-1} and sampling at each 1 °C.

Ligand-induced changes in the molar *ellipticities* of the lectin at 363 K were employed to find the affinity constant (K*a*) for binding of carbohydrates to the lectin as proposed by Greenfield [28,29].

2.9. Antibacterial activity

2.9.1. Microorganisms and culture conditions

Antimicrobial activities were performed using *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 11303. Bacterial cells were grown on Tryptic Soy Agar medium (TSA; Himedia, India) for 24 h at 37 °C. To prepare the bacterial suspension, some isolated colonies of each bacterium were collected from the TSA plates and grown in Tryptic Soy Broth (TSB; Himedia, India) for 24 h at 37 °C under constant agitation. Cell suspensions were prepared in TSB at a cell density of 2×10^6 CFU mL^{-1}, unless otherwise stated.

2.9.2. Bacterial aggregation

S. aureus and *E. coli* and were grown in TSB at 37 °C for 24 h and harvested by centrifugation at 2000 × *g* for 10 min. Agglutination assays were performed as described by Melo et al. [30].

2.9.3. Effects of the lectin on planktonic cells

The effect of ALL on planktonic growth was determined by the broth microdilution method according to guidelines from the National Committee for Clinical Laboratory Standards, M7-A6 [31], with some modifications, as described by Vasconcelos et al. [32].

2.9.4. Biofilm formation assay

Bacterial biofilms were developed on sterile 96-well plates based on the microtiter plate test developed by Stepanovic et al. [33], with modifications. The assay was performed by the addition of 100 μL of each bacterium (2×10^6 CFU mL^{-1}) to each well with 100 μL of different concentrations of lectin (7.8–250 μg mL^{-1}). The microplates were incubated aerobically at 37 °C during 24 h in constant agitation for biofilm development. The biofilms were analyzed by crystal violet staining as an indicator of total biofilm biomass and by counting viable cells.

2.9.4.1. Biofilm mass quantification. Crystal violet staining was used as an indicator of bacterial biofilm biomass. After the growth of biofilm, the wells were washed with 200 μL of 150 mM NaCl to remove weakly adherent cells. Two hundred microliters of 99% methanol were added to each well for 15 min. Methanol was removed, and the plates were allowed to dry at 25 °C. Then, 200 μL of 0.1% (v/v) crystal violet were added, the excess of crystal violet was removed after 5 min, and the plates were washed twice with water. Finally, acetic acid 33% (v/v) was added. The optical density was measured at 590 nm (OD590) on a microtiter plate reader (SpectraMax® I3, Molecular Devices LLC, Sunnyvale, CA, USA).

2.9.4.2. Quantification of number of viable cells in the biofilms. The plates containing biofilm were washed twice with 200 μL of 150 mM NaCl, and biofilm suspensions were removed by sonication for 10 min. Serial decimal dilutions from the obtained suspensions were plated on TSA to verify the number of viable cells in the biofilms. After plating the serial dilution on TSA, plates were incubated at 37 °C in an aerobic incubator for 24 h prior to counting. The total number of colony forming units (CFU) (logCFU mL^{-1}) was determined.

2.9.5. Statistical analysis

Statistical analyses were performed by GraphPad Prism® version 5.0 from Microsoft Windows®. The data from all assays were compared using one-way analysis of variance (ANOVA), with Bonferroni *post hoc* test. The data were considered significant when $p < 0.05$.

3. Results

3.1. Purification of ALL

Crude extract of *A. lactuca* showed strong hemagglutinating activity against human and rabbit erythrocytes, both native and treated with proteases. After ammonium sulfate precipitation, hemagglutinating activity was concentrated in fraction F 0–70. This fraction was loaded onto guar gum column. The unbound proteins washed with TBS showed residual hemagglutinating activity, whereas the retained proteins eluted with glycine buffer showed strong activity against all erythrocytes tested (data not shown).

This procedure increased 110-fold the lectin's specific activity compared to the crude extract. ALL eluted from the column represented 76% of the total hemagglutinating present in the extract (Table 1).

3.2. Hemagglutinating activity and inhibition of ALL

ALL was able to agglutinate all tested erythrocytes with slight preference for untreated erythrocytes from rabbit.

Hemagglutinating activity of ALL was inhibited by the disaccharides α-lactose, β-lactose and lactulose with minimum inhibitory concentrations (MIC) of 25 mM, 50 mM and 50 mM, respectively. The glycoprotein PSM was the most potent inhibitor with MIC of 4 μg.mL^{-1} (Table 2).

The optimum pH for lectin activity was 7. At pH 5 and 6, the activity of ALL decreases, and at pH 4, it was almost entirely lost. Above pH 8, lectin activity was slightly reduced (Fig. 1A). The activity was unaltered until 60 °C. Above of 60 °C, it decreases, and ALL

Table 1
Purification procedure of the lectin from *Aplysina lactuca* (ALL).

Fraction	Protein total (mg)	HU mL^{-1}	Specific activity		Yield (%)	Purification (fold)
			(HU mg^{-1})	Total		
Crude extract	934	128	137	128,000	100	1
F0-70	523.9	1024	278	173,056	135	2
Guar gum	6.5	1024	15,058	98,304	76.8	110

Rabbit erythrocytes in native form were used.

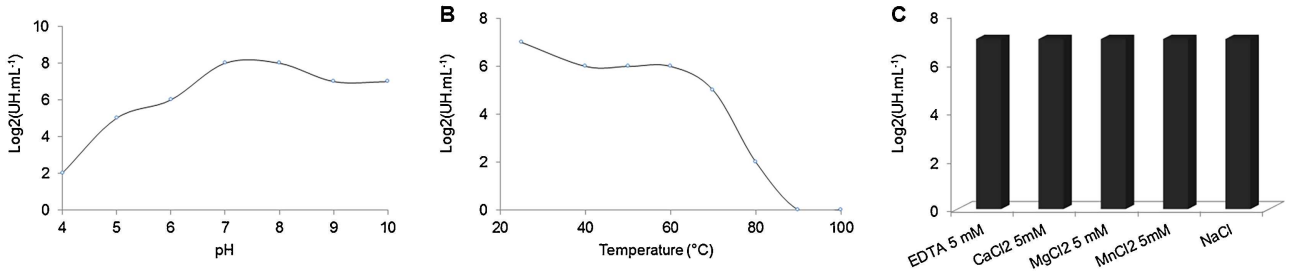

Fig. 1. Properties of the hemagglutinating activity of ALL. Effect of pH (A), temperature (B) and divalent cations and EDTA (C) on the hemagglutinating activity of ALL. Hemagglutinating activity was expressed in logarithm scale as units of titter. All assays were realized in duplicate.

Table 2
Inhibition of the hemagglutinating activity of ALL by sugars and glycoproteins.

Sugars	MIC[a]
α-D-Lactose	25 mM
β-D-Lactose	50 mM
α-D-Lactulose	50 mM
Glycoproteins	
Porcine Stomach Mucin	4 μg.mL^{-1}

The following sugars did not cause inhibition: D-xylose, D-ribose, L-fucose, L-arabinose, L-rhamnose, D-galactose, D-mannose, D-glucose, D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-galacturonic acid, D-fructose, D-sucrose, D-melibiose, D-maltose, D-raffinose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-β-D-thiogalactose, phenyl-β-D-galactopyranoside, 4-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-β-D-galactopyranoside, *O*-nitrophenyl-β-D-galactopyranoside.

[a] Minimum concentration of sugar required for inhibition.

was completely denatured at 90 °C (Fig. 1B). The presence of EDTA and $CaCl_2$ did not affect the activity of ALL (Fig. 1C).

3.3. Molecular mass and sugar content

SDS-PAGE of the pure lectin in the absence of β-ME showed one single band of 28 kDa, which was decomposed to a broad band of 19 kDa after treatment with reducing agent (Fig. 2). On gel filtration, ALL exhibited one single peak, corresponding to 94 kDa (Fig. 3).

ESI–MS deconvoluted spectra of intact lectin revealed one major ion of 31,810 ± 2 Da, with several low intensity ions around this value (Fig. 4A). When ALL was treated with IAA, no shift in mass spectrum was observed, indicating the absence of free-cysteines (data not shown).

On the other hand, when ALL was previously treated with DTT and incubated with IAA (CAM-ALL), two major ions were observed: 15,908 ± 2 Da (ALL-a) and 16,159 ± 2 Da (ALL-b) (Fig. 4B). When previously treated with β-ME and incubated with VP (PE-ALL), two other major ions were observed: 16,054 ± 2 Da and 16,302 ± 2 Da (Fig. 4C). The difference in molecular mass between CAM-ALL and PE-ALL suggests the presence of three cysteines, all of which are involved in disulfide bonds.

Intact ALL submitted to MALDI-ToF analysis (Fig. 5) revealed two major ions of 31,760 ± 2 Da (ALL-A) and 31,946 ± 2 Da (ALL-B).

In addition, ALL is not a glycoprotein, as observed in the Dubois assay.

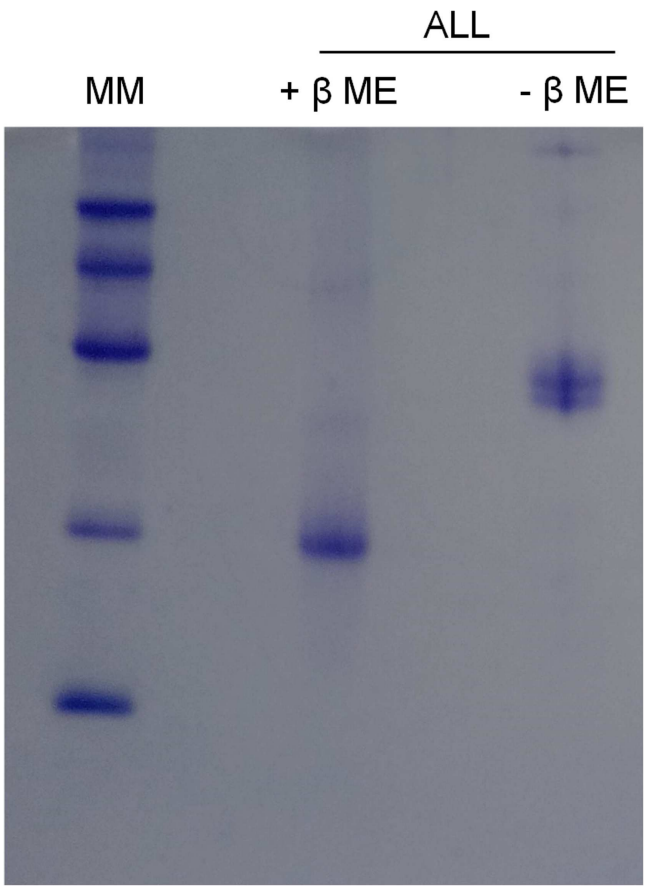

Fig. 2. SDS-PAGE of purified ALL. SDS-PAGE 15%. M) Molecular Marker. 20 μg of ALL were applied in the absence and presence of β-mercaptoethanol.

3.4. MS/MS

After in-gel digestion of ALL, eight tryptic peptides were identified and sequenced (Table 3). Together, these peptides represent 58% of the lectin's amino acid sequence. The partial amino acid sequence of ALL showed 42% of identity and 56% of similarity

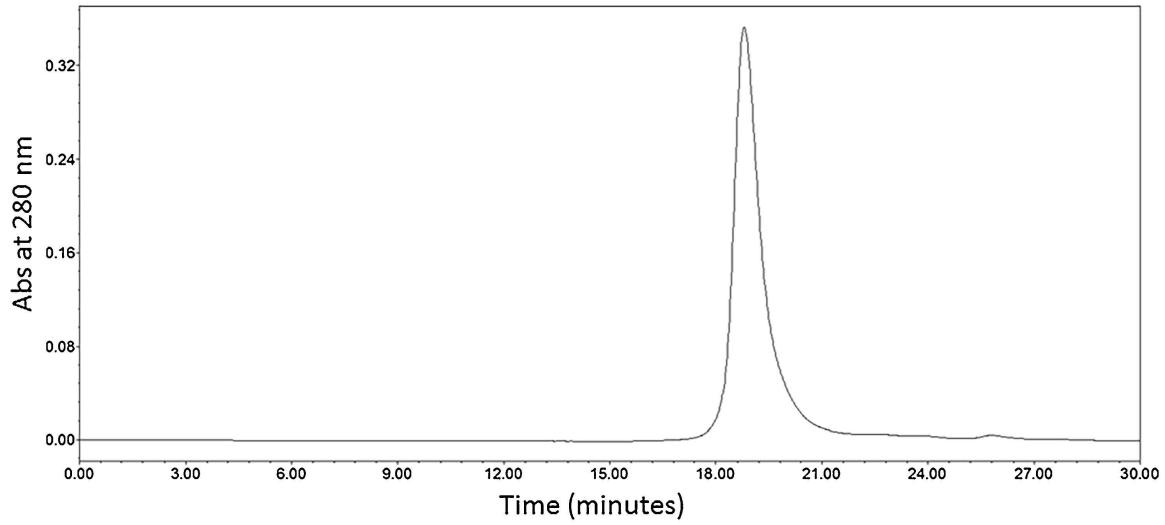

Fig. 3. Size exclusion chromatography of ALL. BioSuite 250 h SEC column (0.78 × 30 cm, 5 um particle size, Waters Corp.) was equilibrated and eluted with Tris-HCl, 50 mM, pH 7.2, containing NaCl 500 mM. Approximately 200 μg of ALL were loaded. UPLC operated at flow of 0.5 mL min^{-1}.

Table 3
Peptides originated by digestion of ALL with Trypsin.

m/z	Name	Sequence	Mass		Δ (Da)
			Observed	Calculated	
786.9293	T1	V[L/I]VSNVQNTVGTT[L/I]K	1571.8430	1571.8883	0.05
643.8115	T2	[L/I]ASESWNPARR	1285.6074	1285.6527	0.05
774.3490	T3	C[L/I]PEQE[L/I]YNVR	1546.6824	1546.7814	0.10
763.3114	T4	VQFDDQGYDAV[L/I]R	1524.6072	1524.7208	0.11
713.3212	T4′	VQFGNSGYDAV[L/I]R	1424.6268	1424.7048	0.08
502.9058	T5	TQFKEVATYTYR	1505.6940	1505.7515	0.06
443.2164	T6	[L/I]PVNTVSR	884.4172	884.5079	0.09
642.3108	T7	Q[L/I]QMVYFN[L/I]K	1282.6060	1282.6743	0.07

Table 4
Affinity constants and free energy of the binding of sugars to ALL at 363 K.

Sugar	K*a* (M^{-1})	α	$-\Delta G_F$(kJ mol^{-1})
Native	–	0.34	−366.1
α-D-lactose	12.5	0.44	−574.9
β-D-lactose	31.9	0.53	−805.5
D-Lactulose	145.5	0.66	−1463.7

α – Fraction folded at 363 K.
ΔG_F – Free energy of folding.

with the galactose-binding lectin isolated from *Axinella polypoides* (P28586.1), lectin I (Fig. 6).

3.5. CD

CD spectra of native ALL exhibited one minimum at 216 nm, suggesting a predominance of β-conformation in its secondary structure (Fig. 7). The prediction method CONTIN [34] indicated that the theoretical secondary structure consisted of 9% α-helix, 35% β-sheet, 23% β-turn and 33% coil.

Thermodynamic parameters of ALL, as determined by CD, revealed the binding constant to the ligands lactulose, α-lactose and β-lactose. The anomeric forms of lactose (α and β) showed close values of K*a*, whereas lactulose appears to be the ligand with which ALL has more affinity (Table 4).

3.6. Antibacterial activity and bacterial aggregation

ALL agglutinated both bacterial cells tested, *S. aureus* and *E. coli* (Fig. 8). On the other hand, the lectin was unable to inhibit the planktonic growth of both species (data not shown).

3.7. Effect of ALL on biofilm formation

ALL significantly reduced the biomass of both bacterial biofilms tested. Biofilm mass of *E. coli* was decreased in all tested concentrations (Fig. 9A). However, the effect on *S. aureus* biofilm showed a concentration-dependent behavior with activity in concentrations ranging from 250 to 15.6 μg mL^{-1} (Fig. 9B). Contact between ALL and both biofilms affected the number of viable cells at all concentrations tested (Fig. 9C and D).

4. Discussion

In this work, we reported the isolation and biochemical characterization of a new lectin from the marine sponge *A. lactuca*. ALL was purified by combining ammonium sulfate precipitation and affinity chromatography on guar gum column. Affinity chromatography on guar gum matrix has been employed to purify several galactophilic lectins, including HGA-2 from the sea cucumber *Holothuria grisea* and PFL from *Ptilota filicina* [23,30].

The genus *Aplysina* has already had two galactophilic lectins isolated from *A. lacunosa* and *A. archeri*. Both are glycoproteins formed by homotetramers with 16 kDa *per* subunit linked by weak interactions, and their hemagglutinating activity is Ca^{2+}-dependent [8].

ALL differs from other *Aplysina* lectins in some features. First, ALL is a trimer of dimers. Two monomers are linked by disulfide bond, and three dimers are linked by weak interactions to form a functional unit of six polypeptide chains. Sponge lectins have shown a wide range of oligomeric organization, including monomeric polypeptide chains, dimers linked by weak interactions, heterotrimers, tetramers linked by disulfide bonds, and large oligomers [4,10,19,35,36].

Second, ALL activity is independent from divalent ions. The presence of divalent ions in the binding site of invertebrate lectins is usual, and several lectins showed this characteristic. Only a few lectins from sponges are Ca^{2+}-dependent [4,8,10,14].

Finally, *A. archeri* lectin and ALL showed distinct specificity [8]. The former was inhibited by several galactosides, especially

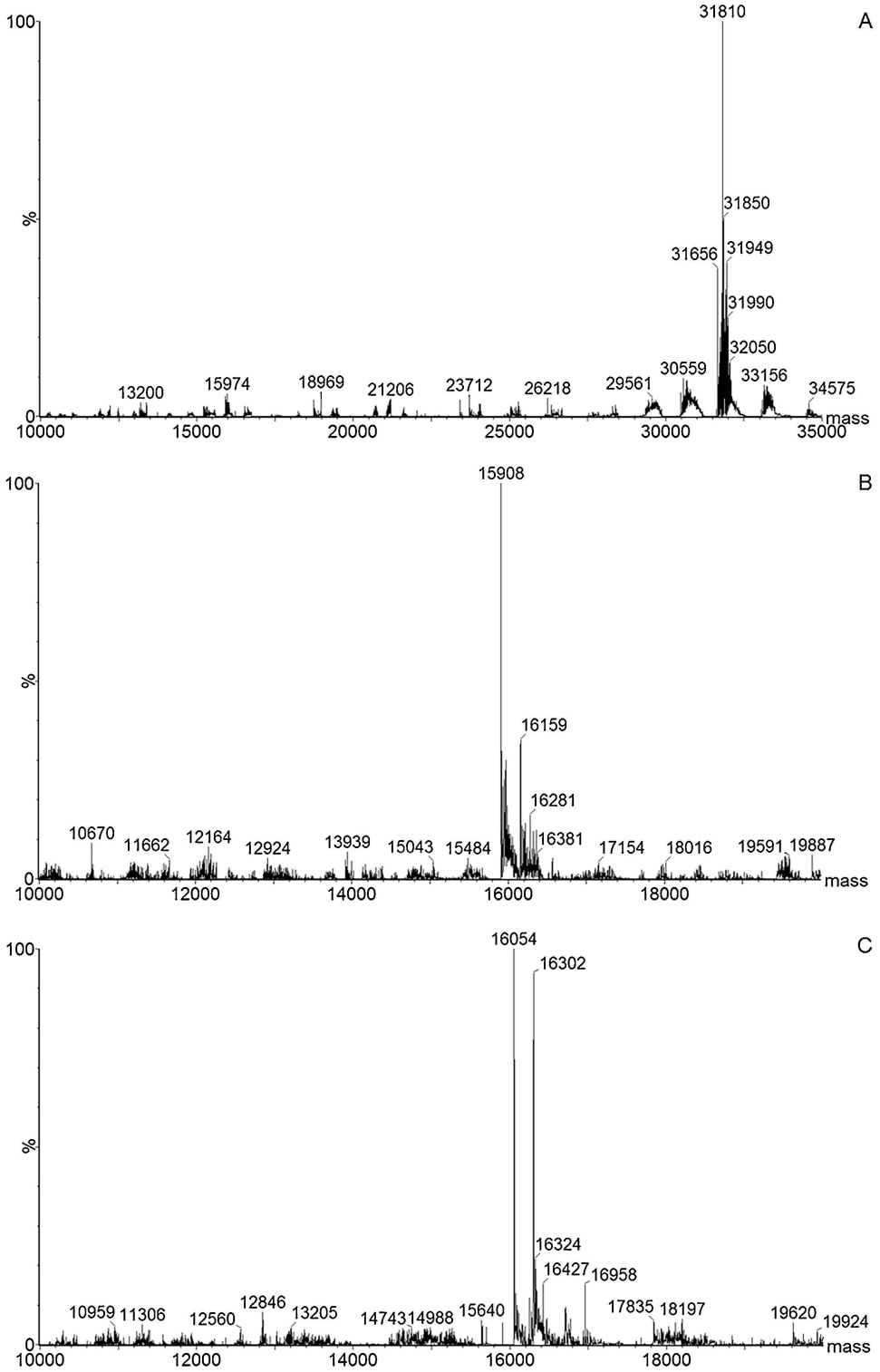

Fig. 4. Molecular mass determination of ALL by ESI–MS. A) Deconvoluted ESI mass spectra of ALL. The lectin (10 pmol μl^{-1}) was dissolved in a solution of ACN 50% containing 0.2% FA and infused into the NanoESI source coupled to an ESI-Q-ToF mass spectrometer. B) Deconvoluted mass spectra of CAM-ALL. C) Deconvoluted mass spectra of PE-ALL.

those that contain a terminal β-linked galactosyl, such as β-lactose, *N*-acetyllactosamine (lacNac) and digalactosyllactose, while thiol-β-D-galactose and β-methyl-D-galactopyranoside were unable to inhibit ALL activity. Indeed, ALL was isolated by guar gum from guar bean, a galactomannan composed of galactosyl linked (α1-6) to mannose backbone, and anomeric forms of the lactose were equally effective in the inhibition assay, which showed very similar K*a* values. Interestingly, these findings, when taken together, indicate that the specificity for terminal β-linked galactosyl is a characteristic that is not shared between *A. archeri* lectin and ALL.

However, ALL showed affinity for lactulose (β-linked galactosyl) and PSM, which presents T-antigen (Galβ1-3GalNac) in its composition, indicating that ALL is able to bind to β-galactosides, but not in an exclusive manner, and may also bind to α-galactosides, such as α-lactose and galactomannan.

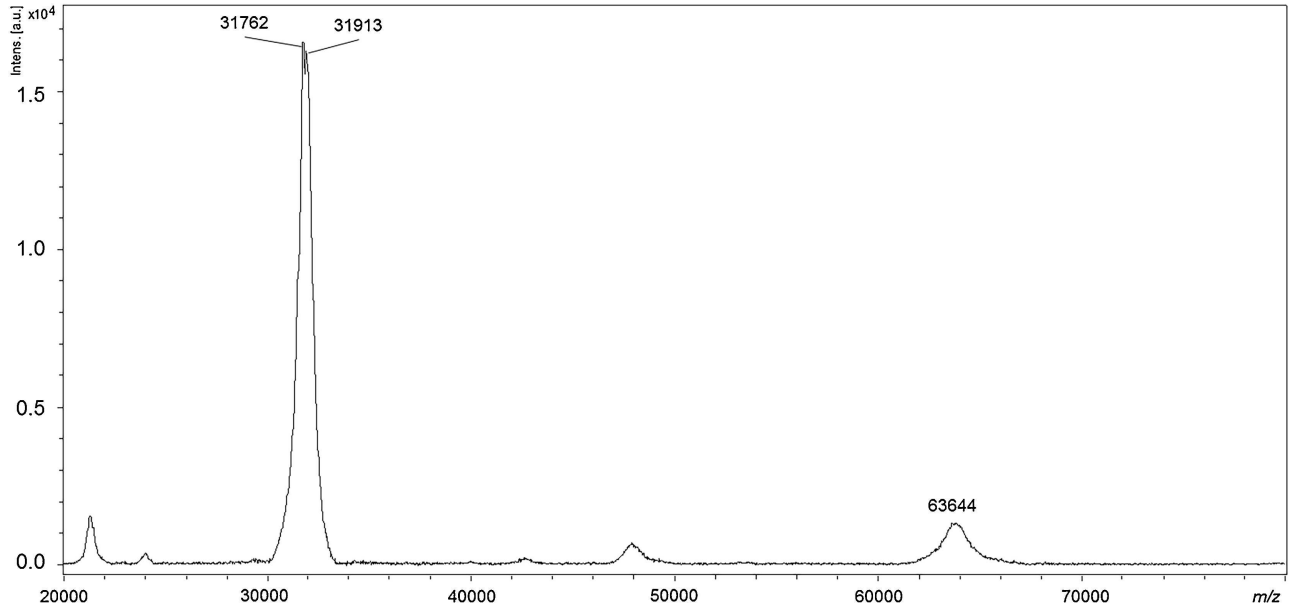

Fig. 5. Molecular mass determination of ALL by MALDI-ToF. The spectra were acquired in linear positive mode and processed with Flex Analysis 3.4 software.

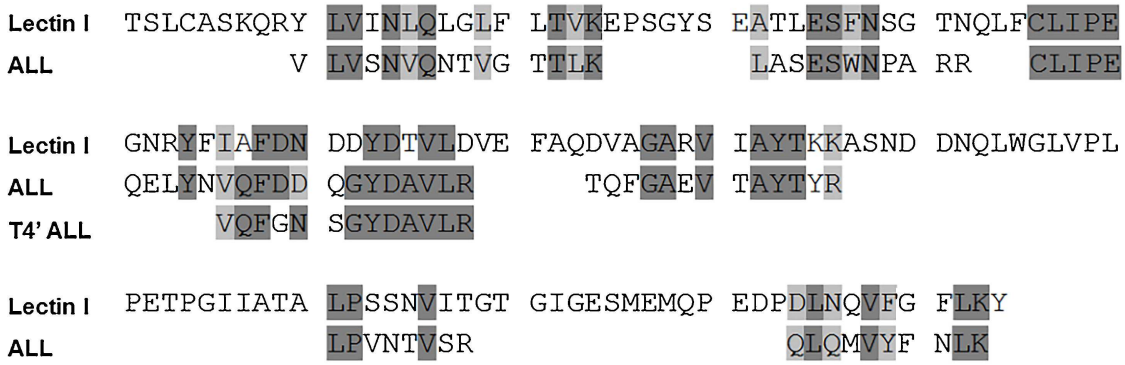

Fig. 6. Comparison of partial amino acid sequence of ALL and Lectin I from *Axinella polypoides*. Black boxes represent identical amino acids; gray boxes represent similar amino acids.

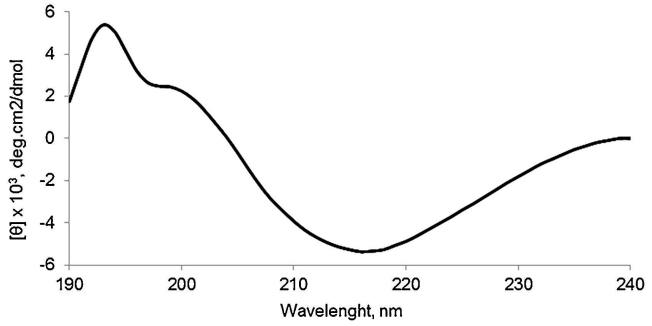

Fig. 7. CD spectra of ALL. Far-UV CD spectra (190–250 nm) of ALL. The cuvette path length was 0.05 cm; protein concentration was 0.2 mg mL^{-1} in PBS, pH 7. Eight acquisition were realized, an average value was calculated and plotted in the figure.

About 60% of the primary structure of ALL was obtained by amino acid sequencing of eight tryptic peptides. These peptides showed similarity with lectin I from *A. polypoides*. Indeed, ALL and lectin I shared some biochemical characteristics. For instance, both are non-glycosylated, their hemagglutinating activity is Ca^{2+}-independent, and their monomeric molecular masses are very close [17,37,38].

A. polypoides lectin I showed one intrachain disulfide bond between cysteines 4 and 46 [17]. ALL has three cysteines *per* subunit, and two of them seem to be involved in one intrachain disulfide bond. Since Cys^{46} appears conserved in ALL, it possible that disulfide bond 4–46 might present in ALL.

Furthermore, isolectins seem be present in both species. In *A. polypoides*, five lectins have been described. Lectins I–IV bind to galactose, whereas lectin V binds to hexuronic acids. Lectin II and V were identified in the first fifteen NH_2-terminal amino acids, and lectins I and II have similar molecular mass values (15,847 ± 10 Da and 16,228 ± 10 Da, respectively), sharing 65% of identity among I and II [17,38].

Like lectins I and II of *A. polypoides*, MS analyses of ALL revealed two distinct isolectins (A and B). When ALL was treated with reducing agents, followed by treatment with alkyl agents, subunits ALL-a and ALL-b were observed. Surprisingly, the combined molecular masses of the subunits did not correspond to the molecular mass of the intact isolectins. The deduced molecular masses of ALL-a and ALL-b are 15,737 ± 2 Da and 15,988 Da ± 2 Da, respectively, totaling hypothetical dimers of 31,474 Da (2x a), 31,725 Da (a + b) or 31,976 Da (2 × b), whereas molecular mass, as determined by MALDI-MS, was 31,760 ± 2 Da and 31,913 ± 2 Da for −A and −B, respectively. Interestingly, the average mass, as determined by ESI–MS, differs from both molecular mass, as determined by

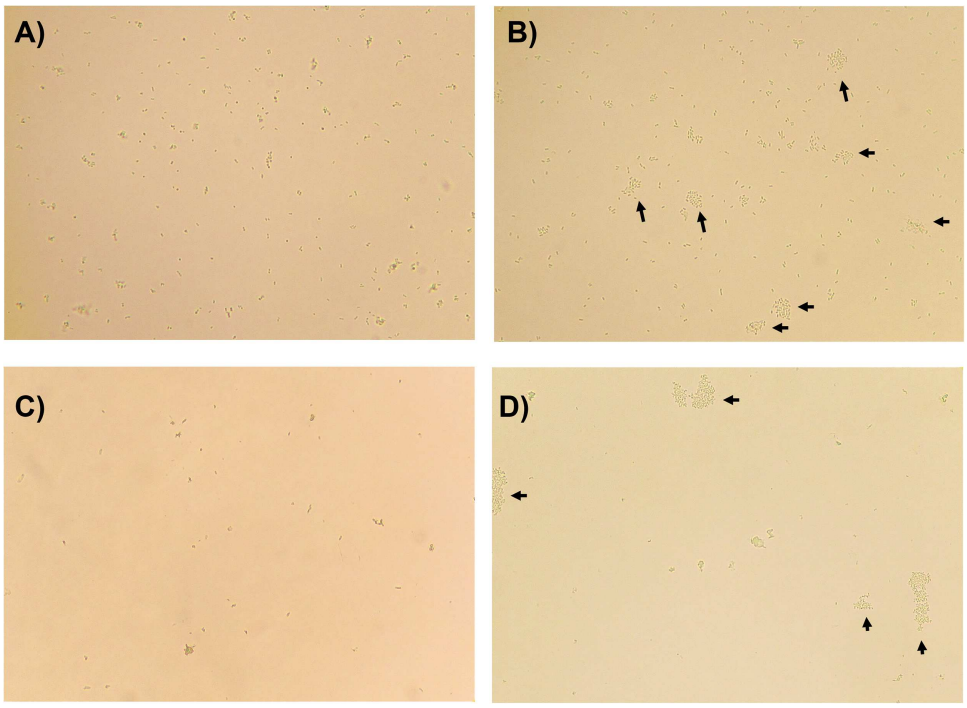

Fig. 8. Agglutination of bacteria by ALL. *S. aureus* incubated with TBS (A) and ALL (B); *E. coli* incubated with TBS (C) and ALL (D). Arrows indicate bacterial agglutination. Agglutinations were realized in duplicated.

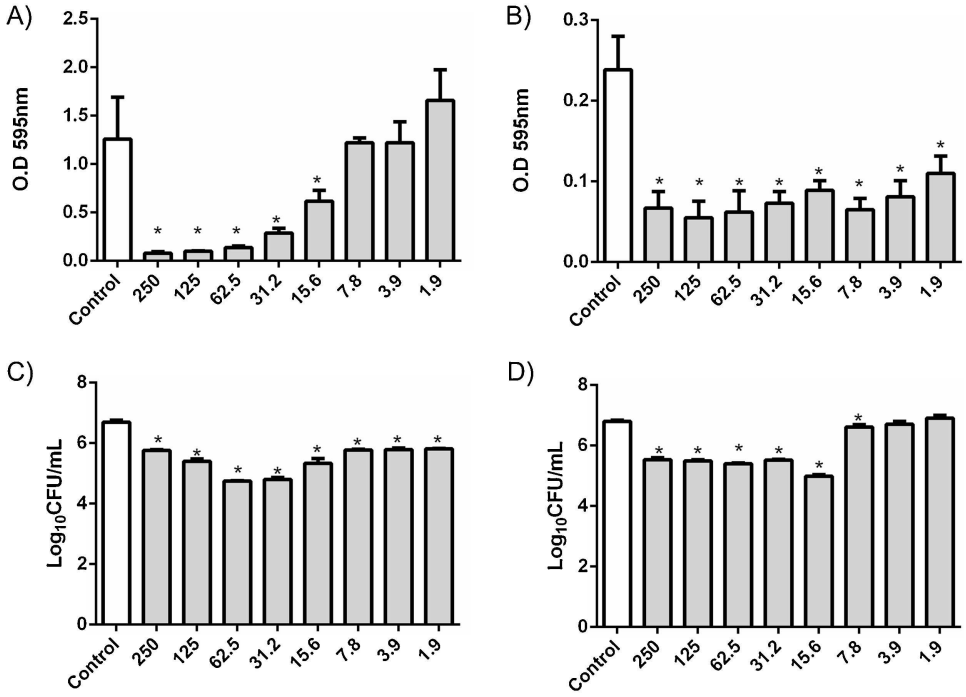

Fig. 9. Biofilm formation of S. aureus and E. coli in the presence of different concentration of ALL. (A and B) Total biomass and (C and D) number of viable cells present in the biofilms. (A) and (C) represents *S. aureus* and (B) and (D) E. coli biofilm. *$p < 0.05$ compared to control (corresponding to 0 μg/mL). Error bars display standard deviations (SD) of the means. The assays were repeated in triplicate on three different occasions.

MALDI-ToF, and molecular mass of the hypothetical dimers. The reason for this divergence is unclear, but it can be speculated that intrinsic properties of the analyte, as well as intrinsic properties of the instrument, may have contributed to these observations.

The major difference between lectins I and II of *A. polypoides* is the insertion of an alanyl-dipeptide in position 131–132 in lectin II. Therefore, lectin II is two amino acids longer than lectin I [38]. In ALL, peptides T4 and T4′ are quite different in their amino acid sequence, which may contribute to the difference between ALL-A and ALL-B.

Despite the common features, the differences between lectin I and ALL are remarkable. In lectin I, subunits are linked by weak interactions, and high affinity for non-reducing terminal β-linked galactosyl was observed [8,37].

Lectin I from *A. polypoides* was initially classified as a $(QxW)_3$-lectin, a family of lectins that included ricin [39]. Currently, ricin and other $(QxW)_3$-lectins are included in the R-type lectin family. Members of the R-type family present repeated domains similar to ricin and, of course, the $(QxW)_3$ motif (Hirabayashi, Dutta, & Kasai, 1998). However, the homology between lectin I and R-type lectins is weak since *Axinella* lectins display just one (QxW) motif, and the alignment of six other conserved amino acids is only achieved by inserting gaps [38].

Axinella lectins and ALL shared a certain level of similarity and, therefore, could be grouped into the same family. However, to accomplish this, the determination of the complete amino acid sequence of ALL is required.

The secondary structure of ALL was predominately formed by β-conformations. Several structural domains of proteins are formed by such predominance, some of which are present in lectins, such as *jelly roll* β-sandwich and β-trefoil, typical domains of L- and R-type lectins, respectively [40].

ALL has the ability to agglutinate Gram-positive and Gram-negative bacterial cells. Several sponge lectins may bind specific glycans on the cell surface of microorganisms, causing bacterial aggregation and/or exhibiting antimicrobial activities [15,41,42]. Moreover, some galactoside-specific lectins are able to cause agglutination of bacterial cells [10,30,43]. Several glycosylated structures present on the cell surface of Gram-positive and Gram-negative bacteria can be recognized by ALL, including peptidoglycan, capsular polysaccharides, teichoic acid and LPS. According to Gardères and coworkers [4], the ability of sponge lectins to bind specific carbohydrates in bacterial cells could potentially be used to develop new antimicrobial agents.

Sponge lectins have shown a large spectrum of antibacterial activities. For instance, the lectin isolated from *Suberites domuncula* displays antibacterial activity against *E. coli* and *S. aureus* [15]. According to Müller and coworkers [44], the *S. domuncula* lectin acts as an antibacterial molecule involved in immune defense. Interestingly, despite causing bacterial agglutination, ALL does not inhibit bacterial growth. Corroborating our results, Kazanjian and Fariñas [45] showed that the lectin from *A. lacunosa* did not exhibit any antibacterial activity on Gram-positive and Gram-negative bacteria. However, the aqueous extract of *A. lacunosa* was able to inhibit bacterial growth. The activity was attributed to the presence of secondary metabolites in the extract [45].

On the other hand, the lectin from *A. lacunosa* strongly reduced *S. aureus* and *E. coli* biofilm formation. Lectins isolated from marine organisms, such as algae marine lectins, have demonstrated the ability to inhibit bacterial biofilm formation [46]. Studies point out that bacterial aggregation may cause a decrease in the number of adherent cells, consequently inhibiting the formation of biofilm [47]. The effect of ALL on bacterial biofilm can be exploited since this could prevent the emergence of drug- resistant strains.

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References

[1] S.H. Barondes, Bifunctional properties of lectins: lectins redefined, Trends Biochem. Sci. 13 (1988) 480–482.
[2] D.C. Kilpatrick, Handbook of animal lectins, properties and biomedical applications, British library, Edinburgh, 2000, pp. 468p.
[3] W.J. Peumans, E.J.M. Van Damme, Lectins as plant defense proteins, Plant Physiol. 109 (1995) 347–352.
[4] J. Gardères, M. Bourguet-Kondracki, L.B. Hamer, R. Batel, H. Schröder, W. Müller, Porifera lectins: diversity, physiological roles and biotechnological potential, Mar. Drugs 13 (8) (2015) 5059–5101.
[5] H. Bretting, C. Donadey, J. Vacelet, G. Jacobs, Investigations on the occurrence of lectins in marine sponges with special regard to some species of the family axinellidae, Comp. Biochem. Phys. B 70 (1981) 69–76.
[6] R.R. Dresch, A.S. Haeser, C. Lerner, B. Mothes, M.M. Vozári-Hampe, A.T. Henriques, Detecção de atividade lectínica e atividade hemolítica em extratos de esponjas (Porifera) nativas da costa atlântica do Brasil, Rev. Bras. Farmacogn. 15 (2005) 16–22.
[7] D. Mebs, I. Weiler, H.F. Heinke, Bioactive proteins from marine sponges: screening of sponge extracts for hemagglutinating, hemolytic, ichthyotoxic and lethal properties and isolation and characterization of hemagglutinins, Toxicon 23 (1985) 955–962.
[8] P.B. Miarons, M. Fresno, Lectins from tropical sponges: purification and characterization of lectins from genus Aplysina, J. Biol. Chem. 275 (2000) 29283–29289.
[9] R.M. Moura, A.A. Melo, R.F. Carneiro, C.R. Rodrigues, P. Delatorre, K.S. Nascimento, S. Saker-Sampaio, C.S. Nagano, A.H. Sampaio, Hemagglutinating/Hemolytic activities in extracts of marine invertebrates from the Brazilian coast and isolation of two lectins from the marine sponge Cliona varians and the sea cucumber Holothuria grisea, An. Acad. Bras. Cienc. 87 (2015) 973–984.
[10] R.M. Moura, A.F. Queiroz, J.M. Fook, A.S. Dias, N.K. Monteiro, J.K. Ribeiro, G.E. Moura, L.L. Macedo, E.A. Santos, M.P. Sales, CvL, a lectin from the marine sponge Cliona varians: isolation, characterization and its effects on pathogenic bacteria and Leishmania promastigotes, Comp. Biochem. Phys. A 145 (2006) 517–523.
[11] A.F.S. Queiroz, R.M. Moura, J.K.C. Ribeiro, I.L. Lyra, D.C.S. Cunha, E.A. Santos, M.P. Sales, Pro-inflammatory effect in mice of CvL, a lectin from the marine sponge Cliona varians, Comp. Biochem. Phys. C 147 (2008) 216–221.
[12] A.F.S. Queiroz, R.A. Silva, R.M. Moura, J.L. Dreyfuss, E.J. Paredes-Gamero, A.C.S. Souza, I.L. Tersariol, E.A. Santos, H.B. Nader, G.Z. Justo, M.P. Sales, Growth inhibitory activity of a novel lectin from Cliona varians against K562 human erythroleukemia cells, Cancer Chemother. Pharm. 63 (2009) 1023–1033.
[13] T. Ueda, Y. Nakamura, C.M. Smith, B.A. Copits, A. Inoue, T. Ojima, S. Matsunaga, G.T. Swanson, R. Sakai, Isolation of novel prototype galectins from the marine ball sponge Cinachyrella sp. guided by their modulatory activity on mammalian glutamate-gated ion channels, Glycobiology 3 (4) (2013) 412–425.
[14] D. Gundacker, S.P. Leys, H.C. Schröder, I.M. Müller, W.E. Müller, Isolation and cloning of a C-type lectin from the hexactinellid sponge Aphrocallistes vastus: a putative aggregation factor, Glycobiology 11 (2001) 21–29.
[15] H.C. Schroder, H. Ushijima, A. Krasko, V. Gamulin, N.L. Thakur, B. Diehl-Seifert, I.M. Muller, W.E.G. Muller, Emergence and disappearance of an Immune molecule, an antimicrobial lectin, in basal metazoa: a tachylectin-related protein in the sponge Suberites domuncula, J. Biol. Chem. 278 (35) (2003) 32810–32817.
[16] H. Stalz, U. Roth, D. Schleuder, M. Macht, S. Haebel, K. Strupat, K. J.Peter-Katalinic, F.G. Hanisch, The Geodia cydonium galectin exhibits prototype and chimera-type characteristics and a unique sequence polymorphism within its carbohydrate recognition domain, Glycobiology 6 (5) (2006) 402–414.
[17] F. Buck, C. Luth, K. Strupat, H. Bretting, Comparative investigations on the amino-acid sequences of different isolectins from the sponge Axinella polypoides (Schmidt), Biochim. Biophys. Acta 1159 (1992) 1–8.
[18] R.F. Carneiro, A.A. Melo, A.S. Almeida, R.M. Moura, R.P. Chaves, B.L. Sousa, K.S. Nascimento, B.S. Cavada, C.S. Nagano, A.H. Sampaio, H-3, a new lectin from the marine sponge Haliclona caerulea: purification and mass spectrometric characterization, Int. J. Biochem. Cell B 45 (2013) 2864–2873.
[19] D.S. Medeiros, T.L. Medeiros, J.K.C. Ribeiro, N.K.V. Monteiro, L. Migliolo, A.F. Uchoa, I.M. Vasconcelos, A.S. Oliveira, M.P. Sales, E.A. Santos, A lactose specific lectin from the sponge Cinachyrella apion: purification, characterization, N-terminal sequences alignment and agglutinating activity on Leishmania promastigotes, Comp. Biochem. Phys. B 155 (2010) 211–216.
[20] I. Pajic, Z. Kljajic, N. Dogovic, D. Sladic, Z. Juranic, M.J. Gasic, A novel lectin from the sponge Haliclona cratera: isolation, characterization and biological activity, Comp. Biochem. Physiol. C 132 (2002) 213–221.
[21] U.S. Pinheiro, E. Hajdu, M.R. Custódio, Aplysina Nardo (Porifera, Verongida, Aplysinidae) from the Brazilian coast with description of eight new species, Zootaxa 1609 (2007) 1–51.
[22] P.S. Appukuttan, A. Surolia, B.K. Bachhawat, Isolation of two galactose-binding proteins from Ricinus communis by affinity chromatography, Indian J. Biochem. Biophys. 14 (1977) 382–384.
[23] A.H. Sampaio, D.J. Rogers, C.J. Barwell, A galactose specific lectin from the red marine alga *Ptilota filicina*, Phytochemistry 48 (5) (1998) 765–769.
[24] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4, Nature 227 (1970) 680–683.

[25] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[26] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, In-gel digestion for mass spectrometric characterization of proteins and proteomes, Nat. Protocols 1 (6) (2006) 2856–2861.
[27] L. Whitmore, B.A. Wallace, Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases, Biopolymers 89 (2008) 392–400.
[28] N.J. Greenfield, Determination of the folding of proteins as a function of denaturants, osmolytes or ligands using circular dichroism, Nat. Protocols 1 (2007) 2733–2741.
[29] N.J. Greenfield, Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions, Nat. Protocols 1 (2006) 2527–2535.
[30] A.A. Melo, R.F. Carneiro, W.M. Silva, R.M. Moura, G.C. Silva, O.V. Sousa, J.S. Saboya, K.S. Nascimento, S. Saker-Sampaio, C.S. Nagano, B.S. Cavada, A.H. Sampaio, HGA-2, a novel galactoside-binding lectin from the sea cucumber Holothuria grisea binds to bacterial cells, Int. J. Biol. Macromol. 64 (2014) 435–442.
[31] National Committee for Clinical Laboratory Standards, Document M7-A6; Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, NCCLS, Wayne, PA, USA, 2003.
[32] M.A. Vasconcelos, F.V. Arruda, V.A. Carneiro, H.C. Silva, K.S. Nascimento, A.H. Sampaio, B.S. Cavada, E.H. Teixeira, M. Henriques, M.O. Pereira, Effect of algae and plant lectins on planktonic growth and biofilm formation in clinically relevant bacteria and yeasts, BioMed. Res. Int. (2014) 9 (ID:365272).
[33] S. Stepanovic, D. Vukovic, I. Dakic, B. Savic, M. Svabic-Vlahovic, A modified microtiter-plate test for quantification of staphylococcal biofilm formation, J. Microbiol. Methods 40 (2) (2000) 175–179.
[34] I.H.M. van Stokkum, H.J.W. Spoelder, M. Bloemendal, R. van Grondelle, F.C.A. Groen, Estimation of protein secondary structure and error analysis from CD spectra, Anal. Biochem. 191 (1990) 110–118.
[35] R.F. Carneiro, A.A. Melo, F.E.P. Nascimento, C.A. Simplicio, K.S. Nascimento, B.A.M. Rocha, S. Saker-Sampaio, R.M. Moura, B.S. Cavada, C.S. Nagano, A.H. Sampaio, Halilectin 1 (H-1) and Halilectin 2 (H-2): two new lectins isolated from the marine sponge Haliclona caerulea, J. Mol. Recognit. 26 (2013) 51–58.
[36] C. Xiong, W. Li, H. Liu, W. Zhang, J. Dou, X. Bai, Y. Du, X. Ma, A normal mucin-binding lectin from the sponge Craniella australiensis, Comp. Biochem. Phys. C 43 (1) (2006) 9–16.
[37] H. Bretting, E.A. Kabat, Purification and characterization of the agglutinins from the sponge Axinella polypoides and a study of their combining sites, Biochemistry 15 (1976) 3228–3236.
[38] F. Buck, C. Schulze, M. Breloer, K. Strupat, H. Bretting, Amino acid sequence of the D-galactose binding lectin II from the sponge Axinella polypoides (Schmidt) and identification of the carbohydrate binding site in lectin II and related lectin I, Comp. Biochem. Phys. B 121 (1998) 153–160.
[39] B. Hazes, The (QxW)3 domain: a flexible lectin sacffold, Prot. Sci. 5 (1996) 1490–1501.
[40] G.R. Vasta, H. Ahmed, E.W. Odom, Structural and functional diversity of lectin repertoires in invertebrates, protochordates and ectothermic vertebrates, Curr. Opin. Struc. Biol. 14 (2004) 617–630.
[41] T. Saito, S. Kawabata, M. Hirata, S. Iwanaga, A novel type of limulus lectin-L6. Purification, primary structure, and antibacterial activity, J. Biol. Chem. 270 (1995) 14493–14499.
[42] B. Fenton, R.A. Espinosa, E.C. Vázquez, B.A. Lozano, N.S. Sánchez, E.G. Hernández, E.Z. Galindo, Purification and characterization of structural and functional properties of two lectins from a marine sponge Spheciospongia vesparia, Indian J. Biochem. Biophys. 50 (2013) 562–569.
[43] R.F. Carneiro, C.S. Teixeira, A.A. Melo, A.S. Almeida, B.S. Cavada, O.V. Sousa, B.A.M. Rocha, C.S. Nagano, A.H. Sampaio, L-rhamnose-binding lectin from eggs of the Echinometra lucunter: amino acid sequence and molecular modeling, Int. J. Biol. Macromol. 78 (2015) 180–188.
[44] W.E. Müller, X. Wang, P. Proksch, C.C. Perry, R. Osinga, J. Gardères, H.C. Schröder, Principles of biofouling protection in marine sponges: a model for the design of novel biomimetic and bio-inspired coatings in the marine environment, Mar. Biotechnol. (NY) 15 (2013) 375–398.
[45] A. Kazanjian, M. Fariñas, Actividades biológicas del extracto acuoso de la esponja Aplysina lacunosa (Porifera: aplysinidae), Rev. Biol. Trop. 54 (2006) 189–200.
[46] E.H. Teixeira, M.H. Napimoga, V.A. Carneiro, T.M. Oliveira, K.S. Nascimento, C.S. Nagano, J.B. Souza, A. Havt, V.P. Pinto, R.B. Gonçalves, W.R. Farias, S. Saker-Sampaio, A.H. Sampaio, B.S. Cavada, In vitro inhibition of oral streptococci binding to the acquired pellicle by algal lectins, J. Appl. Microbiol. 03 (4) (2007) 1001–1006.
[47] W.F. Liljemark, C.G. Bloomquist, G.R. Germaine, Effect of bacterial aggregation on the adherence of oral streptococci to hydroxyapatite, Infect. Immun. 31 (1981) 935–941.

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Antibacterial activity of a new lectin isolated from the marine sponge *Chondrilla caribensis*

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ABSTRACT

A new lectin from the marine sponge *Chondrilla caribensis* (CCL) was isolated by affinity chromatography in Sepharose 6B media. CCL is a homotetrameric protein formed by subunits of 15,445 ±2 Da. The lectin showed affinity for disaccharides containing galactose and mucin. Mass spectrometric analysis revealed about 50% of amino acid sequence of CCL, which showed similarity with a lectin isolated from *Aplysina lactuca*. Secondary structure consisted of 10% α-helix, 74% β-sheet/β-turn and 16% coil, and this profile was unaltered in a broad range of pH and temperatures. CCL agglutinated *Staphylococcus aureus*, *S epidermidis* and *Escherichia coli*, and it was able to reduce biofilm biomass, but showed no inhibition of planktonic growth of these bacteria. CCL activity was inhibited by α-lactose, indicating that Carbohydrate Recognition Domain (CRD) of the lectin was involved in antibiofilm activity.

1. Introduction

Lectins represent a class of proteins with ability to bind to specific sugars in a non-catalytic way. The lectins are ubiquitous proteins, being found in all living organisms. In Porifera phylum, approximately fifty lectins were isolated and biochemically characterized [1].

Interestingly, lectins from marine sponge showed a broad spectrum of biological activities [1]. For instance CaL, a lectin isolated from *Cinachyrella apion*, showed high growth inhibition for HeLa, reducing cell growth at a dose dependent manner [2]. Other lectin, CvL, isolated from *Cliona varians*, inhibited the growth of human leukemia cells, but no effect on heath blood lymphocytes was observed [3].

Moreover, lectins from marine sponges are involved in self-defense of the organism, since several lectins are able to recognize, agglutinate and inhibit growth of bacterial cells and biofilms [1]. ALL, a lectin isolated from *Aplysina lactuca*, agglutinated Gram-positive and Gram-negative bacterial cells, and it were able to reduce the biomass of bacterial biofilms at dose-dependent effect [4]. On the other hand, a lectin from *Cliona varians* (CvL) displays a cytotoxic effect on Gram-positive bacteria, but did not affect Gram-negative bacteria [5].

In addition to marine sponges, others invertebrate lectins have showed interesting biological properties [6,7]. MytiLec isolated from *Mytilus galloprovincialis* showed a dose-dependent cytotoxic effect on human Burkitt lymphoma Raji cells [8], whereas ADEL

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from *Aplysia dactylomela* eggs was able to agglutinate and inhibit biofilm formation of *Staphylococcus aureus* [9].

Chondrilla caribensis Ruetzler, Duran & Piantoni (2007) is a grayish to chestnut and purplish brown sponge commonly found in marine reefs, where it inhabits solid substrate such as rock, conch shells and red-mangrove stilt roots. In this work, we reported the purification and biochemical characterization of new lectin from *C. caribensis* with antibiofilm activity.

2. Material and methods

2.1. Animal collection

C. caribensis specimens were collected in the intertidal zone in the Paracuru beach, Ceará, Brazil. The animals were transported in plastic bags to the lab and stored at −20 °C until use. The species was identified, and a voucher was deposited (ID: UFPEPOR2254) at the Zoology Department of the Universidade Federal de Pernambuco, PE, Brazil. Collections were authorized and certified by responsible environmental institutions (SISBIO ID: 33913-8).

2.2. CCL purification

Frozen sponges were freeze-dried and triturated to a fine powder. Sponge powder was soaked with ten volumes (w:v) of 50 mM Tris-HCl, pH 7.6, containing NaCl 150 mM and PMSF 0.1 mM (TBS), and maintained under agitation for 30 min at room temperature. Then, material was centrifuged at 8000×*g* for 20 min at 4 °C, and the supernatant, named crude extract, was stored at −20 °C until the use.

Crude extract was loaded into HCl-activated Sepaharose™ 6 B column (1.0 × 10.0 cm), previously equilibrated with TBS. Unbound proteins were washed with same buffer and the lectin was recovered through elution with 0.2 M lactose in TBS. Fractions eluted with lactose in TBS were pooled, dialyzed against distillated water, freeze-dried and stored until the use.

Chromatography was conducted at flow rate of 1 mL min^{-1}, and monitored by measurement of absorbance at 280 nm 2-mL fractions were collected.

2.3. Hemagglutinating activity and inhibition

Hemagglutinating activity and inhibition assays was carried according to pre-established methods [10]. Erythrocytes from human (A, B and O) and rabbit were used in their native and protease (trypsin and papain) treated forms.

In the inhibition assay, the following sugars and glycoproteins were used: D-xylose, D-ribose, L-fucose, L-arabinose, L-rhamnose, D-galactose, D-mannose, D-glucose, D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-galacturonic acid, D-fructose, D-sucrose, D-melibiose, α-D-lactose, β-D-lactose, D-lactulose, D-maltose, D-raffinose, methyl-α-D-galactopiranoside, methyl-β-D-galactopiranoside, methyl-β-D-thiogalactose, phenyl-β-D-galactopiranoside, 4-nitrophenyl-α-D-galactopiranoside, 4-nitrophenyl-β-D-galactopiranoside, 2-nitrophenyl-β-D-galactopiranoside, O-nitrophenyl-β-D-galactopiranoside, bovine submaxillary mucin (BSM), porcine fetuin and porcine stomach mucin (PSM) type 2 and 3.

The effect of the pH, temperature and divalent ions on hemagglutinating activity of CCL was evaluated as described by Sampaio, Rogers, & Barwell [10].

2.4. Molecular mass determination and sugar content

Molecular mass of CCL under denaturing conditions was estimated by SDS PAGE [11], in the presence and absence of β-mercaptoethanol. LMW-SDS marker kit (GE Healthcare, UK) was used as standard.

Native molecular mass of CCL was estimated by size exclusion chromatography (SEC) on BioSuite 250 HR SEC column (0.78 × 30 cm, 5 μm particle size, Waters Corp.) coupled to an HClass UPLC system (Waters Corp, MA, USA). 200 μg of CCL were applied into the column, previously equilibrated with TBS. The column was calibrated with BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsnogen (24 kDa), and ribonuclease A (13.7 kDa).

Molecular mass of CCL was determined by Electrospray ionization – Mass Spectrometry (ESI–MS), conform described by Carneiro et al. [4] using a hybrid Synapt HDMS mass spectrometer (Waters Corp., Milford, MA, USA). The instrument was calibrated with [Glu1]fibrinopeptideB fragments. Mass spectra were acquired by scanning at *m*/*z* range from 500 to 3000 at 1 scan/s.

Free and total cysteines in CCL were quantified conform described by Carneiro et al. [4].

Neutral carbohydrate content in CCL was evaluated, as described by Dubois et al. [12], using lactose as the standard.

2.5. Tandem mass spectrometry (MS/MS) of tryptic peptides

Digestion with trypsin and peptide extraction was carried out as described by Shevchenko et al. [13]. Tryptic peptides were separated on a reverse phase C-18 (0.075 × 100 mm) nanocolumn coupled to a nanoAcquity system. The eluates were analyzed in a hybrid mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp. MA, USA). The instrument parameters were adjusted as described by Carneiro et al., [14].

Collision induced dissociation (CID) spectra were manually interpreted, and sequenced peptides were searched online against NCBI and Uniprot protein databanks.

2.6. Circular dichroism analysis

Circular Dichroism (CD) spectroscopic measurement was performed on a Jasco J-815 spectropolarimeter (Jasco International Co., Tokyo, Japan) connected to a peltier with controlled temperature conform described by Carneiro et al., [9].

The effect of the pH on the CCL secondary structure was determined by incubation of the lectin in different pH values (2, 4, 7, 9 and 12) for 1 h followed by CD measurements. The effect of the temperature on the CCL secondary structure was determined by CD measurements in different temperatures (20, 37, 56, 72 and 95 °C). The effect of sugars in the CCL fold was observed by CD measurements in the near UV, conform described by Carneiro et al., [9].

2.7. Antibacterial activity

2.7.1. Strain and culture conditions

Three reference strains from the American Type Culture Collection (ATCC) were used in this study: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Escherichia coli* ATCC 11303.

Bacteria were grown in Trypticase Soy Agar medium (TSA; Himedia, India) and incubated at 37 °C for 24 h. After growth on TSA, isolated colonies were inoculated in 10 mL of Trypticase Soy Broth (TSB; Himedia, India) and incubated under constant agitation

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at 37 °C for 24 h. Then, the bacterial suspensions were prepared in TSB adjusted at a cell density of 2×10^6 cfu/mL.

2.7.2. Bacterial agglutination

The bacteria were grown in TSB at 37 °C for 24 h and harvested by centrifugation at 2000×*g* for 10 min. Agglutination assays were performed as described by Melo et al. [15].

2.7.3. Effects of CCL on planktonic cells

The antibacterial activity of CCL was evaluated by the microdilution method according to the Clinical and Laboratory Standards Institute document M07-A9, with some modifications [16,17].

2.7.4. Effect of CCL on biofilm formation

The methodology used to evaluate the effects of the lectin on biofilm formation was based on the microtiter plate test described by Stepanovic et al. [18] with some modifications. The cellular suspensions adjusted 2×10^6 cells mL^{-1} were transferred to 96-well microtiter plates (100 μL per well) and then added CCL in different concentrations (7.8–250 μg mL^{-1}). To promote biofilm development, the plates were incubated for 24 h at 37 °C. The biofilm formation was evaluated by two distinct methods: biomass quantification by crystal violet staining and enumeration of biofilm-entrapped viable cells.

2.7.4.1. Quantification of the biofilm biomass. The biomass of the biofilms formed in presence and absence of the lectin was analyzed using the crystal violet staining method. For that, the wells containing the biofilms were washed twice with 200 μL with ultrapure water to remove weakly adherent cells and left to air dry. The biofilms were then fixed with 200 μL of 99% methanol for 15 min. After 15 min, the methanol was removed, the wells were dried at room temperature and then 200 μL of crystal violet stain (1%, v/v) were added to the wells and after 5 min, the excess of crystal violet was removed. After the staining step, the plates were washed and air dried for approximately 20 min. Lastly, 200 μL of acetic acid (33%, v/v) were added to each well to solubilize the bound crystal violet. The absorbance of each well was measured at 590 nm (OD_{590}) using a microplate reader (SpectraMax® I3).

2.7.4.2. Quantification of biofilm-entrapped cells. In order to determine viability of biofilm-entrapped cells treated and untreated with CCL, the wells were washed twice with 200 μL of ultrapure water and the biofilm suspensions removed by sonication for 10 min and then serially diluted and plated on TSA [16]. After incubation at 37 °C for 24 h, the total number of colony-forming units (CFUs) was quantified and expressed as CFU/mL.

2.7.5. Effect of lactose on antibiofilm activity of CCL

To verify the role of the carbohydrate recognition domain (CRD) on the antibiofilm activity of CCL, the lectin (250 μg mL^{-1}) was incubated with 100 mM α-lactose at 37 °C for 1 h. After incubation, the biofilm of *S. aureus* and *S. epidermidis* were developed in presence of CCL with or without α-lactose. The biofilm formation was evaluated by biomass quantification as described previously.

2.7.6. Statistical analysis

Statistical analyses were performed by GraphPad Prism® version 5.0 for Microsoft Windows®. Data from all assays were compared using one-way analysis of variance (ANOVA), with Bonferroni post hoc test. Data were considered significant when $P < 0.05$.

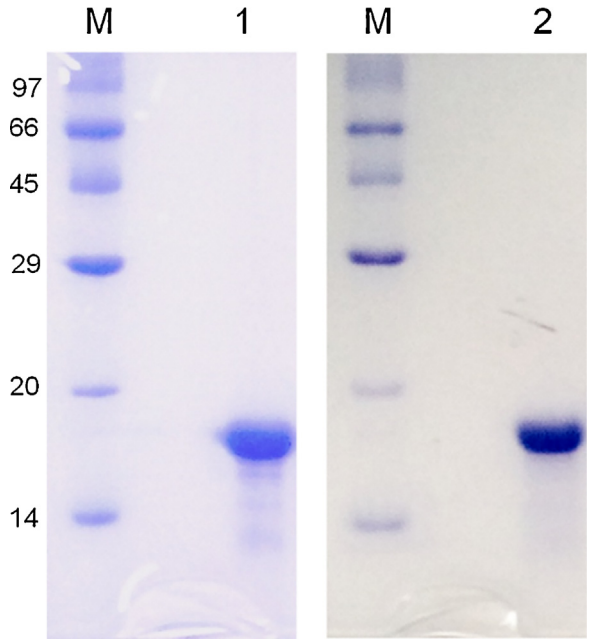

Fig. 1. SDS-PAGE of purified CCL. SDS-PAGE 15%. M) Molecular Marker. 20 μg of CCL were applied in the absence (1) and presence of β-mercaptoethanol (2).

3. Results

3.1. CCL purification

The crude extract of *C. caribensis* showed hemagglutinating and hemolytic activity against all tested erythrocytes. In affinity chromatography, CCL was adsorbed in the Sepharose matrix, and it was successfully recovered by elution with lactose (data not shown). Nevertheless, hemagglutinating activity was observed in non-retained fractions. CCL was purified 21 times and represented 34% of the total hemagglutinating activity of the extract (Table 1)

In SDS PAGE, under non-reducing and reducing conditions, CCL exhibited one single band with apparent molecular mass of 17 kDa (Fig. 1).

3.2. Hemagglutinating and inhibition

CCL agglutinated all tested erythrocytes with preference for rabbit erythrocytes. Moreover, enzyme treated-erythrocytes were better agglutinates than those non-treated (Table 2).

Among all tested sugars, only lactose, lactulose at concentration of 100 mM, fetuin, BSA, PSM type 2 and 3 were able to inhibit CCL hemagglutinating activity (Table 3).

Table 1
Purification of CCL.

Fraction	Volume (mL)	Titer (HU mL^{-1})	Protein total (mg)	Hemagglutinating activity total (HU)	Specific activity (HU mg^{-1})	Purification (fold)	Yield (%)
Crude extract	50	128	395	6400	16.2	1	100
CCL	17	128	6.3	2176	346	21	34

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Table 2
Hemagglutinating activity of CCL against distinct erythrocytes.

Erythrocyte/treatment	Non-treated	Enzyme-treated	
		Trypsin	Papain
Human A	256	512	512
Human B	256	512	512
Human O	256	512	512
Rabbit	512	1024	1024

Suspension of erythrocytes were prepared at 3%; Hemagglutination was expressed in titer of hemagglutination.

Table 3
Inhibitory substance of CCL.

Sugar	MIC[a]
α-lactose (Galβ1 → 4α-Glc)	100 mM
β-lactose (Galβ1 → 4β-Glc)	100 mM
Lactulose (Galβ1 → 4Fru)	100 mM
Glycoproteins	
BSM	32 μg mL^{-1}
Fetuin	16 μg mL^{-1}
PSM type 2	8 μg mL^{-1}
PSM type 3	8 μg mL^{-1}

[a] Minimal Inhibition Concentration.

3.3. Effect of the pH, temperature and divalent cations on hemagglutinating activity

Maximum activity of CCL was observed at pH 9, below and above pH 9 hemagglutinating activity decreases, at pH 10 residual activity was observed, while at pH 4 there was complete loss of activity (data not shown).

CCL is a high thermostable protein. Only after boil for 60 min its activity was fully lost. EDTA, $CaCl_2$ and $MnCl_2$ did not affect CCL activity.

3.4. Molecular mass determination and sugar content

ESI–MS revealed molecular mass of 15,445 ±2 Da with slight differences around this value, indicating the presence of micro-heterogeneities in the amino acid sequence of CCL (Fig. 2).

No cysteine was found in CCL structure, since no mass shift were observed by ESI–MS after treatment with iodoacetamide (IAA) and dithiothreitol followed of IAA (data not shown).

In size exclusion chromatography, CCL was eluted as a sharp and symmetrical peak of 54 kDa, suggesting an oligomeric structure composed of four identical 15 kDa subunits linked by weak interactions (Fig. 3).

The phenol-sulfuric acid assay indicated that CCL is a glycoprotein with 5.2% of neutral carbohydrate.

3.5. MS/MS

Six peptides were sequenced by MS/MS (Table 4). Together, these peptides correspond to approximately 50% of the amino acid sequence of CCL. Peptides T1, T2 and T-4 showed slight similarity with peptides from ALL, a lectin isolated from marine sponge *Aplysina lactuca*. T1 and T2 showed 75% and 62% of identity with T6 from ALL, whereas T4 presented 50% of identity with ALL T5 (data not shown).

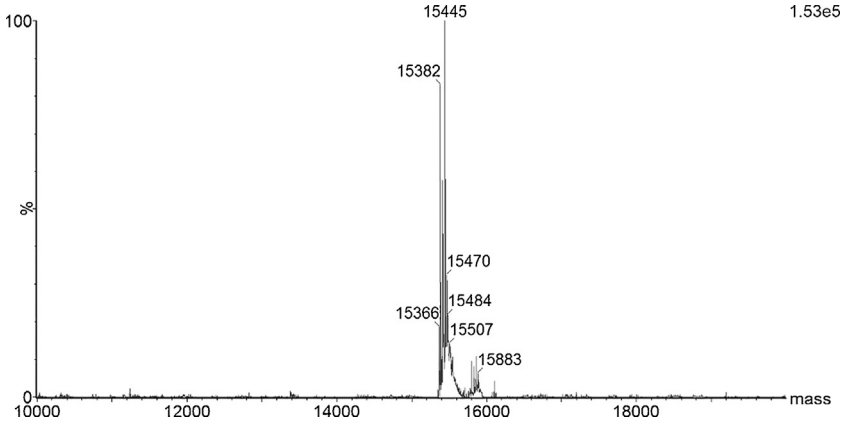

Fig. 2. Molecular mass determination of CCL by Electrospray ionization mass spectrometry (ESI–MS). A) Deconvoluted ESI–MS of CCL. The lectin (10 pmol μL-1) was dissolved in Acetonitrile 50% containing 0.2% formic acid and infused into the NanoESI source coupled to an ESI-Q-ToF mass spectrometer.

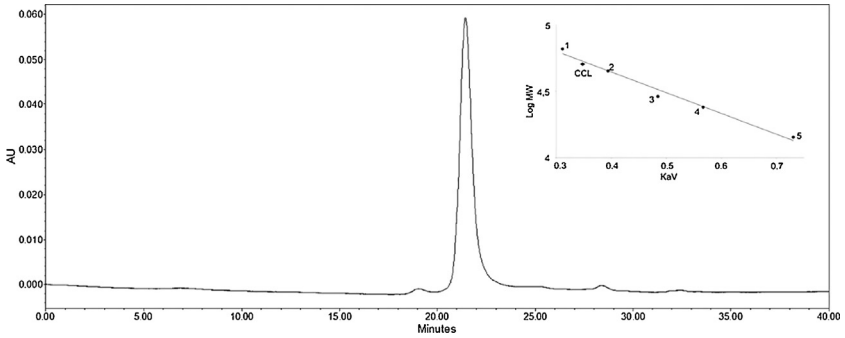

Fig. 3. Size exclusion chromatography of CCL. Approximately 200 μg of CCL were loaded into BioSuite 250 HR SEC column (0.78 × 30 cm, 5 μm particle size, Waters Corp.). The column was equilibrated and eluted with 50 mM Tris-HCl, pH 7.2, containing NaCl 150 mM. UPLC operated at flow of 0.5 mL min-1. Insert. Column calibration: 1. BSA (66 kDa); 2. Ovoalbumin (45 kDa); Carbonic anhydrase (29 kDa); 4. Trypsinogen (24 kDa); 5. Lisozyme (14,3 kDa).

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Table 4
Peptides originated by digestion of CCL with trypsin.

Peptide	Amino acid Sequence	Mass		Δ (Da)
		Observed	Calculated	
T-1	[L/I]PVNSVQR	911.47	911.52	0.05
T-2	[L/I]PVNSVK	755.34	755.45	0.11
T-3	GVGQSATAVYTAPGDGR	1605.74	1605.77	0.03
T-4	S[L/I]DGHFA[L/I][L/I]ADG[L/I]QVATYDHR	2298.05	2298.13	0.13
T-5	VATVS[L/I]PR	841.46	841.50	0.04
T-6	TD[L/I]N[L/I][L/I]DADGGYV[L/I]HEDYR	2196.00	2196.04	0.04

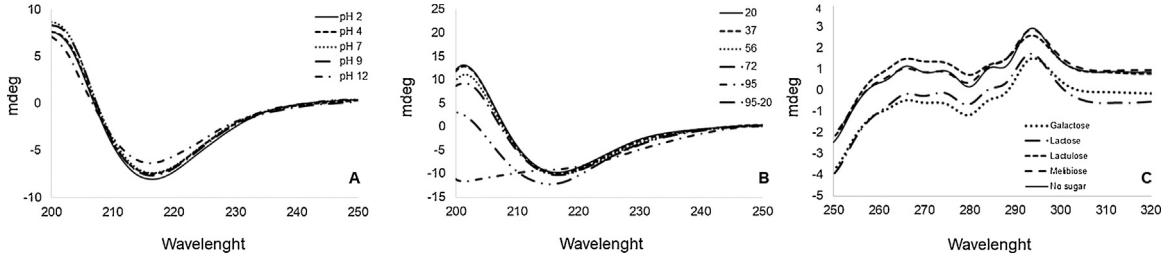

Fig. 4. CD spectra of CCL. (A) Far-UV CD spectra (200–250 nm) of CCL incubated at different pH. (B) Far-UV CD spectra (200–250 nm) of CCL incubated at different temperatures (°C). (C) Near-UV spectra (250–320 nm) of CCL in the presence of sugars.

3.6. Dichroism circular

CD spectra of native CCL exhibited one minimum at 217 nm, suggesting a predominance of β-conformation in its secondary structure. The prediction method CONTIN [19] indicated that the theoretical secondary structure consisted of 10% α-helix, 74% β-sheet/β-turn and 16% coil.

No significant modifications in the maximum and minimum absorption were observed when CCL was incubated in pH 2–10. The secondary structure of the lectin at pH 12 was slightly affected (Fig. 4A)

No significant changes in secondary structure were observed between 20 °C and 75 °C, but after heating at 95 °C deep modifications were detected in the maximum and minimal absorptions, indicating loss of secondary structure. Curiously, when a CCL 95 °C heated-aliquot was cooled at 20 °C, and CD measurements were performed, it was observed a partial recuperation of secondary structure (Fig. 4B).

CD measurements in the far-UV revealed that sugars galactose, α-lactose, melibiose and lactulose caused distinct changes in the minimum and maximum absorption values (Fig. 4C).

3.7. Antibacterial activity and bacterial agglutination

CCL was able in agglutinate all strain cells tested, *S. aureus*, *S epidermidis* and *E. coli* (Fig. 5). However, CCL did not show inhibition of planktonic growth (data not shown).

3.8. Effect of CCL on biofilm formation

The results of total activity of CCL on biofilm formation are shown in Fig. 6. For total biomass quantification, CCL caused significant reductions in the total biomass in the highest concentrations tested, compared to the negative control (Fig. 6A, C and E). Regarding number of biofilm-associated cells, after contact of the adhered cells with CCL for 24 h no reduction in the number of cells was observed (Fig. 6B, D and F).

3.9. Effect of lactose on CCL antibiofilm activity

In general, the reduction of biofilm biomass caused for CCL was abolished in presence of 100 mM α-lactose (Fig. 7). As expected CCL at 250 μg mL^{-1} caused significate reduction of biomass biofilm of *S. aureus* and *S. epidermidis*. However, in presence of 100 mM α-lactose the activity of CCL on *S. aureus* was completely abolished, CCL incubated with lactose did not show significant difference compared with control (biofilm formed without CCL) (Fig. 7A). Regarding *S. epidermidis* biofilm, the activity of CCL was partially inhibited (Fig. 7B). Interestingly, the biomass biofilm of both species was increased in presence of 100 mM α-lactose.

4. Discussion

A new lectin from marine sponge *C. caribensis* was successfully purified by affinity chromatography on HCl-activated Sepharose.

Besides hemagglutinating activity, *C. caribensis* crude extracts showed strong hemolytic effect. After heat treatment (boiling of the extract at 100 °C for five minutes), hemolytic effect was not observed, but hemagglutinating activity remains. Galactose-containing carbohydrates (data not shown) weakly inhibited the lectin present in the boiled material, therefore, affinity chromatography was chosen to performer purification.

HCl-activated Sepharose was chosen because it is an excellent media for isolation of galactose-binding lectins, as reported elsewhere. [5,9,20,21].

Marine sponge are excellent sources of galactophilic lectins and therefore, several lectins have been isolated from marine sponges throughout affinity chromatography using galactose and its derivatives [5,22,23,24], including a galactose-binding lectin isolated from *Chondrilla nucula* (CN lectin) [25].

CN lectin and CCL shared some properties: 1) both lectins are homotetrameric, formed by four identical subunits linked by weak interactions; 2) they are stable at variation of pH and temperature and 3) they are glycoproteins. These lectins seem to diverge in an important aspect: the sugar specificity.

CCL was only inhibited by galactose-containing disaccharides, such as lactose and lactulose. Moreover, some *O*-linked glycoproteins composed of galactose, GalNAc and fucose residues [26] were able to inhibit CCL, while CN lectin was inhibited by monosac-

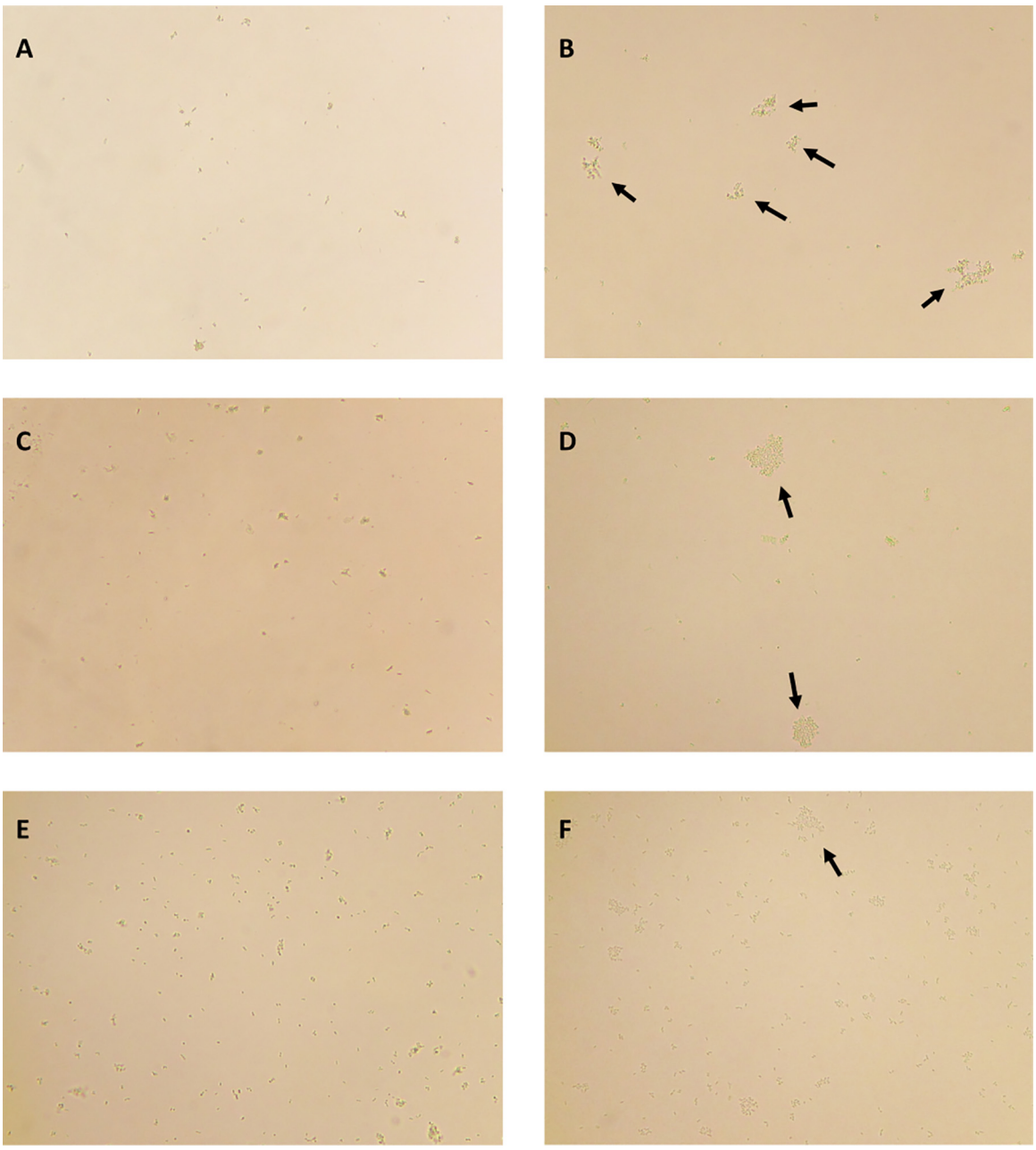

Fig. 5. Agglutination of Gram-positive and Gram-negative bacteria by CCL. S. aureus (A) S. epidermidis (C) and E. coli (E) cells incubated with TBS; S. aureus (B) S. epidermidis (D) and E. coli (F) cells incubated with CCL at (500 μg mL-1). Arrows indicate bacterial agglutination.

charaides, including galactose and GalNAc [25]. Recently, we have isolated a lectin from marine sponge *Aplysina lactuca* (ALL), which has similar inhibition profile that one showed by CCL [4].

Besides, resembling specificities ALL and CCL share similarity of amino acid sequence. Currently, ALL is an orphan lectin, but it showed slight identity and shares some common properties with lectin-I from marine sponge *Axinella polypoide* [4]. Possibly, ALL, lectin-I and CCL are members of a new lectin family, but to consolidate this family complete amino acid sequence of ALL and CCL is required.

The carbohydrates that inhibit CCL hemagglutinating activity showed high values of MIC, but, interestingly, some these sugars causes great changes in CCL tertiary structure, conform observed in CD measurements, suggesting that the interaction between CCL and carbohydrates may be deeper than the inhibition assay indicates. For instance, galactose showed no inhibitory effect in hemagglutinating inhibition assay, but its presence alters considerably CCL profile in CD measurement. On the other hand, lactulose caused inhibition of CCL, but showed slight effect on the CD assay.

Maximum values in far-CD were observed at wavelengths that aromatic amino acids usually absorb (*i.e.* 280 nm for Tryptophan, 260 nm for Tyrosine, and 240 nm for Phenylalanine). Modifications in the absorption of these wavelengths in the presence of sugars suggest that these amino acids are involved in carbohydrate recognition. In fact, several lectins have aromatic amino acids in their active sites; these residues directly interact with sugars by weak interactions [27,28].

Moreover, CD measurements helps us to understand the thermostability showed by CCL, which was just inactivated after boiling

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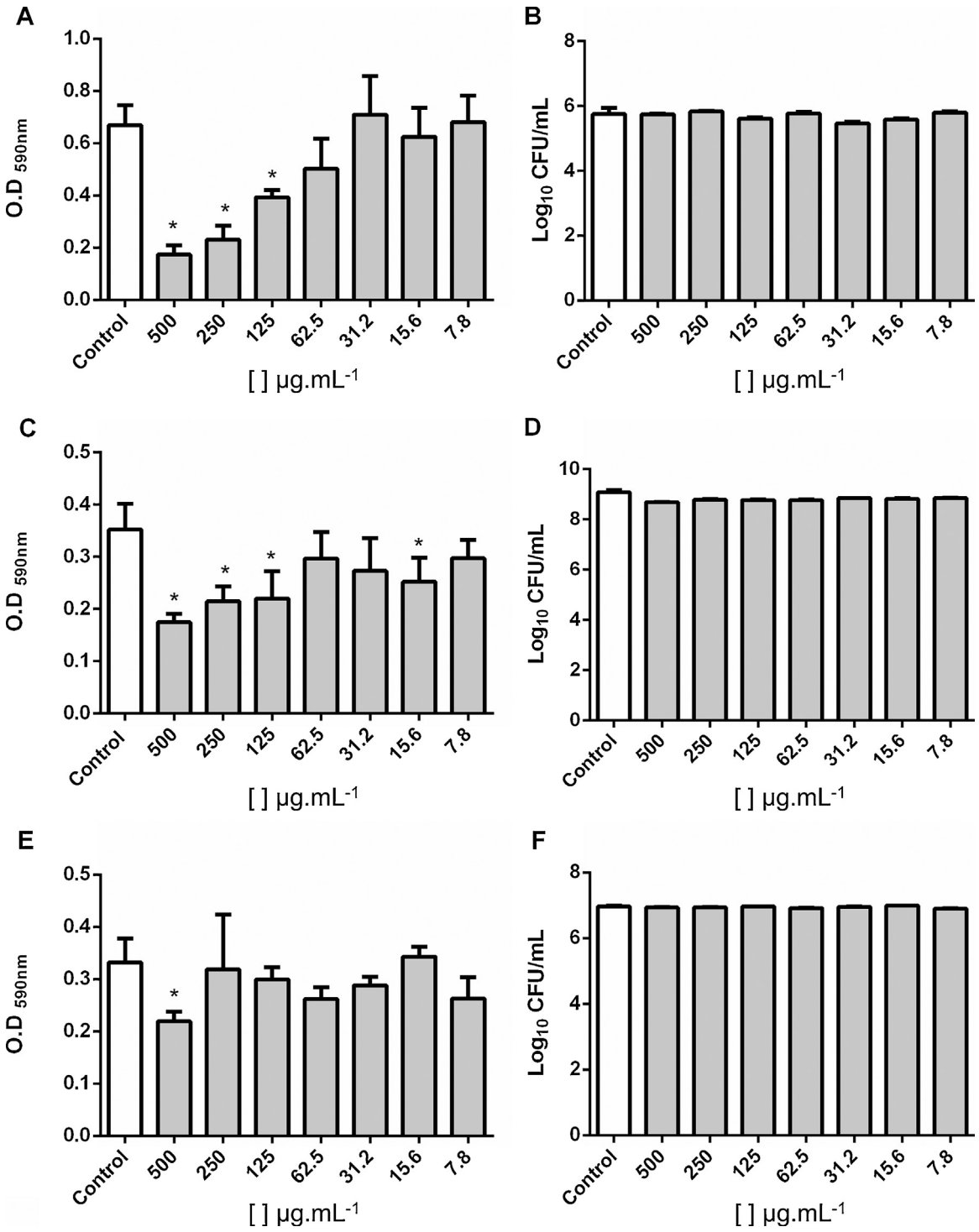

Fig. 6. Activity of CCL against biofilm formation (24 h) of Gram-positive and Gram-negative bacteria. Total biomass quantification measuring the intensity of crystal violet stain for *S. aureus* (A), *S. epidermidis* (C) and *E. coli* (E) biofilms; colony forming units per mL for *S. aureus* (B), *S. epidermidis* (D) and *E. coli* (F) biofilms. *Significantly different ($p < 0.05$) compared to the control group. Error bars display standard deviation (SDs) of the means.

for 1 h, whereas most lectins from marine source retain their activities in temperatures below 60 °C [14,29,30,31].

Commonly, protein stability is associated to some peculiar structural feature, such as disulfide bonds, but CCL possess no disulfide bond, second observed in ESI–MS. Then, it is possible that weak interactions compensate the absence of disulfide bonds through of the maintenance of a globular and compact structure even if the lectin was exposed to high temperatures. CCL is rich in β-conformations and few structures disordered were observed. Several lectins are rich in β-conformations, for instance the β-sandwich structure is probably the structure most commonly found in lectins. To our knowledge, few lectins showed β-conformations rates greater than CCL.

Sponge lectins have shown agglutination and potential antimicrobial activity against Gram-positive and Gram-negative bacteria [4,5,22,32]. In fact, some lectins may recognize and bind to glycans on cell surfaces, cell wall polysaccharides and interact with bacterial lipopolysaccharides (LPS) or with the extracellular matrix of

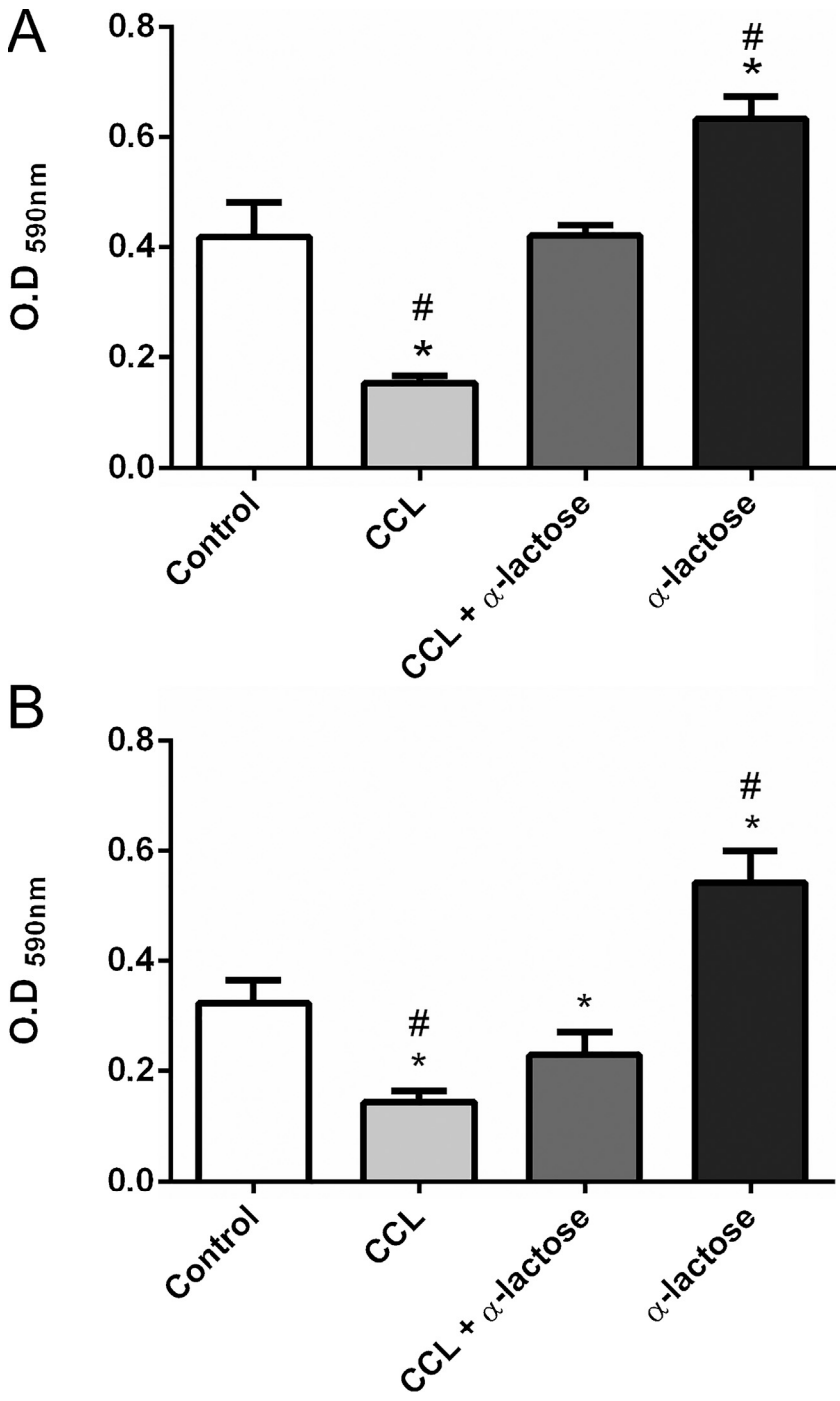

Fig. 7. Inhibitory effect of lactose on biomass biofilm reduction caused by CCL. Total biomass quantification measuring the intensity of crystal violet stain for *S. aureus* (A) and *S. epidermidis* (B) in presence of CCL (500 μg mL^{-1}); CCL (500 μg mL^{-1}) incubate with 100 mM α-lactose or 100 mM α-lactose alone. *Significantly different ($p < 0.05$) compared to the control group. # Significantly different ($p < 0.05$) compared to the CCL + α-lactose group. Error bars display standard deviation (SDs) of the means.

microorganisms [33–36]. Furthermore, according Gardères [1] the ability of sponge lectins to bind specific carbohydrates in bacterial cells could potentially be used to develop new antimicrobial agents.

In this study, CCL did not inhibited the planktonic growth, but caused agglutination of Gram-positive and Gram-negative bacteria. Others lectins have been similar activity [4,9]. ADEL caused agglutination in bacterial cells of *S. aureus*, however, did not inhibit the planktonic growth [9]. Interestingly, ALL agglutinated *S. aureus* and *E. coli* cells and was not able to inhibit the planktonic growth [4].

CCL showed a significant reduction of the total biomass on *S. aureus*, *S. epidermidis* and *E. coli* biofilms. However, corroborating with planktonic results, CCL did not reduced the viability of the biofilm-entrapped cells. Biofilms are described as microbial communities adhered to each other covered with a polymeric extracellular matrix produced by microorganisms themselves [37]. The biofilm resistance is reportedly up to 10 ± 1000 fold greater when compared with planktonic cells, protection conferred by biofilm matrix polymers [38]. The biomass of the biofilm consists of both bacterial cells and the biofilm matrix and in most biofilms

whereas the matrix can account for over 90% of the total biomass [39].

CCL activity was inhibited by α-lactose, suggesting that the CRD is involved in the antibiofilm activity. According Paiva et al. [40] the antibacterial activity of lectins occur due the specific recognition of components of the bacterial surface. Some studies reported that lectins could inhibit biofilm formation by interaction with bacterial cells constituents of the biofilm and alter the expression of genes associated with virulence and biofilm formation [16,41,42].

Thus, according to the results obtained in this study, reduction of the biofilm biomass caused by CCL can be considered a promising activity, considering that its effect could prevent the emergence of drug resistant strains, by the fact it does not eliminate pathogens directly.

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References

[1] J. Gardères, M.-L. Bourguet-Kondracki, B. Hamer, R. Batel, H. Schröder, W. Müller, Porifera Lectins: diversity, physiological roles and biotechnological potential, Mar. Drugs 13 (2015) 5059–5101.
[2] L. Rabelo, N. Monteiro, R. Serquiz, P. Santos, R. Oliveira, A. Oliveira, H. Rocha, A.H. Morais, A. Uchoa, E. Santos, A lactose-binding lectin from the marine sponge *Cinachyrella apion* (Cal) induces cell death in human cervical adenocarcinoma cells, Mar. Drugs 10 (2012) 727–743.
[3] A.F.S. Queiroz, R.A. Silva, R.M. Moura, J.L. Dreyfuss, E.J. Paredes-Gamero, A.C.S. Souza, I.L. Tersariol, E.A. Santos, H.B. Nader, G.Z. Justo, M.P. Sales, Growth inhibitory activity of a novel lectin from *Cliona varians* against K562 human erythroleukemia cells, Cancer Chemother. Pharm. 63 (2009) 1023–1033.
[4] R.F. Carneiro, P.H.P. LimaJr, R.P. Chaves, R. Pereira, A.L. Pereira, M.A. Vasconcelos, U. Pinheiro, E.H. Teixeira, C.S. Nagano, A.H. Sampaio, Isolation, biochemical characterization and antibiofilm effect of a lectin from the marine sponge *Aplysina lactuca*, Int. J. Biol. Macromol. 99 (2017) 213–222.
[5] R.M. Moura, A.F. Queiroz, J.M. Fook, A.S. Dias, N.K. Monteiro, J.K. Ribeiro, G.E. Moura, L.L. Macedo, E.A. Santos, M.P. Sales, CvL, a lectin from the marine sponge Cliona varians: isolation, characterization and its effects on pathogenic bacteria and Leishmania promastigotes, Comp. Biochem. Physiol. A 145 (2006) 517–523.
[6] L. Wang, L. Wang, D. Zhang, F. Li, M. Wang, M. Huang, H. Zhang, L. Song, A novel C-type lectin from crab *Eriocheir sinensis* functions as pattern recognition receptor enhancing cellular encapsulation, Fish Shellfish Immun. 34 (2013) 832–842.
[7] Y. Xiu, Y. Wang, J. Bi, Y. Liu, M. Ning, H. Liu, S. Li, W. Gu, W. Wang, Q. Meng, A novel C-type lectin is involved in the innate immunity of *Macrobrachium nipponense*, Fish Shellfish Immun. 50 (2016) 117–126.
[8] Y. Fujii, N. Dohmae, K. Takio, S.M.A. Kawsar, R. Matsumoto, I. Hasan, Y. Koide, R.A. Kanaly, H. Yasumitsu, Y. Ogawa, S. Sugawara, M. Hosono, K. Nitta, J. Hamako, T. Matsui, Y. Ozeki, A lectin from the mussel *Mytilus galloprovincialis* has a highly novel primary structure and induces glycan-mediated cytotoxicity of globotriaosylceramide-expressing lymphoma cells, J. Biol. Chem. 287 (2012) 44772–44783.
[9] R.F. Carneiro, R.C.F. Torres, R.P. Chaves, M.A. Vasconcelos, B.L. Sousa, A.C.R. Castelo, F.V. Arruda, M.N.C. Matos, H. Mattews-Cascon, V.N. Freire, E.H. Teixeira, C.S. Nagano, A.H. Sampaio, Purification, biochemical characterization, and amino acid sequence of a novel type of lectin from *Aplysia dactylomel*a eggs with antibacterial/antibiofilm potential, Mar. Biotechnol. 19 (2017) 49–64.
[10] A.H. Sampaio, D.J. Rogers, C.J. Barwell, A galactose-specific lectin from the red marine alga *Ptilota filicina*, Phytochemistry 48 (1998) 765–769.
[11] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4, Nature 227 (1970) 680–683.
[12] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[13] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, In-gel digestion for mass spectrometric characterization of proteins and proteomes, Nat. Protoc. 1 (2006) 2856–2861.
[14] R.F. Carneiro, A.A. Melo, A.S. Almeida, R.M. Moura, R.P. Chaves, B.L. Sousa, K.S. Nascimento, S. Saker-Sampaio, J.P.M.S. Lima, B.S. Cavada, C.S. Nagano, A.H. Sampaio, H-3, a new lectin from the marine sponge *Haliclona caerulea*: Purification and mass spectrometric characterization, Int. J. Biochem. Cell B 45 (2013) 2864–2873.
[15] A.A. Melo, R.F. Carneiro, W.M. Silva, R.M. Moura, G.C. Silva, O.V. Sousa, J.S. Saboya, K.S. Nascimento, S. Saker-Sampaio, C.S. Nagano, B.S. Cavada, A.H. Sampaio, HGA-2, a novel galactoside-binding lectin from the sea cucumber *Holothuria grisea* binds to bacterial cells, Int. J. Biol. Macromol. 64 (2012) 435–442.
[16] M.A. Vasconcelos, F.V. Arruda, V.A. Carneiro, H.C. Silva, K.S. Nascimento, A.H. Sampaio, B.S. Cavada, E.H. Teixeira, M. Henriques, M.O. Pereira, Effect of algae and plant lectins on planktonic growth and biofilm formation in clinically relevant bacteria and yeasts, Biomed. Res. Int. (2014), 365272 (9).
[17] CLSI, Metodologia dos Testes de Sensibilidade a Agentes Antimicrobianos por Diluição para Bactéria de Crescimento Aeróbico: Norma Aprovada – Sexta Edição vol. 23, 2003.
[18] S. Stepanovic, D. Vukovic, I. Dakic, B. Savic, M. Svabic-Vlahovic, A modified microtiter-plate test for quantification of staphylococcal biofilm formation, J. Microbiol. Meth 40 (2000) 175–179.
[19] I.H.M. van Stokkum, H.J.W. Spoelder, M. Bloemendal, R. van Grondelle, F.C.A. Groen, Estimation of protein secondary structure and error analysis from CD spectra, Anal. Biochem. 191 (1990) 110–118.
[20] N.I. Belogortseva, V.I. Molchanova, A.V. Kurika, A.S. Skobun, V.E. Glazkova, Isolation and characterization of new GalNAc/Gal-specific lectin from the sea mussel *Crenomytilus grayanus*, Comp. Biochem. Physiol. C 119 (1998) 45–50.
[21] H. Bretting, E. Kabat, Purification and characterization of the agglutinins from the sponge *Axinella polypoides* and a study of their combining sites, Biochemistry 15 (1976) 3228–3236.
[22] S.M. Kawsar, Y. Fujii, R. Matsumoto, T. Ichikawa, H. Tateno, J. Hirabayashi, H. Yasumitsu, C. Dogasaki, M. Hosono, K. Nitta, J. Hamako, T. Matsui, Y. Ozeki, Isolation, purification, characterization and glycan-binding profile of a D-galactoside specific lectin from the marine sponge, *Halichondria okadai*, Comp. Biochem. Physiol. B 150 (2008) 349–357.
[23] P.B. Miarons, M. Fresno, Lectins from Tropical Sponges: purification and characterization of lectins from genus *Aplysina*, J. Biol. Chem. 275 (2000) 29283–29289.
[24] T. Ueda, Y. Nakamura, C.M. Smith, B.A. Copits, A. Inoue, T. Ojima, S. Matsunaga, G.T. Swanson, R. Sakai, Isolation of novel prototype galectins from the marine ball sponge *Cinachyrella* sp. guided by their modulatory activity on mammalian glutamate-gated ion channels, Glycobiology 23 (2013) 412–425.
[25] H.C. Schröder, Z. Kljajic, B.E. Weiler, M. Gasic, G. Uhlenbruck, B. Kurelec, W.E.G. Muller, The galactose specific lectin from the sponge *Chondrilla nucula* displays anti human immunodeficiency virus activity in vitro via stimulation of the 2'-5' oligoadenylate metabolism, Antivir. Chem. Chemoth. 1 (1990) 99–105.
[26] D.L. Slomiany, K. Meyer, Isolation and structural studies of sulfated glycoproteins of hog gastric mucin, J. Biol. Chem. 247 (1972) 5062–5070.
[27] D.M. Freymann, Y. Nakamura, P.J. Focia, R. Sakai, G.T. Swanson, Structure of a tetrameric galectin from *Cinachyrella* sp. (ball sponge), Acta Crystallogr. D 68 (2012) 1163–1174.
[28] A. Kita, M. Jimbo, R. Sakai, Y. Morimoto, K. Miki, Crystal structure of symbiosis related lectin from octocoral, Glycobiology 25 (2015) 1016–1023.
[29] Y. Imamichi, Y. Yokoyama, Purification, characterization and cDNA cloning of a novel lectin from the jellyfish *Nemopilema nomurai*, Comp. Biochem. Physiol. 156 (2010) 12–18.
[30] S. Kawsar, R. Matsumoto, Y. Fujii, H. Yasumitsu, C. Dogasaki, M. Hosono, K. Nitta, J. Hamako, T. Matsui, N. Kojima, Y. Ozeki, Purification and biochemical characterization of a D-galactose binding lectin from Japanese sea hare (*Aplysia kurodai*) eggs, Biochem. USRR 74 (2009) 709–716.
[31] D.S. Medeiros, T.L. Medeiros, J.K.C. Ribeiro, N.K.V. Monteiro, L. Migliolo, A.F. Uchoa, I.M. Vasconcelos, A.S. Oliveira, M.P. Sales, E.A. Santos, A lactose specific lectin from the sponge Cinachyrella apion: purification, characterization, N-terminal sequences alignment and agglutinating activity on Leishmania promastigotes, Comp. Biochem. Physiol. B 155 (2010) 211–216.
[32] H.C. Schroder, H. Ushijima, A. Krasko, V. Gamulin, N.L. Thakur, B. Diehl-Seifert, I.M. Muller, W.E.G. Muller, Emergence and disappearance of an immune molecule, an antimicrobial lectin, in basal metazoa: a tachylectin-related protein in the sponge *Suberites domuncula*, J. Biol. Chem. 278 (2003) 32810–32817.
[33] T. Miki, O. Holst, W. Hardt, The bactericidal activity of the C-type lectin regiii against gram-negative bacteria involves binding to lipid A, J. Biol. Chem. 287 (2012) 34844–34855.
[34] L.M. Purish, L.G. Asaulenko, D.R. Abdulina, S.I. Voychuk, G.A. Iutynskaya, Lectin binding analysis of the biofilm exopolymeric matrix carbohydrate composition of corrosion aggressive bacteria, Appl. Biochem. Micro. 49 (2013) 458–463.
[35] W.S. Shin, D. Park, S.C. Kim, H.Y. Park, Two carbohydrate recognition domains of Hyphantria cunea lectin bind to bacterial lipopolysaccharides through O-specific chain, FEBS Lett. 467 (2000) 70–74.
[36] M. Strathmann, J. Wingender, H. Flemming, Application of fluorescently labelled lectins for the visualization and biochemical characterization of polysaccharides in biofilms of *Pseudomonas aeruginosa*, J. Microbiol. Meth. 50 (2002) 237–248.
[37] T. Bjarnsholt, O. Ciofu, M. Givskov, Applying insights from biofilm biology to drug development — can a new approach be developed, Nature 12 (2013) 791–808.
[38] D. Davies, Understanding biofilm resistance to antibacterial agents, Nat. Rev. Drug Discov. 2 (2003) 114–122.

[39] H. Flemming, J. Wingender, The biofilm matrix, Nat. Rev. Microbiol. 8 (2010) 623–633.
[40] P.M.G. Paiva, F.S. Gomes, R.A. Napoleão Sá, L.C.B.B. Coelho, Antimicrobial activity of secondary metabolites and lectins from plants, in: Méndez-Vilas (Ed.), Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex Research Center, Badajoz, 2010, pp. 396–406.
[41] M.C. Moura, D. Trentin, T. Napoleão, M. Primon-Barros, A. Xavier, N. Carneiro, P. Paiva, A. Macedo, L. Coelho, Multi-effect of the water-soluble *Moringa oleifera* lectin against *Serratia marcescens* and *Bacillus* sp.: antibacterial, antibiofilm and anti-adhesive properties, J. Appl. Microbiol. 123 (2017) 861–874.
[42] T.T.A. Cavalcante, V.A. Carneiro, C.C. Neves, H. de, S. Duarte, M.G. de, Q. Martins, F.V.S. Arruda, M.A. de Vasconcelos, H.S. dos Santos, R.M.S. da Cunha, B.S. Cavada, E.H. Teixeira, A ConA-like lectin isolated from *Canavalia maritima* seeds alters the expression of genes related to virulence and biofilm formation in *Streptococcus mutans*, Adv, Biosci. Biotechnol. 4 (2013) 1073–7078.

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