Universidade Federal de Pernambuco
Centro de Biociências
Programa de Pós-Graduação em Bioquímica e Fisiologia

MYCHELY SHEILA MELO LUNA

BIOPROSPECÇÃO DE METABÓLITOS SECUNDÁRIOS DE *Libidibia ferrea* var*. ferrea*

RECIFE
2016

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia, Área de Concentração BIOQUÍMICA E FISIOLOGIA, da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de doutora em Bioquímica e Fisiologia.

Orientadora: Prof[a]. Dra. Maria Tereza dos Santos Correia

RECIFE
2016

Catalogação na Fonte:
Bibliotecário Bruno Márcio Gouveia, CRB-4/1788

Luna, Mychely Sheila Melo
Bioprospecção de metabólitos secundários de *Libidibia ferrea* var. *ferrea* / Mychely Sheila Melo Luna. – Recife, 2016.

94 f. : il.

Orientadoras: Maria Tereza dos Santos Correia, Maria Vanusa da Silva
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Pós-graduação em Bioquímica e Fisiologia, Recife, 2016.

Inclui referências e anexos

1. Plantas medicinais 2. Caatinga 3. Agentes anti-infecciosos I. Correia, Maria Tereza dos Santos (orient.) II. Silva, Maria Vanusa da III. Título.

581.634 CDD (22.ed.) UFPE/CB-2017-531

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Aprovada em: 29/09/2016

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Aos meus pais, meu marido e aos meus amigos pelo carinho e amor a mim dedicados e especialmente a meu sobrinho João Fernando por ser motivo de grande alegria em minha vida.

AGRADECIMENTOS

Agradeço a Deus pelo dom da vida, por guiar meus passos, por me conceder perseverança para chegar até o final e por ter me dado tantas oportunidades e bênçãos.

A minha família, por todo amor e carinho.

Ao meu marido, Paulo Luna, pelos momentos maravilhosos compartilhados e por todo incentivo, principalmente nos momentos mais difíceis.

A Professora Maria Tereza dos Santos Correia, minha orientadora, pelos ensinamentos, exemplo de pessoa e profissional e por todo o carinho dedicado a mim, desde a iniciação científica.

A professora Márcia Vanusa da Silva, minha coorientadora, por toda dedicação na realização deste trabalho.

A todos os funcionários e professores do Departamento de Bioquímica e Programa de Pós Graduação em Bioquímica e Fisiologia.

Aos amigos do Laboratório de Bioproteínas por todos os momentos, conhecimentos compartilhados, apoio e amizade.

Ao grupo da Caatinga pelos momentos compartilhados e contribuição científica.

Aos amigos que conheci através da universidade, especialmente Raiana, Clóvis, Thiago e Priscila por todo carinho, amizade, apoio.

Por fim, a todos que participaram direta ou indiretamente desta jornada.

“A persistência é o caminho para o êxito”

Charles Chaplin, 1997.

RESUMO

Libidibia ferrea var. *ferrea,* popularmente conhecida como pau-ferro*,* é uma planta de grande ocorrência na região semiárida brasileira, muito utilizada pelas populações locais como forrageira e para fins medicinais. O ecossistema Caatinga tem sido alvo de busca de compostos naturais por ser uma região rica em diversidade animal e vegetal e por abrigar diversas espécies endêmicas exclusivamente brasileiras. Diante disto, o presente estudo objetivou identificar e isolar, a partir de folhas e frutos de *L. ferrea*, fitoquímicos bioativos contra bactérias patogênicas humanas e com potencial antioxidante. Folhas de pau-ferro foram submetidas à extração em série eluotrópica utilizando ciclohexano, clorofórmio, acetato de etila e metanol como solventes. A análise fitoquímica destes extratos por cromatografia em camada delgada revelou a presença de alcalóides, derivados cinâmicos, flavonóides, terpenos e taninos. Através da técnica de microdiluição em caldo, os extratos orgânicos foram avaliados contra bactérias gram-positivas e gram-negativas, mostrando-se ativos com concentração inibitória mínima (CIM) variando de 0.39mg/mL a 12.5mg/mL. Entre os extratos testados, o ciclohexânico (LFCH) apresentou melhor inibição das bactérias testadas e maior rendimento, por isso foi fracionado em sílica gel. Os constituintes químicos de LFCH e suas frações foram identificados por GC-MS, revelando a presença de ácidos graxos, alcanos, terpenos e esteróides. As frações foram ativas contra cepas de *Staphylococcus aureus* com CIM variando de 0.625mg/ml a 10mg/ml. Além disso, foram preparados extratos aquosos brutos de folhas (LFAQ) e frutos (LFRAQ) de *L. ferrea* que posteriormente foram avaliados contra a formação de biofilme de *Staphylococcus aureus.* Tanto extratos dos frutos como das folhas, na concentração de 2.5 mg/mL, inibiram a formação de biofilme de *S. aureus* em 71.1% e 46.4%, respectivamente. Por ter apresentado melhor efeito antibiofilme sobre a bactéria testada, LFRAQ foi fracionado em um sistema HPLC-DAD-MS, obtendo-se oito frações que foram testadas contra formação de biofilme de *S. aureus*. Entre as frações avaliadas, F2 e F4 exibiram efeito antibiofilme com inibição de 52.7% e 76.3%, respectivamente. O extrato bruto das folhas foi ainda avaliado quanto seu potencial antioxidante, evidenciando alto poder de captura de radicais livres pela metodologia colorimética do DPPH, tendo como composto majoritário o ácido elágico, identificado por cromatografia líquida de alta eficiência. LFAQ foi também capaz de proteger eritrócitos humanos da hemólise causada por radicais livres e apresentou efeito radioprotetor em embriões de *Biomphalaria Glabrata* contra danos causados pela radiação gama. Portanto, folhas e frutos de *L. ferrea* podem ser considerados fontes de bioprospecção para novas moléculas bioativas com efeito antibacteriano e antibiofilme, e potencial antioxidante.

Palavras-chaves: Atividade antibacteriana. Atividade antioxidante. Caatinga.

ABSTRACT

Libidibia ferrea var. ferrea, popularly known as “pau-ferro”, which is widely distributed in semi-arid region of northeast Brazil, generally used by the local populations as fodder plant and for medicinal purposes. The Caatinga ecosystem has been main target for search of natural compounds because is a region with high diversity in plants and animals furthermore harbouring several exclusively endemic species, particularly plants. The aim of the present study was to identify and isolate from the leaves and fruits of *L. ferrea*, bioactive phytochemicals compounds against human pathogenic bacteria and antioxidant activity. Leaves of “pau-ferro” were submitted to eluotropic series extraction using cyclohexane, chloroform, ethyl acetate and methanol as solvent. Phytochemical analysis of these extracts by thin-layer chromatography (TCH) showed the presence of alkaloids, cinnamic acid derivatives, flavonoids, terpenes and tannins. Using broth microdilution tecnique, organic extracts were evaluated against gram-positive and Gram-negative bacteria, showing effective with minimum inhibitory concentration (MIC) ranging from 0.39 mg/mL up to 12.5 mg/mL. Between the extracts tested, the cyclohexanic (LFCH) presented best result in bacterial inhibition assay and higher yield, therefore it was fractioned in silica gel. The chemical constituents of LFCH and its fractions were identified by GC-MS, revealing the presence of fatty acids, alkanes, terpenes and steroids. The fractions were active against strains of *Staphylococcus aureus* with MIC ranging from 0.625 mg/ml up to 10 mg/ml. Further, aqueous crude extracts were prepared from leaves (LFAQ) and fruits (LFRAQ) of *L. ferrea* which subsequently have been assessed against biofilm formation of *Staphylococcus aureus*. Both fruit and leaf extracts, in concentration of 2.5 mg/mL inhibited the formation of biofilms of *S. aureus* in 71.1% and 46.4%, respectively. The LFRAQ eventually presented a best antibiofilm effect on the bacteria, consequently it was split in an HPLC-DAD-MS system, getting eight fractions that were tested against biofilm formation of *S. aureus*. Among the evaluated fractions, F2 and F4 exhibited antibiofilm effect with inhibition of 52.7% and 76.3%, respectively. The crude extract of the leaves was still valuated about its antioxidant potential, demonstrate a high power of capture of free radicals by DPPH methodology, taking into majority compound the ellagic acid, identified by high-performance liquid chromatography. LFAQ was also able to protect human erythrocyte hemolysis caused by free radicals and presented radioprotective effect on embryos of *Biomphalaria Glabrata* against damage caused by gamma radiation. Therefore, leaves and fruits of *L. ferrea* can be considered sources of bioprospecting for new bioactive molecules with antibacterial, antibiofilm effect and antioxidant potential.

Key-words: Antibacterial activity. Antioxidant activity. Caatinga.

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1 INTRODUÇÃO

O efeito terapêutico de plantas medicinais tem sido descrito ao longo dos anos através do conhecimento popular e passado de geração em geração. Esse efeito deve-se à produção de substâncias ativas por espécies vegetais incluindo os metabólitos secundários, grupo de compostos de estruturas complexas, como alcalóides, terpenos, compostos fenólicos e seus derivados. A diversidade, em termos de estruturas e propriedades químicas, na qual essas substâncias ocorrem na natureza, podem servir para o desenvolvimento de um grande número de produtos naturais de interesse industrial e farmacológico. Diversas atividades biológicas têm sido atribuídas aos compostos secundários, tais como antioxidante, antimicrobiana, anti-inflamatória, antitumoral, entre outras (ROMAGNOLO et al., 2012; BELHADJ et al., 2016).

Na região semiárida nordestina há várias plantas medicinais, com destaque para *Libidibia ferrea* var. *ferrea*, árvore conhecida vulgarmente por pau-ferro, pertencente à família Fabaceae (FLORA DO BRASIL, 2014). O pau-ferro é utilizado na medicina popular como antiasmático, antidiabético, antitérmico e antidiarréico (MAIA, 2004). Estudos farmacológicos comprovam que preparações a partir de frutos e casca desta planta tem ação anti-inflamatória, analgésica e antimicrobiana. O potencial terapêutico de *L. ferrea* está relacionado à presença de diversos compostos fitoquímicos, especialmente os fenóis (FERREIRA; SOARES, 2015).

Embora as indústrias química e farmacêutica tenham produzido uma grande variedade de antibióticos, frequentemente tem sido observado o aumento de microorganismos resistentes aos medicamentos disponíveis no mercado. Esse aumento está relacionado a diversos fatores, incluindo a formação de biofilmes. Biofilmes microbianos podem ser definidos como um sistema biológico formado por comunidades de células agregadas, organizadas e funcionais embebidas em matriz extracelular composta por substâncias poliméricas produzidas pelas próprias células, possibilitando sua aderência a superfícies bióticas ou abióticas (TOLKER-NIELSEN; MOLIN, 2000). Estima-se

que 80% das infecções humanas estejam associadas a biofilmes bacterianos, especialmente aquelas que envolvem implantes biomédicos, como próteses e cateteres (RÖMLING; BALSALOBRE, 2012) Com isso, bactérias em biofilme podem causar infecções crônicas, as quais são caracterizadas por inflamação persistente e forte dano tecidual. Nesse contexto, aumenta-se a importância da descoberta de compostos terapêuticos alternativos, que atinjam alvos diferentes daqueles utilizados pelos fármacos usados rotineiramente e que apresentem baixa toxicidade.

Muitas evidências bioquímicas, biológicas e clínicas sugerem o envolvimento do estresse oxidativo induzido por radicais livres na patogênese de várias doenças e no envelhecimento acelerado. Radicais livres em excesso podem danificar as células através da oxidação de lipídios, proteínas e DNA (COVARRUBIAS et al., 2008). Por esta razão, maior atenção aos antioxidantes naturais tem sido observada, pois estes podem atuar prevenindo o organismo humano contra esses agentes oxidantes, retardando o progresso de muitas doenças crônicas. As plantas possuem uma larga variedade de moléculas capturadoras de radicais livres, incluindo os compostos fenólicos (PENG et al.,2015; ACHAT et al., 2016). Silva e colaboradores (2011) observaram a partir do extrato hidroalcoólico de frutos de *L. ferrea* um alto teor de fenóis relacionado com elevada ação antioxidante *in vitro.*

Considerando o potencial botânico da Caatinga, as relevantes propriedades farmacológicas do pau-ferro e a necessidade de encontrar novos compostos bioativos, este trabalho se propôs a fornecer dados fitoquímicos e biológicos de extratos de folhas de *L. ferrea*, tecido pouco estudado, avaliando seu potencial antibacteriano, antioxidante e seu efeito protetor em eritrócitos humanos e em embriões de *Biomphalaria glabrata* e, mais adiante sua atuação contra danos ao DNA. Com base em estudos recentes que promoveram a ação antibiofilme de frutos de *L. ferrea* contra diversos patógenos orais (SAMPAIO et al., 2009) e contra cepas de *Staphylococus epidermidis* (TRENTIN et al., 2011), avaliamos também seus efeitos na formação de biofilmes por cepas de *Staphylococus aureus*.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 PLANTAS MEDICINAIS DA CAATINGA

As plantas medicinais têm apresentado um importante papel na terapêutica, pois parte dos medicamentos prescritos mundialmente é de origem vegetal (RATES, 2001). O conhecimento sobre o uso das plantas na cura de doenças faz parte da cultura popular e é de grande interesse científico possibilitando a descoberta de novos fármacos, dada a grande variedade de seus constituintes químicos. Desta forma, sempre houve, ao longo da história, a necessidade de se estudar o conhecimento e uso das plantas por grupos humanos de diferentes culturas, resgatando informações essenciais à descoberta de substâncias biologicamente ativas que possam ser utilizadas na produção de medicamentos.

Fitogeograficamente, a caatinga ocupa cerca de 11% do território nacional, distribuindo-se pelos estados da Bahia, Sergipe, Alagoas, Pernambuco, Paraíba, Rio Grande do Norte, Ceará, Piauí e Minas Gerais. Abrangendo uma área de aproximadamente 800.000 km^2, o ecossistema Caatinga é considerado uma das 37 grandes regiões geográficas do planeta (AGUIAR et al. 2002), possuindo a vegetação mais heterogênea dentre os ecossistemas brasileiros (RIZZINI, 1997). Nessa região, o clima é semiárido, quente (temperatura média variando entre 24 ºC e 26 ºC) e com pluviosidade variando entre 250 e 800 mm anuais, podendo-se distinguir duas estações: uma chuvosa, com três a cinco meses, e uma seca, com sete a nove meses (MAIA, 2004). É considerado uma das 37 grandes regiões geográficas do planeta (AGUIAR et al., 2002), possuindo a vegetação mais heterogênea dentre os biomas brasileiros (RIZZINI, 1997).

A vegetação de caatinga é constituída, especialmente, de espécies lenhosas e herbáceas, de pequeno porte, na maioria das vezes dotada de espinhos, sendo, geralmente, caducifólias, perdendo suas folhas no início da estação seca, e de cactáceas e bromeliáceas. As famílias mais frequentes são Caesalpinaceae, Mimosaceae, Euphorbiaceae, Fabaceae e Cactaceae, sendo os gêneros *Senna, Mimosa* e *Pithecellobium* com maior número de espécies (SAMPAIO et al., 1994). Muitas espécies são economicamente importantes como forrageiras para caprinos, bovinos, muares e ovinos, destacam-se: o

angico (*Anadenanthera macrocarpa* Benth), o pau-ferro (*Libidibia ferrea* var. *ferrea* Mart. ex. Tul.), a catingueira (*Caesalpinia pyramidalis* Tul.), a catingueira rasteira (*Caesalpinia microphylla* Mart.), a canafistula (*Senna spectabilis* var. *excelsa* (Sharad) H.S.Irwin & Barnely), o marizeiro (*Geoffraea spinosa* Jacq.), o mororó (*Bauhinia* sp.), o sabiá (*Mimosa caesalpinifolia* Benth.), o rompe-gibão (*Pithecelobium avaremotemo* Mart.) e o juazeiro (*Zizyphus joazeiro* Mart.) (ALBUQUERQUE; BANDEIRA, 1995).

Varias espécies são também empregadas na medicina popular, pois diversos tecidos tais como cascas, raízes e folhas são comercializadas livremente. Entre elas, destacam-se a *Myracrodruon urundeuva* (cicatrizante), *Annona sp*. (antidiarréico), *L. ferrea* (antiasmática), *Caesalpinia pyramidalis* Tul. (antidiarréica), *Anadenanthera macrocarpa* Benth. (adstringente), *Ziziphus joazeiro* Mart. (gastroprotetor), *Selaginella convuluta* Spring (diurético), entre outras (AGRA, 1996).

Diante da velocidade do fenômeno de devastação da Caatinga, unidades de conservação têm sido criadas. Atualmente, a caatinga conta com 47 unidades de conservação que somam 4.956km^2, aproximadamente 6,4% do ecossistema (SILVA et al., 2004). Dentre essas unidades temos o Parque Nacional do Catimbau, localizado no Sertão Pernambucano, distribuída entre os municípios de Buíque, Ibimirim e Tupanatinga, com área de 607 km^2.

Dessa forma, esse ecossistema tem passado a ser alvo de muitas pesquisas recentes, pois a grande diversidade de compostos químicos presentes nos vegetais da Caatinga, uma vez que estão expostas a ambientes extremos, são importantes instrumentos na busca de novas substâncias ativas e na estimulação de medidas de conservação e uso de suas áreas.

2.2 *Libidibia ferrea* var. *ferrea/*

Libidibia ferrea (Mart. *ex* Tul.)L.P.Queiroz var. *ferrea* é uma árvore que pertence à família Fabaceae e que cresce em todo o Brasil, largamente distribuída nas regiões Norte e Nordeste, principalmente em Pernambuco e no Ceará , sendo conhecida popularmente como pau-ferro, jucá, ibirá-obi, imirá-itá, muirá-obi, muiré-itá (LORENZI, 2002).

A espécie *L. ferrea* apresenta três variedades: *ferrea*, *leiostachya* e *parvifolia.* A variedade *ferrea* ocorre predominantemente no Nordeste, sendo mais comum em regiões da Caatinga, onde é conhecida principalmente por Jucá. Diferentemente, as variedades *leiostachya* e *parvifolia* são bastante semelhantes e características da Mata Atlântica, (DUCKE, 1953; RIZZINI; MATTOS FILHO, 1968). O Pau-ferro possui tronco curto de 40 a 60 cm de diâmetro com manchas claras (Figura 1A-C) chegando atingir a altura de 10-15m. Suas folhas são compostas, bipinadas de 15-19 cm de comprimento, com 5-11 pinas opostas e folíolos em número de 8-24 por pina (figura 1B). Possui flores amarelas pequenas e em forma de cachos (Figura 1B), fruto marrom escuro, na forma de vagem indeiscente (Figura 1D). O pau-ferro é uma árvore que tem diversas utilidades, sendo usada como medicamento, na indústria madeireira, na ornamentação pública, na restauração florestal de áreas degradadas, como forrageira para os animais e na fabricação de tinturas naturais (LORENZI, 2002; NASCIMENTO et al., 2002).

Figura 1. *Libidibia ferrea* (Mart. *ex* Tul.) L.P.Queiroz var. *ferrea*. A-árvore; B-folhas e flores; C-caule; D-fruto

Fonte: adaptado de https://appverde.wordpress.com/ (2016).

Na medicina popular, diversas propriedades terapêuticas foram atribuídas ao pau-ferro, incluindo o tratamento de feridas, contusões, combate à asma, diarréia e anemia (BRAGA, 1960; AGRA, 2007). Estudos recentes comprovaram propriedades medicinais tanto de *L. ferrea* var *ferrea* como das

outras variedades. Nakamura e colaboradores (2002a, 2002b) estudaram dois componentes extraídos de *L. ferrea*, var *ferrea*, o ácido gálico e metil galato, que apresentaram atividade anti-tumoral e inibitória frente ao vírus Epstein-Barr. Nozaki e colaboradores (2007) isolaram o Pauferrol A que exibiu atividade inibitória a DNA topoisomerase II e induziu a apoptose em células leucêmicas humanas (HL60). O potencial antioxidante de extratos dos frutos foi avaliado, apresentando significativo poder redutor do radical peróxido e inibição da degradação do DNA (SILVA et al., 2011). Além disso, extratos de frutos do pau-ferro apresentaram atividade antimicrobiana frente a patógenos orais (SAMPAIO et al., 2009), e ainda inibiram a formação de biofilme de *Staphylococcus epidermidis* (TRENTIN et al., 2011). Recentemente, um enxaguante bucal fitoterápico contendo extrato dos frutos foi preparado, mostrando-se estável e com qualidade farmacológica, porém apresentou moderada citotoxicidade frente a fibroblastos (VENÂNCIO et al., 2015).

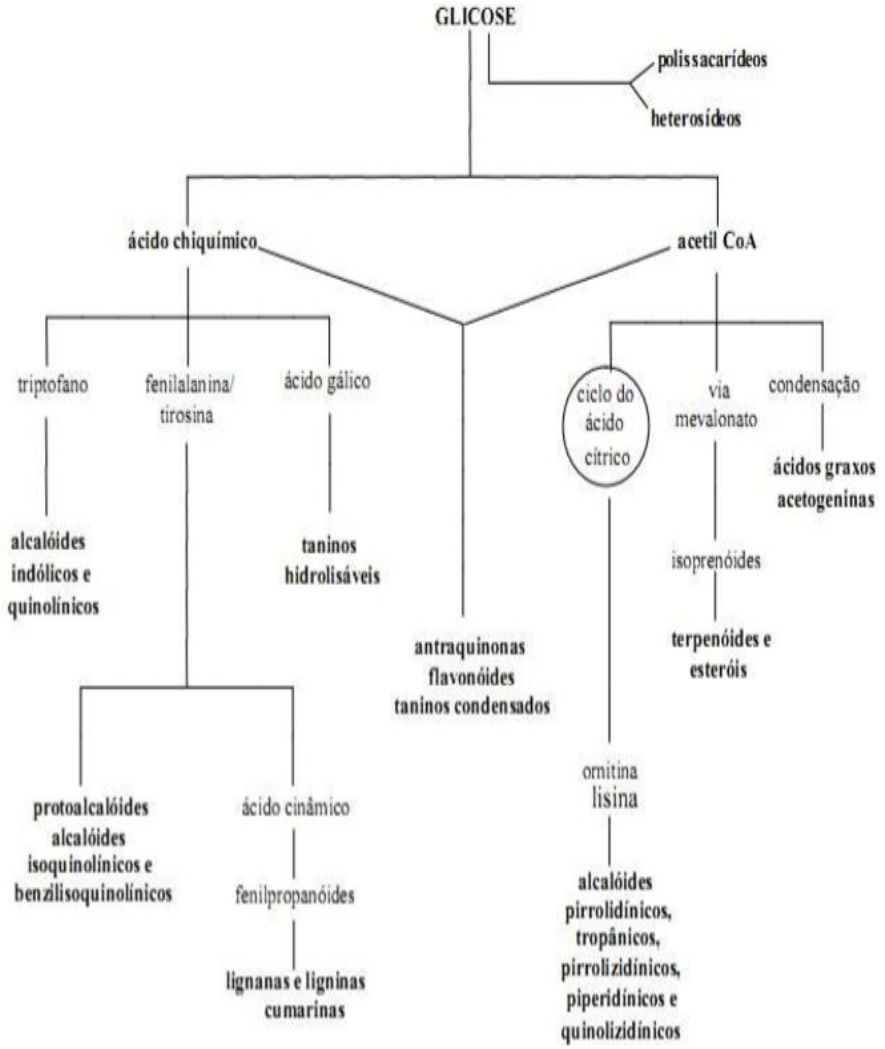
2.3 METABÓLITOS SECUNDÁRIOS DE PLANTAS

As plantas produzem diversas classes de compostos orgânicos que estão geralmente associados aos processos de respiração, transporte e diferenciação. Estas substâncias são conhecidas como metabólitos secundários e têm como função proteger a planta contra herbivoria, ataque de patógenos, bem como beneficiá-la na competição com outros vegetais. Os metabólitos secundários podem ainda favorecer a atração de polinizadores e dispersores de sementes, e regulam a relação de simbiose entre plantas e microorganismos. Além disso, a produção destes componentes pode proteger a planta de fatores externos como variações de temperatura, umidade, exposição à radiação ultravioleta (UV) e deficiência de nutrientes minerais (ATSATT, 1976; LUCKNER, 1984; EVANS, 1989).

A origem dos metabólitos secundários pode ser resumida a partir do metabolismo da glicose, através de dois intermediários principais, o ácido chiquímico e o acetato. São classificados de acordo com as suas rotas biossintéticas, divididos em três grandes grupos: compostos fenólicos, terpenos e alcalóides. Os compostos fenólicos são derivados do ácido chiquímico e do ácido mevalônico, os terpenos são produzidos a partir do ácido mevalônico ou

piruvato e 3-fosfoglicerato e os alcalóides, compostos secundários nitrogenados, são produzidos a partir de aminoácidos aromáticos, os quais são derivados do ácido chiquímico e de aminoácidos alifáticos (Figura 2). Flavonóides, taninos e ligninas fazem parte dos compostos fenólicos; saponinas, carotenóides e a maioria dos hormônios vegetais são terpenos; nicotina e cafeína são alguns exemplos de alcalóides. (BELL; CHARLWOOD, 1980).

Figura 2. Ciclo biossintético dos metabólitos secundários.



Fonte: Simões et al (1999).

2.3.1 Compostos fenólicos

Os compostos fenólicos são caracterizados por possuírem pelo menos um anel aromático ligado a um ou mais grupos hidroxilas (OH^-), podendo ter

vários grupos substituintes, como carboxilas, metoxilas, estruturas cíclicas não aromáticas, entre outras (RIBÉREAU-GAYON, 1968). A tabela 1 mostra a classificação dos compostos fenólicos considerando sua estrutura básica.

Tabela 1. Classificação dos compostos fenólicos.

Estrutura Básica	Classe de Compostos Fenólicos
C6	Fenóis simples, benzoquinonas
C6-C1	Ácidos fenólicos
C6-C2	Acetofenonas e ácidos fenilacéticos
C6-C3	Fenilpropanóides; ácidos cinâmicos e compostos análogos, fenilpropenos, cumarinas e isocumarinas
C6-C4	Naftoquinonas
C6-C1-C6	Xantonas
C6-C2-C6	Estilbenos, antraquinonas
C6-C3-C6	Flavonóides e isoflavonóides
$(C6-C3)_2$	Lignanas
$(C6-C3-C6)_2$	Biflavonóides
$(C6)_n$	Melaninas vegetais
$(C6-C3)_n$	Ligninas
$(C6-C1)_n$	Taninos hidrolisáveis
$(C6-C3-C6)_n$	Taninos condensados

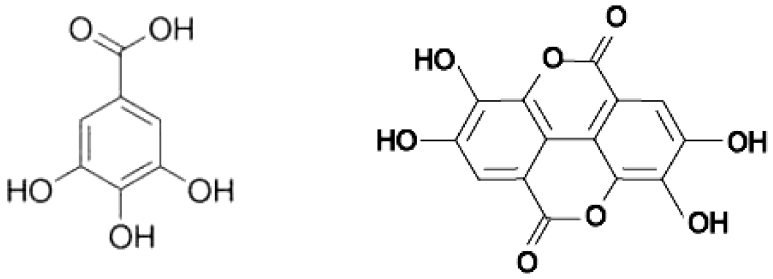
Fonte: Adaptado de Carvalho et al.(2004).

Esses compostos estão envolvidos principalmente na sinalização entre plantas e outros organismos e na proteção contra radiação UV. No que se refere à sinalização entre plantas e outros organismos, pode se incluir nesse item a relação entre os vegetais e seus agentes polinizadores, sendo a coloração das flores um dos principais atrativos. Exemplos de compostos que as plantas utilizam para colorir suas flores são as antocianinas (flavonóide). A maioria dos compostos fenólicos ocorre complexada a carboidratos (mono e polissacarídeos), proteínas e outros componentes vegetais (ROBBINS, 2003), resultando em uma grande variedade de fenóis na natureza, os quais são organizados em classes, sendo os ácidos fenólicos, os taninos e os flavonóides

considerados os principais compostos fenólicos (BALASUNDRAM; SUDRAM; SAMMAN, 2006).

Os ácidos fenólicos são estruturalmente fenóis simples que incluem dois grupos: ácidos hidroxibenzóicos e os ácidos hidroxicinâmicos. O primeiro corresponde ao maior grupo de ácidos fenólicos encontrados na natureza. Neste grupo, destacam-se os ácidos gálico e elágico (Figura 3), que possuem atividade antioxidante, geralmente, determinada pelo número de hidroxilas presentes na molécula, além da proximidade do grupo –COOH com o grupo fenil (HARBORNE, 1998). O ácido elágico apresenta-se como um dos ácidos fenólicos de maior relevância, e tem sido reportado em vários estudos pela sua capacidade antioxidante. Este composto é um hidrólito da elagitanina, produto da condensação do ácido gálico, que pode estar presente nas plantas na forma de ácido elágico livre, de ácido elágico glicosídeo ou de elagitaninos, que é a mais comum (ZAFRILLA, 2001). Sua ação antioxidante foi comprovada por testes *in vitro* através da captura de radicais livres, quelação de íons e inibição da peroxidação do ácido linoleico (KILIC et al., 2014). Além disso, segundo Ya-Mei (2005) o ácido elágico foi capaz de reduzir o estresse oxidativo podendo ser útil na prevenção da aterosclerose.

Figura 3. Estrutura química do ácido gálico e do ácido elágico.



Ácido gálico Ácido elágico

Fonte: adaptado de https://.cienciabrasil.blogspot.com.br/ (2008).

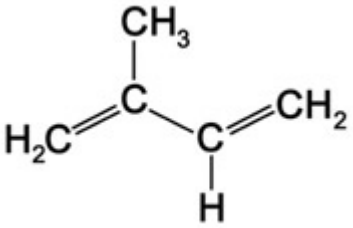
Em geral os compostos fenólicos são multifuncionais como antioxidantes, pois atuam de diversas formas: combatendo radicais livres pela doação de um átomo de hidrogênio de um grupo hidroxila da sua estrutura

aromática, quelando metais de transição como Fe^{+2} e o Cu^{+}, reparando a lesão de células a moléculas atacadas por radicais livres, interrompendo a reação de propagação dos radicais livres na oxidação lipídica (EBELER, 2008; FERLAZZO et al., 2016). Desta maneira, essas substâncias podem ser empregadas na indústria de cosméticos sendo incorporadas a formulações farmacêuticas diversas, como fotoprotetores, para minimizar os efeitos oxidativos das EROS, formando produtos cada vez mais eficientes e sustentáveis (GUARATINI et al., 2009).

2.3.2 Terpenos

Os terpenos ou terpenóides são compostos que ocorrem em todas as plantas e compreendem uma classe de metabólitos secundários com uma grande variedade estrutural. São formados pela fusão de unidades de cinco carbonos que quando submetidos a altas temperaturas, podem se decompor em isoprenos, podendo referir-se, ocasionalmente, a todos os terpenos como isoprenóides (TAIZ et al., 2004). Os terpenos podem ser classificados de acordo com o número de isoprenos (Figura 4) que constituem: hemiterpenos (C5), monoterpenos (C10), sesquiterpenos, (C15), diterpenos (C20), triterpenos (C30) e carotenos (C40) (OLIVEIRA et al., 2003).

Figura 4. Estrutura química do isopreno.



Os hemiterpenos são classificados como o menor grupo dos terpenos e seu representante mais conhecido e estudado é o isopreno, um produto volátil liberado de tecidos fotossinteticamente ativos (CROTEAU et al., 2000). Os monoterpenos são formados por duas unidades de isopreno. Devido a sua baixa massa molecular, estes costumam ser voláteis, sendo os constituintes das essências voláteis e óleos essenciais, atuando na atração de polinizadores, principalmente. Os sesquiterpenos são encontrados nos óleos

essenciais e em hormônios vegetais, constituindo a maior classe de terpenóides (OLIVEIRA et al*.,* 2003; GAÍNZA et al., 2015). Os diterpenos compreendem um grande grupo de compostos não voláteis, possuindo uma vasta gama de atividades diferentes que incluem os hormônios, ácidos resínicos e agentes anticancerígenos e antimicrobianos (ROBBERS et al., 1997; CROTEAU et al*.*, 2000; OLIVEIRA et al., 2003). Os triterpenos formam os componentes das resinas, látex, ceras e cutícula das plantas. Entre os triterpenos encontra-se uma importante classe de substâncias, os esteróides, os quais são componentes dos lipídios de membrana e precursores de hormônios esteróides em mamíferos, plantas e insetos. Outra classe importante de triterpenos são as saponinas, que nas plantas desempenham um importante papel na defesa contra insetos e microorganismos (PERES 2004). Os carotenóides são pigmentos responsáveis pelas colorações amarela, laranja, vermelha e púrpura dos vegetais, apresentando função essencial na fotossíntese e, especialmente, na pigmentação de flores e frutos. Os politerpenóides são aqueles com mais de oito unidades de isopreno, ou seja, com mais de 40 carbonos na sua estrutura, como os longos polímeros encontrados na borracha (ROBBERS et al., 1997; CROTEAU et al., 2000; OLIVEIRA et al., 2003).

Os terpenos apresentam inúmeras atividades biológicas de interesse farmacêutico, dentre elas estão as ações anestésica (VALERIO et al., 2007; ZHANG et al., 2010), citotóxica (CHEN et al., 1990; KHIEV et al., 2012; FERNANDES, 2013), anti-helmintica (NIRMAL et al., 2007; GAÍNZA et al., 2015) e, mais notadamente, antimicrobiana (GALVÃO et al., 2012 ; ABDEL-RAOUF et al. 2015).

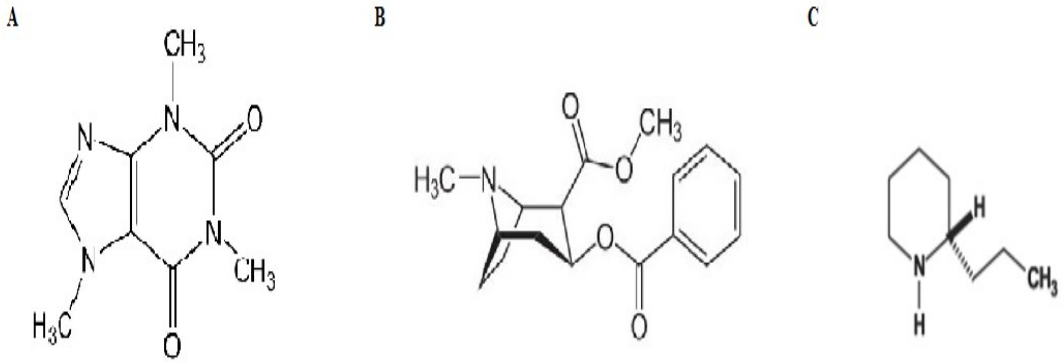
2.3.3 Alcalóides

Os alcalóides são substâncias de caráter alcalinoencontrados predominantemente nas angiospermas e são originados, geralmente, a partir de aminoácidos. Porém tais substâncias ocorrem também em microrganismos (fungos) e animais marinhos. Basicamente são constituídos por um átomo de nitrogênio, oriundos dos aminoácidos e um anel heterocíclico. A maioria dos alcalóides é derivada de aminoácidos como a ornitina, a lisina, a tirosina e o triptofano. A partir dos aminoácidos alifáticos, têm-se os alcalóides pirrolidínicos e tropânicos (Ornitina) e os piperidínicos (Lisina), e dos

aminoácidos aromáticos, têm-se os alcalóides isoquinolínicos (Tirosina) e os indólicos (Triptofano) (DEWICK, 1997). Essa classe de metabólitos é conhecida pela presença de substâncias que possuem acentuado efeito no sistema nervoso, sendo muitas delas largamente utilizadas como venenos e alucinógenos (PERES, 2004). Podem ser classificados em alcalóides verdadeiros (Figura 5A), protoalcalóides (Figura 5B) e pseudoalcalóides (Figura 5C). Os alcalóides verdadeiros são formados pelo átomo de nitrogênio pertencente ao anel heterocíclico, enquanto os protoalcalóides, o nitrogênio não pertence ao anel heterocíclico. Os pseudoalcalóides são os compostos nitrogenados cujos precursores não são aminoácidos, mas sim outras substâncias como os terpenos e esteróides (HENRIQUES et al., 2004).

Diversas atividades biológicas são atribuídas aos alcalóides, destacando-se os efeitos antiespasmódico, analgésico e anti-inflamatório (ZHAO et al., 2010; QIONG et al., 2016).

Figura 5. Estrutura química dos alcaloides.



A: alcalóide verdadeiro (Cafeína); B: protoalcalóide (Cocaína); C: pseudoalcalóide (Coniína).

2.4 INFECÇÕES BACTERIANAS E RESISTÊNCIA A ANTIBIÓTICOS

Os avanços tecnológicos relacionados aos procedimentos invasivos, diagnósticos e terapêuticos, e o aparecimento de microrganismos multirresistentes aos antimicrobianos usados rotineiramente na prática

hospitalar tornaram as infecções hospitalares um grave problema de saúde pública.

Nas últimas décadas a resistências a antimicrobianos tem aumentado significativamente em todo o mundo, especialmente em hospitais. O uso intensivo de antibióticos aumentou drasticamente a frequência de resistência dos patógenos humanos, reduzindo a possibilidade de tratamento eficaz das infecções, elevando o risco de complicações e morte para o paciente (WOODFORD; LIVERMORE 2009; ANDERSSON; HUGHES, 2010). Entre as espécies mais associadas à resistência a antimicrobianos estão *Staphylococcus aureus* resistente à meticilina (conhecido como MRSA), *Acinetobacter baumannii*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Clostridium difficile*, *Escherichia coli* e *Klebsiella pneumoniae* (BARBOUR, 2009; THEURETZBACHER et al. 2012; PEACOCK AND PATERSON, 2015).

Infecções por MRSA têm sido descritas mundialmente, tratando-se, portanto de uma das bactérias mais disseminadas em todo o mundo. Nos Estados Unidos, estudos realizados pelos Centros para Controle e Prevenção de Doenças (CDC) avaliaram que o número de infecções por MRSA estaria próximo de 100 mil por ano, com cerca de 20 mil casos fatais (PEACOCK; PATERSON, 2015). Estudo realizado entre países da América Latina (México, Argentina e Brasil) mostrou incidência média de 26,5% de MRSA como agente de infecção comunitária de trato respiratório, sendo 31,3% no Brasil (MENDES et al., 2003). Outros tipos de bactérias resistentes relacionadas com surtos mundiais de infecções hospitalares são as produtoras de enzimas ß-lactamases. A produção dessas enzimas, em geral, é o mecanismo de resistência mais prevalente e importante na família Enterobacteriaceae. ß-lactamases atuam rompendo e inativando o anel betalactâmico, importante componente da estrutura química dos antimicrobianos β-lactâmicos. Estas enzimas podem ser classificadas com base na estrutura molecular e na homologia da sequência de aminoácidos, resultando em quatro grandes grupos: A-carbapenemases do tipo serina, B- Metalo- ß-lactamases, C- AmpC ou cefalosporinases, D-oxacilinases (AMBLER, 1980). As carbapenemases de classe A já foram identificadas nas bactérias *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa e Acinetobacter baumannii* e são

conhecidas como KPC (sigla de *Klebsiella pneumoniae*-carbapenemases) por terem sido encontradas inicialmente nessa bactéria (YIGIT et al., 2008). No Brasil, bactérias produtoras dessa enzima foram registradas pela primeira vez em 2005, mas somente em 2010 elas passaram a causar surtos mais graves no país, pois segundo a Agência Nacional de Vigilância Sanitária (ANVISA), 246 pacientes foram contaminados, em vários estados, e 19 casos de morte foram relatados (BRASIL, 2013).

2.4.1 Biofilmes microbianos

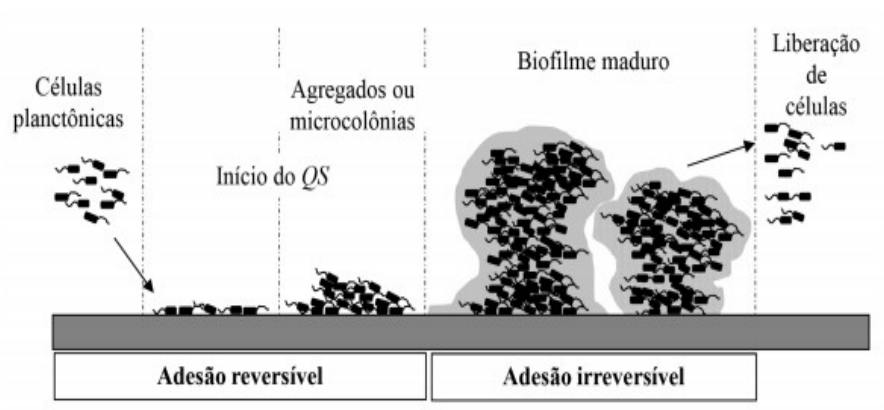
A formação de biofilmes é considerada um dos fatores que elevam a resistências aos antimicrobianos. Biofilmes microbianos podem ser definidos como um sistema biológico formado por comunidades de células agregadas, organizadas e funcionais embebidas em matriz extracelular composta por substâncias poliméricas produzidas pelas próprias células, a qual possibilita a aderência a superfícies bióticas ou abióticas (BJARNSHOLT, 2013).

O desenvolvimento do biofilme microbiano em superfícies é um processo dinâmico que envolve etapas de adesão, crescimento e produção de matriz extracelular (Figura 6). A adesão bacteriana seja em uma superfície abiótica (inanimada, como plásticos e metais) ou biótica (como células e tecidos animais ou vegetais), é o primeiro estágio na formação de biofilmes e é considerado um processo bastante complexo (DUNNE, 2002).

Considerando superfícies abióticas, a atração inicial das células bacterianas planctônicas (primeiro estágio) à superfície ocorre aleatoriamente, através do movimento browniano e da força gravitacional ou, de modo dirigido, via quimiotaxia e motilidade, através de flagelos e pili (O'TOOLE; KOLTER, 1998). Após o primeiro estágio, as bactérias devem manter o contato com a superfície. Porém, para que desenvolvam um biofilme maduro, é necessário que a adesão se torne irreversível. Para tal, precisam se multiplicar e ancorar seus apêndices, o que caracteriza o segundo estágio. Depois da aderência irreversível à superfície, as bactérias emitem sinais químicos que auxiliam na comunicação entre as células bacterianas. Uma vez que a intensidade do sinal excede um determinado nível de limiar, os mecanismos genéticos subjacentes para produção de exopolissacarídeos são ativados (STOODLEY et al., 2002).

Esse processo de comunicação encontrado em muitas bactérias patogênicas, que acopla a transcrição de genes específicos com a densidade celular bacteriana, é referido como Quorum sensing (QS) (SIMÕES et al., 2009; AHMAD et al., 2011). Com isso, são formados agregados celulares e a motilidade é diminuída quando os mesmos formam camadas com espessura superior a cerca de 10 µm. O terceiro estágio de desenvolvimento resulta na produção de matriz exopolimérica e consequente formação de microcolônias. O quarto estágio se inicia quando o biofilme alcança sua espessura final. Durante esta etapa, o biofilme adquire arquitetura complexa com canais e poros, formando uma estrutura tridimensional. Esses canais funcionam como um sistema circulatório de entrega de nutrientes, da interface para o interior do biofilme e de remoção de restos metabólitos (HALLSTOODLEY COSTERTON; STOODLEY, 2004). O ultímo estágio caracteriza-se pela dispersão celular e fechamento do ciclo. A dispersão é o termo geralmente utilizado para descrever o destacamento de células (individuais ou em grupos) de um biofilme (BAYLES, 2007).

Figura 6. Estágios do desenvolvimento dos biofilmes



Fonte: TRENTIN; GIORDANI; MACEDO, (2013).

A adesão bacteriana e a consequente formação de biofilme possuem um papel importante na patogênese, representando um grande obstáculo para a saúde humana, sendo causa comum de infecções persistentes (COSTERTON; STEWART; GREENBERG, 1999). As estratégias para o combate de biofilmes podem, basicamente, ser divididas em dois segmentos: a inibição da formação de biofilmes e a erradicação ou tratamento de biofilmes já formados.

Considerando superfícies abióticas, o bloqueio da adesão celular bacteriana à superfície pode ser realizado pelo uso profilático de antibióticos podendo reduzir a incidência de infecções associadas a biofilmes em dispositivos implantáveis, por exemplo. A inibição da formação de biofilmes, após a etapa da adesão primária, pode ser obtida através da interferência na sinalização intercelular bacteriana. O uso de moléculas inibidoras do sistema QS (QSI), as quais competem com o receptor das moléculas sinalizadoras ou de enzimas conhecidas como Quorum Quenching (QQ), as quais degradam as moléculas de sinalização, podem bloquear a comunicação celular bacteriana (MARTIN; HOVEN; COOK, 2008). Dessa maneira, a produção de EPS, dentre outras atividades, é inibida, o que dificulta a manutenção da estrutura tridimensional dos biofilmes (adesão irreversível) (LAZAR, 2011), e a associação desses interferentes do sistema QS com antimicrobianos tradicionais pode aumentar a efetividade dos fármacos correntemente utilizados, facilitando o controle de infecções bacterianas relacionadas a biofilmes. Para erradicar biofilmes já formados, em fase de estudo, principalmente, encontram-se enzimas e algumas outras moléculas capazes de desintegrar a matriz (EPS) que engloba as células bacterianas. A intenção não é necessariamente inibir o crescimento bacteriano, mas, sim, perfurar a estrutura do biofilme (através de ruptura enzimática), sendo útil em combinação com um agente antimicrobiano para o tratamento de infec- ções associadas a biofilmes. A diversificada constituição química da matriz do biofilme, incluindo material protéico, DNA extracelular e polissacarídeos, torna o EPS suscetível à degradação por uma série de enzimas exogenamente adicionadas (como proteinase K, tripsina e DNAse I) (BOLES; HORSWILL, 2011).

A busca por substâncias capazes de combater a formação e o desenvolvimento de biofilmes tem demonstrado que metabólitos secundários possuem tais propriedades (BAZARGANI AND ROHLOFF, 2016). O mecanismo de ação pelo qual esses compostos atuam interferindo na formação ou desenvolvimento de biofilmes é ainda desconhecido. No entanto, sabe-se que uma das formas de ação de muitos destes compostos é atuar na interferência do QS. Óleos essenciais como os de *Eucalyptus globulus* e *Eucalyptus radiata* demonstraram ter influência na formação de biofilmes,

através da inibição do quorum- sensing regulado pelo pigmento violaceína (LUÍS, et al., 2016). Recentemente, alguns trabalhos têm demonstrado a ação antimicrobiana e antibiofilme de plantas nativas do bioma Caatinga. Extratos e frações obtidas de folhas de *Pityrocarpa monilifomis* demonstraram atividade antibacteriana significativa contra *Staphylococcus aureus* e efeito sinérgico com os antibióticos cloranfenicol, tetraciclina, eritromicina e estreptomicina (SILVA et al.,2013). Trentin e colaboradores (2011) investigaram a atividade antibiofilme de extratos de 24 espécies vegetais da Caatinga usadas na medicina popular, destacando-se *Bauhinia acuruana, Pityrocarpa monilifomis e Commiphora leptophloeos* inibindo a formação de biofilme por *Staphylococcus epidermidis.*

2.5 METABÓLITOS REATIVOS DO OXIGÊNIO E COMPOSTOS ANTIOXIDANTES

Metabólitos reativos do oxigênio (ROS, do termo em inglês: *reactive oxygen species*), radicais livres e oxidantes são termos usados, para identificar os intermediários químicos reativos do metabolismo do oxigênio (HALLIWELL; GUTTERIDGE, 2003). Os mecanismos de geração de ROS ocorrem, normalmente, nas mitocôndrias, membranas celulares e no citoplasma. Tais mecanismos podem, especialmente, ser favorecidos pelos íons ferro e cobre. A mitocôndria, por meio da cadeia transportadora de elétrons, é a principal fonte geradora de radicais livres. Em condições fisiológicas do metabolismo celular aeróbio, o O_2 sofre redução tetravalente, com aceitação de quatro elétrons, resultando na formação de H_2O. Durante esse processo, são formados intermediários reativos como: os radicais superóxido ($O2^-$) e hidroxila (OH^-), o não radical peróxido de hidrogênio (H_2O_2), cujas características são mostradas abaixo:

- Radical superóxido ($O2^-$)

É um radical livre, formado a partir do oxigênio molecular pela adição de um elétron. Sua formação ocorre espontaneamente, especialmente na membrana mitocondrial, através da cadeia respiratória. É também produzido por flavoenzimas, lipoxigenases e cicloxigenases. Sua formação ocorre em

quase todas as células aeróbicas e são produzidos durante a ativação máxima de neutrófilos, monócitos e eosinófilos. É um radical pouco reativo e não tem a habilidade de penetrar membranas lipídicas, agindo, portanto, apenas no compartimento onde é produzido (NORDBERG; ARNÉR, 2001).

- Radical hidroxila (OH^-)

É considerado o radical livre mais reativo em sistemas biológicos, sendo capaz de causar mais danos do que qualquer outro ROS. É formado a partir do peróxido de hidrogênio em uma reação catalisada por íons metais (Fe++ ou Cu+), denominada reação de Fenton (FERREIRA; MATSUBARA, 1997; NORDBERG; ARNÉR, 2001). O principal alvo da ação do radical hidroxila é o DNA (que sofre quebra da dupla cadeia) e os ácidos graxos insaturados (que sofrem peroxidação lipídica) (HALLIWELL; GUTTERIDGE, 2003; THOMAS et al., 2009).

- Peróxido de hidrogênio (H_2O_2)

O H2O2 não é um radical livre, mas um metabólito do oxigênio extremamente deletério porque participa como intermediário na reação que produz o OH^-; tem vida longa e é capaz de atravessar membranas biológicas (FERREIRA; MATSUBARA, 1997; NORDBERG; ARNÉR, 2001). Uma vez produzido, o H2O2 é removido por um dos três sistemas de enzimas antioxidantes: catalase, glutationa peroxidase e peroxiredutases (NORDBERG; ARNÉR, 2001).

Os radicais livres em baixos níveis e em condições normais possuem um papel importante em seres vivos. Um exemplo de suas funções no organismo é na resposta imune a infecções. Os fagócitos em geral possuem um mecanismo de defesa contra corpos estranhos onde ocorre um alto consumo de oxigênio, geralmente denominado queima ou explosão respiratória. Nesse processo, o oxigênio consumido é convertido em ânion superóxido através do complexo da NADPH oxidase, que é usado para eliminar bactérias e partículas engolfadas pelos fagócitos, no processo chamado de fagocitose (DRÖGE, 2002; HALLIWELL; GUTTERIDGE, 2007). Há evidências de que os ROS também desempenham um papel importante na sinalização celular (RAY et al., 2012).

O excesso de radicais livres no organismo, seja de origem exógena ou endógena, é combatido por antioxidantes produzidos pelo próprio corpo ou

adquiridos através da dieta. Quando a geração de radicais livres é maior que a sua degradação pelas defesas antioxidantes, um desequilíbrio é gerado no organismo, denominado estresse oxidativo, que tem um papel importante na patogênese de muitas doenças, pois com passar dos anos, os danos causados aos componentes celulares se acumulam, contribuindo para a degeneração de células somáticas e indução de doenças crônico-degenerativas, especialmente associadas com o envelhecimento, destacando-se câncer, doenças cardiovasculares, pulmonares, inflamatórias, mal de Parkinson, mal de Alzheimer e catarata (SCALBERT et al., 2005; FEARON et al., 2009, CIENCEWICKI et al., 2008; MOREIRA et al., 2008; DESAI et al., 2010).

Do ponto de vista biológico, define-se antioxidantes como compostos que protegem sistemas biológicos contra efeitos potencialmente lesivos de processos ou reações que promovem a oxidação de macromoléculas ou de estruturas celulares. Os antioxidantes podem atuar de forma enzimática, como por exemplo, as enzimas: superóxido dismutase (SOD); catalase (CAT), peroxiredoxinas (Prx), glutationa (GSH), glutationa redutase (GR) e glutationa peroxidase (GPX) ou não enzimático, como principalmente vitaminas, polifenóis, e outros (AMAROWICZ et al., 2004). Adicionalmente, os antioxidantes podem ser subdivididos em sintéticos e naturais. Os sintéticos são comumente usados na indústria alimentícia, para prevenir a deterioração oxidativa, aumentando a vida de prateleira de alimentos lipídicos. São exemplos de antioxidantes sintéticos: o butilhidroxitolueno (BHT), o butilhidroxianisol (BHA), o propilgalato (PG) e a terciobutilhidroxinona (TBHQ) (BARREIROS; DAVID, 2006). No entanto, propriedades carcinogênicas têm sido apontadas para os antioxidantes sintéticos, aumentando a necessidade de pesquisas na busca de antioxidantes naturais (Chludil et al., 2008).

O uso de antioxidantes naturais tem aumentado com as descorbertas das propriedades dos componentes que são produzidos pelas plantas através do metabolismo secundário. Atribui-se a presença de compostos fenólicos, com destaque aos flavonóides, a atividade antioxidante dos componentes produzidos pelos vegetais. Esses componentes podem atuar como agentes redutores, sequestradores de radicais livres, quelantes de metais e /ou exibir mais de uma dessas funções simultaneamente (JIANG; XIONG, 2016).

3 OBJETIVOS

3.1 GERAL

Investigar os metabólitos secundários de folhas e frutos de *Libidibia ferrea* var. *ferrea* e avaliar atividades biológicas de extratos e frações semi-purificadas.

3.2 ESPECÍFICOS

a) Obter extratos com solventes de diferentes polaridades (ciclohexano, clorofórmio, acetato de etila e metanol) a partir de folhas de *Libidiba ferrea*;

b) Determinar a composição fitoquímica dos extratos obtidos;

c) Avaliar a atividade antibacteriana dos extratos frente aos patógenos Gram-positivos (*Bacillus subtilis* e *Staphylococcus aureus*) e Gram-negativos (*Escherichia coli*, *Pseudomonas aeruginosa* e *Proteus vulgaris*);

d) Efetuar o fracionamento do extrato de melhor atividade por cromatografia em coluna em sílica gel, analisar por cromatografia gasosa acoplada a espectrometria de massas (CG-EM) extrato e frações obtidas e posteriormente avaliar a atividade antibacteriana das frações frente ao patógeno mais sensível ao extrato;

e) Obter extratos brutos aquosos a partir de folhas e frutos de *L. ferrea* e analisar a constituição química de ambos por cromatografia líquida de alta eficiência (CLAE);

f) Avaliar o efeito dos extratos aquosos na formação de biofilme de *S. aureus*, fracionando o extrato de melhor atividade por CLAE-EM;

g) Avaliar a toxicidade do extrato aquoso de folhas em modelos de *Artemia salina* e embriões de *Biomphalaria glabrata;*

h) Determinar a atividade antioxidante *in vitro* do extrato aquoso de folhas pelo método de captura do radical DPPH;

i) Avaliar os efeitos protetores do extrato aquoso de folhas por meio dos ensaios: radioproteção em embriões de *B.glabrata,* inibição da degradação do DNA induzida pelo reagente de Fenton e inibição da hemólise eritrocitária induzida por agente oxidante.

4 RESULTADOS

4.1 ARTIGO 1 - EVALUATION OF ANTIBACTERIAL ACTIVITY BY *Libidibia ferrea* var*. ferrea* EXTRACTS AGAINST HUMAN PATHOGENIC STRAINS

Evaluation of antibacterial activity by *Libidibia ferrea* var*. ferrea* extracts against human pathogenic strains

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ABSTRACT

Objective: This study evaluated the antibacterial potential and characterized the chemical composition of extracts from *Libidibia ferrea* leaves collected from Vale do Catimbau, Pernambuco- Brazil.

***Materials and methods*:** Extracts of leaves were prepared using solvents of various polarities in order to extract a wide range of phytochemicals. TLC and GC-MS were used to determine the chemical constituents of extracts and fractions. The antibacterial activity of these extracts was evaluated by micro-dilution assay, against Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) as well as Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*) bacteria.

***Results*:** Phytochemical analysis of crude extracts from *L. ferrea* revealed presence of alkaloids, flavonoids, cinnamic derivates, terpenes and tannins. Generally, the cyclohexanic (LFCH), chloroform (LFCF) and ethyl acetate (LFEA) extracts demonstrated the best activities, exhibiting the highest average activity for all pathogens. Of these, LFCH was the most active with MIC ranging from 0.39 mg/ml to 12.5 mg/ml. The results obtained for the fractions were lower than the ones obtained with the crude extract LFCH. Some other notable activity was observed for the chloroform and ethyl acetate extracts against *Staphylococcus aureus* with an MIC values of 0.19 mg/mL and 0.39 mg/mL respectively. The GC–MS analysis revealed that the major compound present in the cyclohexanic extract was heptacosane.

***Conclusion*:** *Libidibia ferrea* var. *ferrea* leaves extracts have antibacterial potential that can inhibit *in vitro* growth of human pathogens.

Keywords: Caatinga, *Libidibia ferrea* var*. ferrea,* eluotropic extraction, antibacterial activity.

1. Introduction

The caatinga is a semi-arid vegetation occurring only Brazil northeast that covering a vast area of 844,453 km^2. Marked by a severe climate with accentuated dryness, Caatinga may be source of new active substances because the vegetation is constantly subjected to environmental stresses and must develop different survival strategies to compete for the limited water resource (Sampaio et al., 2002).

Various plants from Caatinga have been used in folk medicine and in production of herbal products but there are few studies relating these plants to their pharmacological properties. Several studies have been demonstrated that Caatinga plants are a rich source of biotechnology compounds, in special with remarkable antimicrobial activity (Silva et.al., 2013a; Trentin et al., 2014).

Libidibia ferrea var. *ferrea* is a leguminous tree native to Brazil and is endemic in northern and northeastern regions mainly in Caatinga ecosystem. Commonly known as “pau-ferro”, this plant is used in folk medicine for therapeutical purposes including respiratory tract diseases, dysentery, diabetes, anemia and inflammations (Bragança, 1996).

This study represent a phytochemical composition analysis and evaluate the antibacterial properties of *L. ferrea* leaves collected from the Caatinga, an ecosystem characterized by extreme climatic conditions considered a potential source of new bioactive natural products.

2. Material and methods

2.1 Plant material and extraction

Libidibia ferrea var. *ferrea* leaves were collected at Vale do Catimbau, Pernambuco- Brazil, a preservation area of Caatinga bioma, at non-raining season. Botanical identification was confirmed at the herbarium of Instituto Agronômico de Pernambuco (IPA), where the voucher was deposited under number 84035.

The air-dried leaves (340g) were extracted using an eluotropic series of solvents (in the following order: cyclohexane, chloroform, ethyl acetate and methanol) at room temperature (25°C) for 12h under constant agitation. All extracts from leaves (cyclohexane: LFCH; chloroform: LFCF; ethyl acetate:

LFEA; methanol: LFML) were concentrated until the complete removal of the solvent on a rotating evaporator at 45◦C (under reduced pressure) to yield 2.66%, 0.47%, 1.07%, 11.9% respectively. For phytochemical analysis the extracts were dissolved in ethanol, while they were solubilized in a sterile dimethylsulfoxide solution (DMSO; 10% in water) for antibacterial assay.

2.2 Phytochemical analysis

An aliquot (10µL) of each extract (1 mg/mL in ethanol) obtained from leaves of *L. ferrea* was subjected to qualitative phytochemical analysis to ascertain the presence of secondary metabolites such as: tannins, alkaloids, flavonoids, cinnamic derivatives, terpenes (Harborne, 1998; Wagner and Bladt, 1996; Roberts et al. 1957). The compounds classes were visualized as aid thin layer chromatography (TLC) on silica gel 60 F254 (Merck, Germany), different systems of development and adequate visualization techniques were used: Dragendorff, NEU-PEG, KOH-Ethanol, Liebermann-Burchard and vanillinsulfuric acid.

2.3 Antibacterial activity

2.3.1 Microorganisms

The antimicrobial activity of *L. ferrea* leaves extracts and its fractions were tested against the following microorganisms: *Bacillus subtilis* (UFPEDA86), *Staphylococcus aureus* standard strain (UFPEDA02) and clinical isolates (UFPEDA 660 - vaginal discharge; UFPEDA 663 - catheter tip; UFPEDA 676 - prosthesis secretion; UFPEDA 712 - wound secretion transplant), *Escherichia coli* (UFPEDA 224), *Pseudomonas aeruginosa* (UFPEDA416), *Proteus vulgaris* (UFPEDA740). All strains were provided by Departamento de Antibióticos, Universidade Federal de Pernambuco (UFPEDA) and maintained in Nutrient Agar (NA) and stored at 4°C. The cultures were adjusted turbidimetrically at a wavelength of 600 nm to 1×10^8 colony forming units (CFU)/ml (0.5 in McFarland scale).

2.3.2 Determination of the Minimum inhibitory concentration (MIC) and the Minimum bactericidal concentration (MBC)

MIC was determined by the microdilution method (CLSI, 2011). A serial dilution of the extract/fraction was made in 96-well plates to obtain concentrations ranging from 25 to 0.04 mg/mL in Mueller Hinton Broth (MHB).

Following that, each well received 10µL of microorganism suspensions and the plates were incubated at 37°C for 24 h. After incubation, the optical density at 600 nm (OD600) was measured using a spectrophotometer for microplates Epoch™. Commercially available antibiotic was used as positive control for bacteria (ampicillin) and sterile DMSO aqueous solution (10%) was used as negative control. Assays were carried out in triplicate and the results expressed in MIC (mg/mL). MIC was expressed as the lowest concentration which inhibited growth, judged by lack of turbidity in the well.

Minimal bactericide concentration (MBC) was determined starting from the results of MIC assay. Inoculations (10 µL) from the wells in which the sample inhibited bacterial growth were transferred to petri plates containing Mueller Hinton agar. The number of CFU grown in plates was determined after incubation at 37 ◦C for 24 h. The MBC corresponded to the minimum concentration of extract/fraction in which no bacterial growth was observed.

2.3 Fractionation procedures

The antibacterial test showed that cyclohexanic extract exhibited greater inhibition of tested microorganisms when compared with the other extracts. Then, LHCH (100 mg) was chromatographed on a 42 x 5 cm column chromatography on silica gel (70e230 mesh ASTM) using C_6H_{14}; C_6H_{14}-CH_2CL_2 mixtures (9:1, 1:1, v/v) and CH_2CL_2 solvents for elution, corresponded the fractions F1, F2, F3 and F4, respectively.

The extract and fractions were analyzed by Gas chromathography - mass spectrometry **(**Shimadzu GCMS-QP5050A) on Central Analítica, Universidade Federal de Pernambuco, Brazil. The components of extract/fractions were identified based on the comparison of their retention indices and mass spectra with the fragmentation patterns from data base Library.

3. Results

3.1 Extraction and phytochemical analysis

Different solvent systems were used for the extraction of lipophilic and hydrophilic compounds from *Libidibia ferrea* leaves. Types of secondary

metabolites revealed by phytochemical assay are listed in **Table 1**. Flavonoids were observed in all extracts. The presence of cinnamic derivatives and tannins was observed in LFEA, LFML. Alkaloids were observed only at LFEA. Terpenes were found in LFCH and LFCF. Carbohydrates, specifically sucrose, were observed only in LFEA.

3.2 Antibacterial activity

Extracts of *L. ferrea* leaves were evaluated for their antimicrobial effects against 5 common human pathogens. Two strains are Gram positives (*B. subtilis*, and *S. aureus*) and Three are Gram negative (*E. coli*, *P. vulgaris* and *P. aeruginosa*). The MIC, MBC and MBC/MIC ratio values of the extracts/fractions against pathogenic bacteria are presented in **Table 2.** Overall, all extracts from leaves of *L. ferrea* exhibited antimicrobial activity with broad spectrum, as they inhibited all tested bacteria. Furthermore, the best antibacterial results observed were provided by the extracts obtained using cyclohexane and chloroform with MIC ranging from 0.39 mg/mL to 6.25 mg/mL. Fractionation of LFCH also leads to active fractions, showing inhibitory effect with MIC ranging from 2.5 mg/ml to 10 mg/ml. Reference drug Clindamycin inhibited bacterial growth with MIC values ranging from 0.019 to 0.19 mg/ml.

3.3 Fractionation and GC-MS analysis

GC-MS analysis of LFCH and fractions as summarized in **Table 3**. Cyclohexanic extract from *L. ferrea* leaves has long chain alkanes as constituents. The major components identified by GC-MS were heptacosane (53.2%), octacosane (17.34%) and docosane (16.8%) shows in **Figure 1**.

Three semipurified fractions and heptacosane compound were obtained from fractionation of LFCH. GC-MS analysis identified several compounds, including fatty acids, terpenes, steroids and alkanes. The first fraction (F1) sesquiterpenes, nerolidol (34.2%) and globulol (65.1%) as major constituents. Fraction F2 is composed of two fatty acids, palmitic acid and linoleic acid (**Figure 2**). Two fatty acids were identified at fraction F2 (**Figure 3**), palmitic acid (7.1%) and linoleic acid (92.9%).Several compounds were identified in fraction 3 as carboxylic acids, steroids and terpenes (sesquiterpene, and diterpene). The most abundant of these compounds were Phytol (25.2%), pyrrolidine (16.8%), benzoic acid (11.4%), hexanoic acid (11.1%) and γ-sitosterol (9.5%). The last fraction eluted with dichloromethane allowed isolate

the heptacosane, this alkane was identified as major compound of cyclohexanic extract (**Figure 4**).

4. Discussion

Preliminary phytochemical screening in others studies revealed that fruits of *L. ferrea* contained terpenoids, saponins, tannins, coumarins and flavonoids (Dias et.al., 2013; Sampaio et.al., 2009). These compounds may be responsible for the antimicrobial activity observed as previously demonstrated (Sampaio et.al., 2009; Silva et.al., 2013b) The extracts LFCH and LFCF exhibited more significant antibacterial activity can be explained by the presence of terpenes and flavonoids, compounds found abundantly in plants with antimicrobial activity.

The antimicrobial activity is considered significant when MIC ≤ 1.56 mg/mL (Silva et al, 2016). On this basis, LFCH had significant antibacterial activity against several tested pathogens including *E. coli, B. subtilis, P.aeruginosa* and *S. aureus* (UFPEDA02 and UFPEDA663) with MIC values of 1.56 mg/mL, 0.78 mg/mL , 1.56 mg/mL, 0.39 mg/mL and 0.78mg/mL, respectively. LFCF best inhibited *B. subtilis* and *S. aureus* (MIC = 0.78 mg/mL). LFEA was more active against *S. aureus* (UFPEDA02 and UFPEDA663) with MIC's to 0.78 mg/mL and 0.39 mg/mL. Antimicrobial substances are considered bacteriostatic agents when the MBC/MIC> 4 ratio, and bactericides when MBC/MIC ≥ 4 (Gatsing et al., 2009). The MBC/MIC ratio ranging from 1 to 2 for LFCF, 1 to 4 for LFCH and LFML , 2 to 4 for LFEtAc. Therefore, most of the extracts exhibited bactericidal activity against the bacteria tested.

The highest levels of antibacterial activity of the extracts were observed against *Staphylococcus aureus* and *Bacillus subtilis*, Gram-positive bacteria species. Previous studies suggested that Gram-negative bacteria are less susceptible to active compounds present in the extracts due to the presence of the outer membrane consisting of lipoproteins and lipopolysaccharides, which is selectively permeable and thus regulates access of various molecules (Urzua et al, 1998; Briers and Lavigne, 2015). Our results show that increase of antibacterial activity was observed with decreasing polarity of extracts, this suggest that compounds present in less polar extracts are responsible for the potential activity.

In this study, we reported for the first time a high resolution GC–MS method for the evaluation of the chemical constituents *L. ferrea* leaf's. This analysis revealed the various phytoconstituents present in the cyclohexanic extract such heptacosane, major compound, has been described as one of the major component of essential oil isolated from *Dieffenbachia picta* with antibacterial activity (Oloyede et al., 2011). GC–MS analysis for semipurified fractions from LFCH revealed several compounds such as sesquiterpenes, nerolidol and globulol. Tan et al (2008) reported antimicrobial activity of *Eucalyptus globulus Labill* fruits and suggested that globulol was the main compound bioactive present in ethanol extract. Brehm & Johnson (2003) showed that nerolidol sensitized *S. aureus* and *E. coli*, increasing susceptibility of these microorganisms to important antimicrobial agents as ciprofloxacin and clindamycin.

Fractions from cyclohexanic extract exhibited less antibacterial activity when compared with the crude extract (**Table 2**). All fractions exhibited MIC and MBC ranging from 5 mg/ml to 10 mg/ml for clinical isolates but the fraction F3 against standard strain was capable of inhibiting bacterial growth at concentration of 0.625mg/ml. This can be explained by the presence of the phytol, a terpene that showed antibacterial activity against *Mycobacterium tuberculosis* (Rajab et al., 1998) and *Staphylococcus aureus* (Inoue et.al 2005) in previous studies. The antimicrobial activity of compounds present in LFCH was reduced after fractionation, suggesting the possibility of synergism between molecules present in the extract.

The results of the present investigation complement the ethnobotanical use of the *L. ferrea* leaves which possess several phytoconstituents with antibacterial activity mainly against *Staphylococcus aureus,* a important human pathogen related in nosocomial infections.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors acknowledge the support from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). Authors also acknowledge the cooperation from the inhabitants of the areas of study, the financial support from NANOBIOTEC–Brasil from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for authorizing collections in PARNA do Catimbau and the curator of Herbarium IPA (Instituto Agronômico de Pernambuco) for allowing access to the collection. We express our gratitude to Dr. Alexandre Gomes Silva (Instituto Nacional do Semiarido - INSA) for performing the collection of botanical material.

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Table 1: Screening of phytochemicals of *Libidibia ferrea* var. *ferrea* leaves extracts.

Extract[1]	Tannins	Flavonoids	Alkaloids	Carbohydrates	Cinnamic derivatives	Terpenes
LFCH	-	+	-	-	-	+
LFCF	-	+	-	-	-	-
LFEA	+	+	+	+	+	+
LFML	+	+	-	-	+	-

[1]*Libidibia ferrea* var. *ferrea* leaf extracts: Cyclohexanic extract, LFCH; chloroform extract, LFCF; ethyl acetate extract, LFEA; methanol extract, LFML . (+) Present; (-) Absent.

Table 2. Antibacterial Activity of extracts and fractions from leaves of *L. ferrea* against Gram-positive and Gram-negative bacteria.

Name of the bacterial pathogens	MICs, MBCs and MBC/MIC ratio (in parenthesis)																	
	Reference drug		Extracts								Fractions							
	Clin		LFCH		LFCF		LFEA		LFML		F1		F2		F3		F4	
B. subtilis	0.039	0.039 (1)	0.78	1.56 (2)	0.78	0.78 (1)	1.56	6.25 (4)	3.12	6.25 (2)	nt	nt	nt	nt	nt	nt	nt	nt
E. coli	0.019	0.039 (2)	1.56	6.25 (4)	6.25	6.25 (1)	6.25	12.5 (2)	12.5	-	nt	nt	nt	nt	nt	nt	nt	nt
P. vulgaris	0.039	0.039 (1)	3.12	12.5 (4)	6.25	12.5 (2)	12.5	25.0 (2)	12.5	-	nt	nt	nt	nt	nt	nt	nt	nt
P. aeruginosa	0.190	0.390 (2)	1.56	3.12 (2)	3.12	3.12 (1)	1.56	3.12 (2)	6.25	12.5 (2)	nt	nt	nt	nt	nt	nt	nt	nt
S. aureus	0.019	0.078 (4)	0.39	0.78 (2)	0.78	1.56 (2)	0.78	3.12 (4)	0.39	1.56 (4)	5	10 (2)	2.5	5 (2)	0.625	5 (8)	2.5	10(4)
UFPEDA660[1]	0.156	0.312 (2)	3.12	6.25 (2)	6.25	6.25 (1)	6.25	12.5 (2)	12.5	12.5 (1)	5	10 (2)	5	-	5	-	5	-
UFPEDA663[1]	0.039	0.039 (1)	0.78	0.78 (1)	0.19	0.39 (2)	0.39	0.78 (2)	6.25	12.5 (2)	-	-	10	-	10	-	5	10 (2)
UFPEDA676[1]	0.019	0.078 (4)	3.12	12.5 (4)	3.12	3.12 (1)	1.56	3.12 (2)	6.25	6.25 (1)	-	-	5	-	5	-	5	-
UFPEDA712[1]	0.019	0.039 (2)	3.12	12.5 (4)	1.56	3.12 (2)	3.12	12.5 (4)	0.78	6.25 (8)	-	-	5	-	5	-	10	-

MIC, Minimal Inhibitory Concentration; MBC, Minimal Bactericidal Concentration; MIC and MBC values are expressed in mg/mL. Tested samples [Cyclohexanic extract, LFCH; chloroform extract, LFCF; ethyl acetate extract, LFEA; methanol extract, LFML; Clindamicyn, Clin]. ; (-): MBC >25 mg/mL for extract and MBC >10mg/mL for fraction; nt: not tested; tested concentration range: 0,04 - 25 mg/mL for extracts and fractions 0,01 - 10 mg/mL. [1]Source of *S. aureus* strains: vaginal discharge, catheter tip, prosthesis secretion, wound secretion transplant, (respectively).

Table 3. Phytochemicals identified in the cyclohexanic extract and fractions of *L.ferrea* by GC–MS.

Name of the compound	Retention time (min)	Relative area (%)
Cyclohexanic extract		
octadecanal	28.740	5.07
n-dodecanal	28.858	3.76
octacosane	30.377	17.34
docosane	30.504	16.86
pentadecane	43.032	3.77
heptacosane	53.220	53.21
Fraction 1		
pentadecanoic acid	16.883	0.59
globulol	23.208	65.12
Fraction 2		
palmitate acid	23.700	7.10
linolenic acid	27.983	92.90
Fraction 3		
n-decanal	15.958	1.76
14-methyl-pentadecanoat	16.797	6.87
1,2-benzenedicarboxylic acid	17.994	4.26
phytol	19.037	25.25
hexanoic acid	21.001	11.18
1,2-benzenedicarboxylic acid	22.652	4.97
benzoic acid	26.222	11.49
pyrrolidine	26.549	16.82
γ- Sitosterol	39.027	9.48
2,4-pyridinedicarboxylic acid	40.617	6.93
viridiflorol	40.683	0.97
Fraction 4		
heptacosane	53.093	100

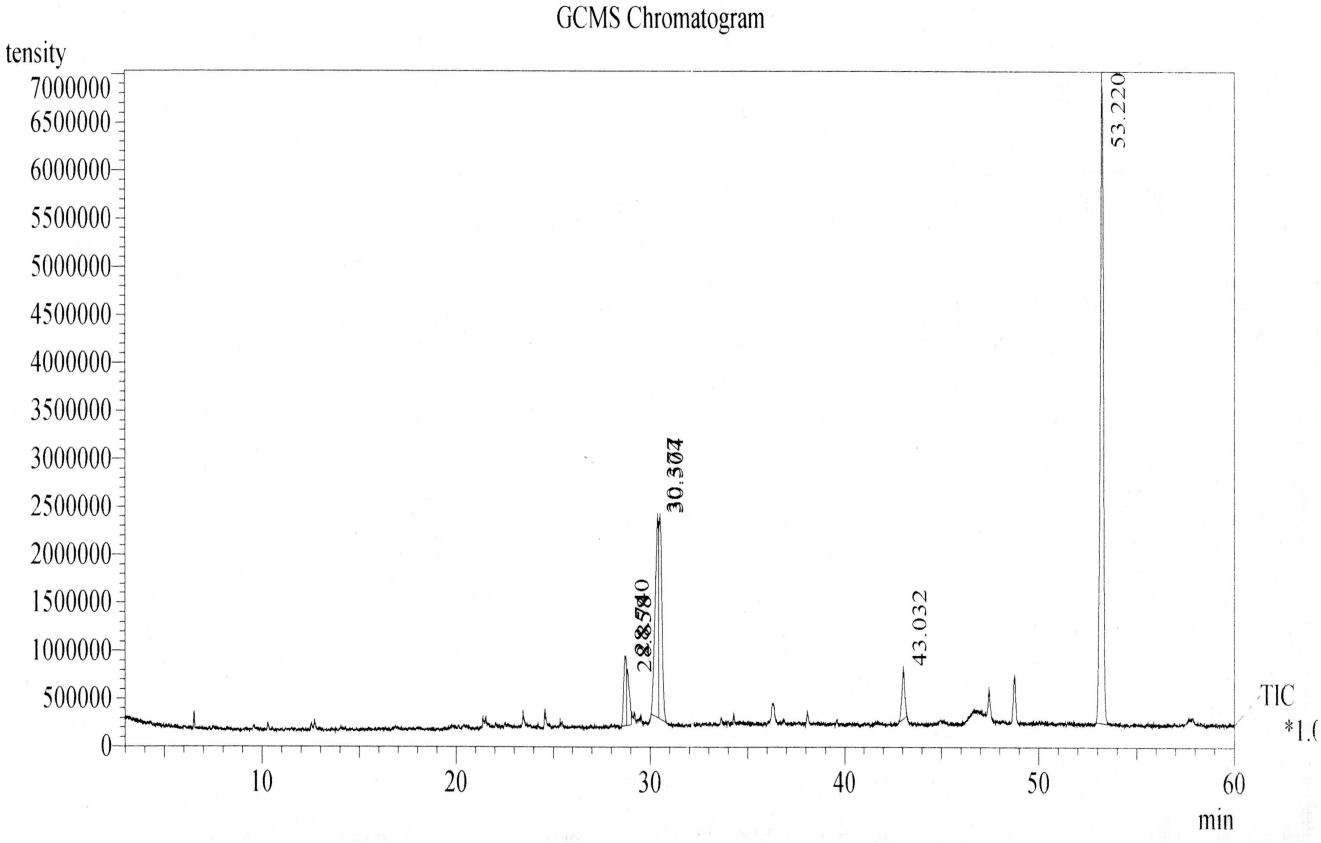

Figure 1. GC–MS chromatogram of cyclohexanic extract of *L. ferrea* leaves.

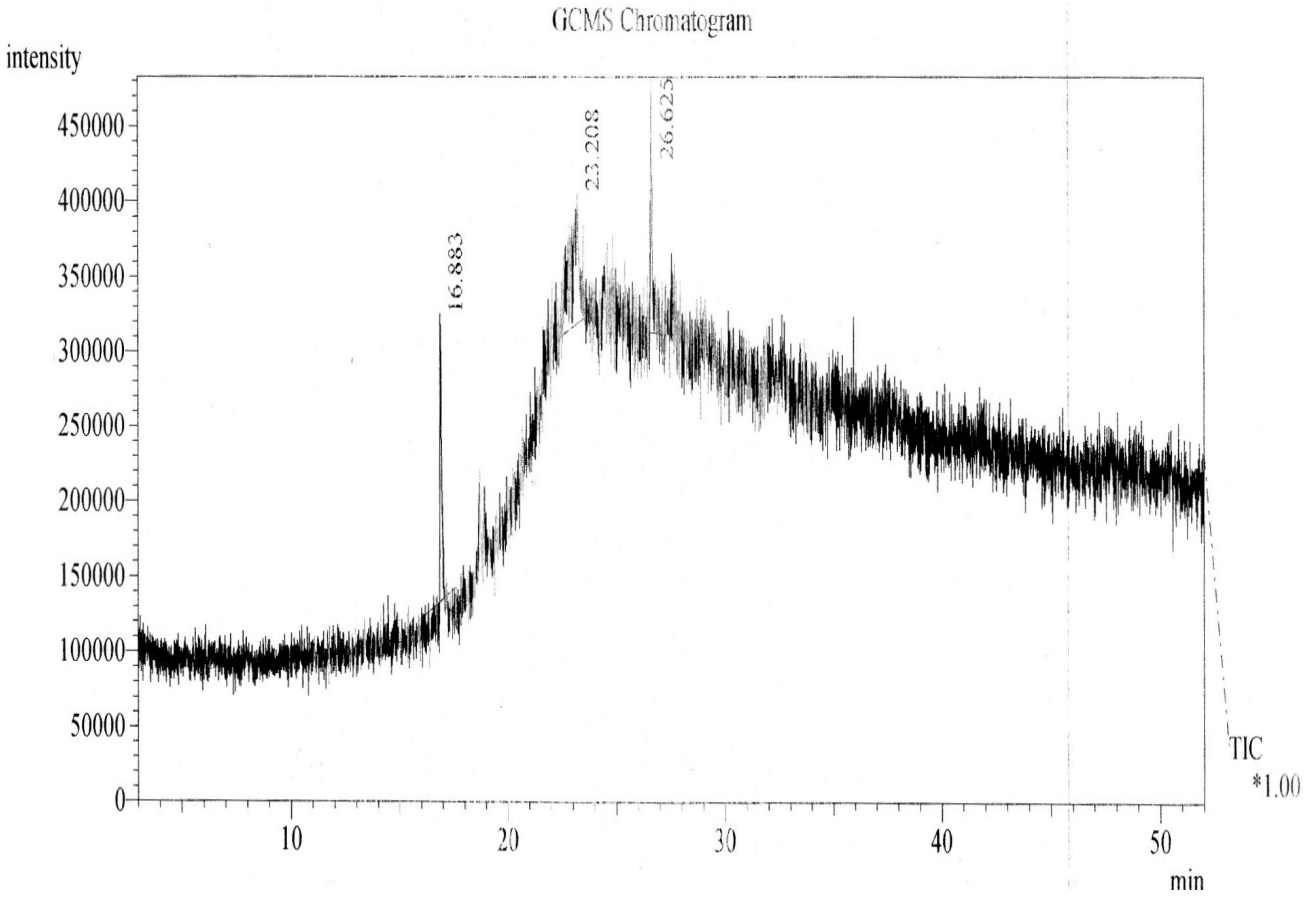

Figure 2. GC–MS chromatogram of fraction 1 from cyclohexanic extract of *L. ferrea* leaves.

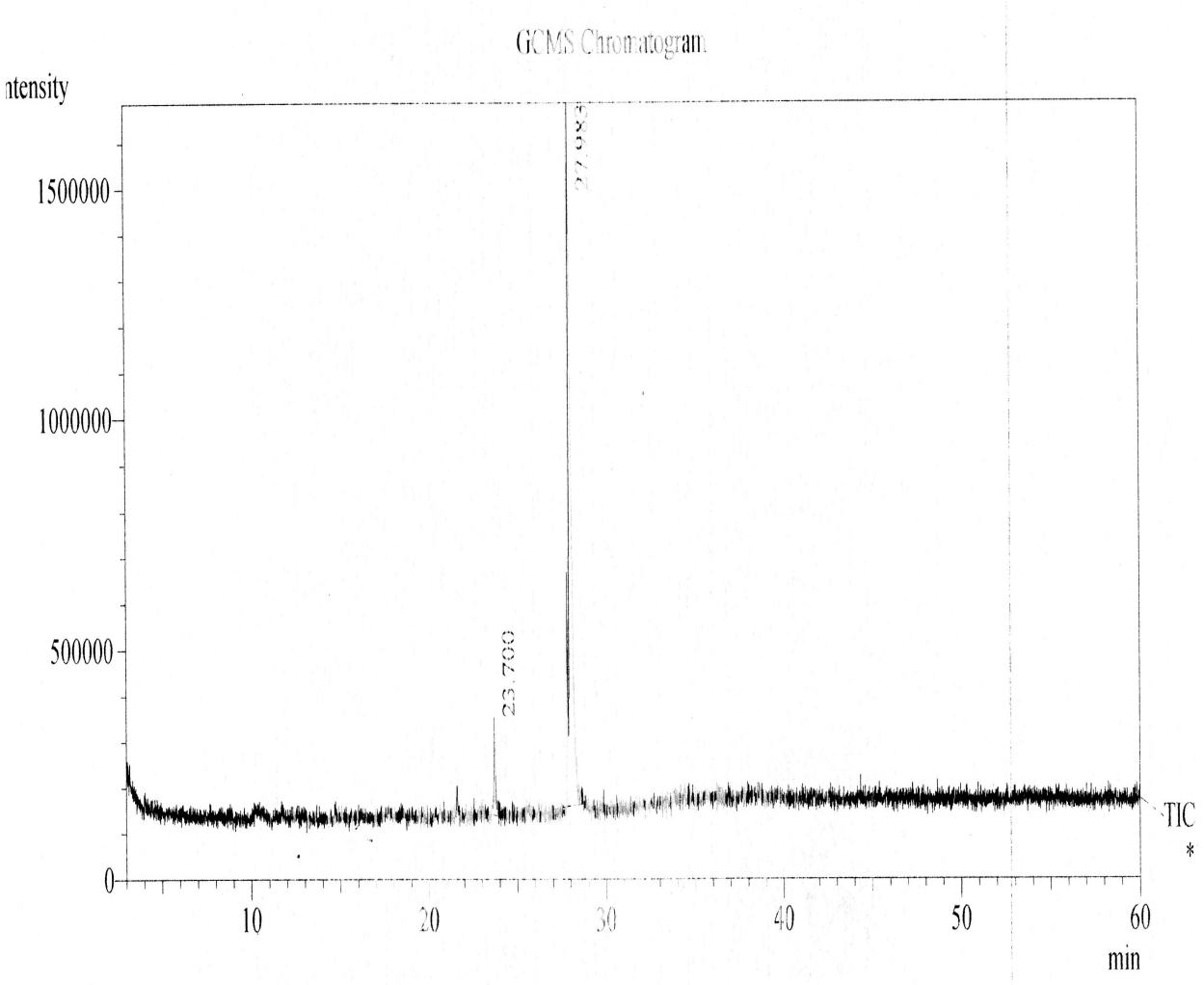

Figure 3. GC–MS chromatogram of fraction 2 from cyclohexanic extract of *L. ferrea* leaves.

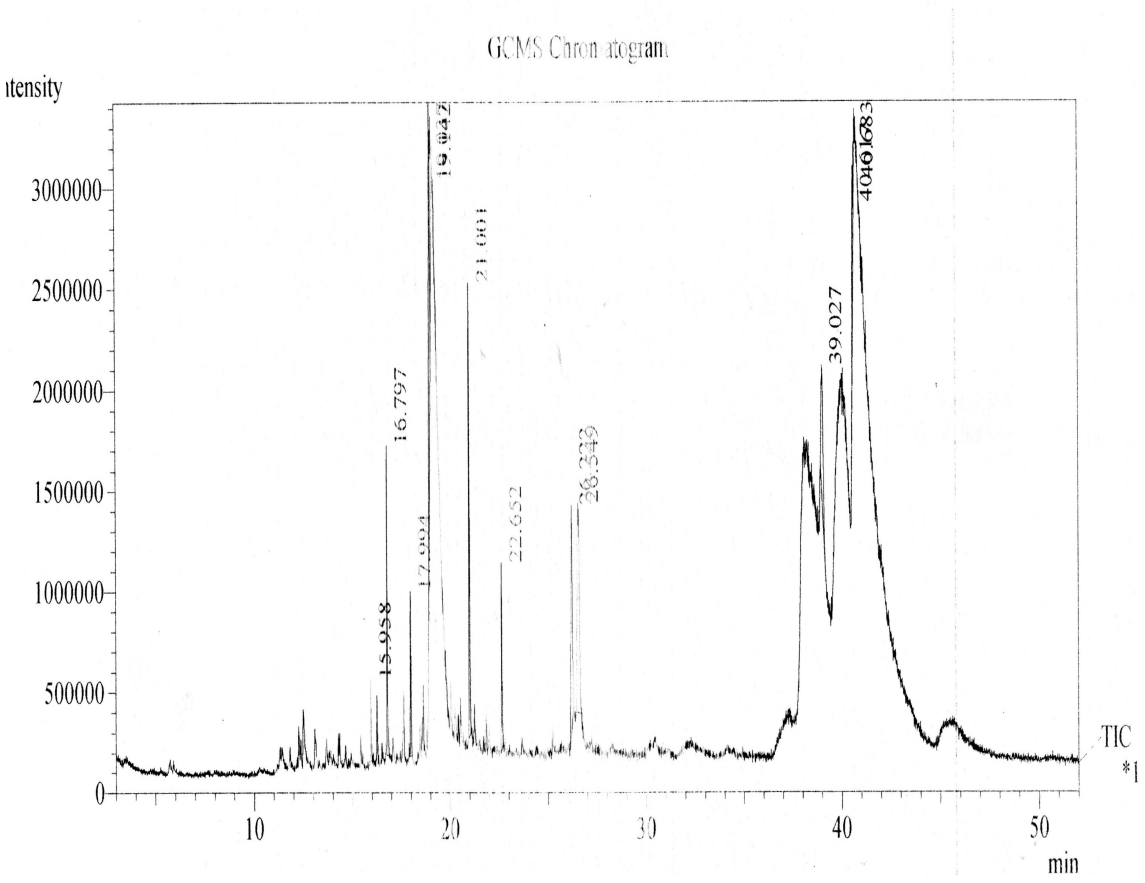

Figure 4. GC–MS chromatogram of fraction 3 from cyclohexanic extract of *L. ferrea* leaves.

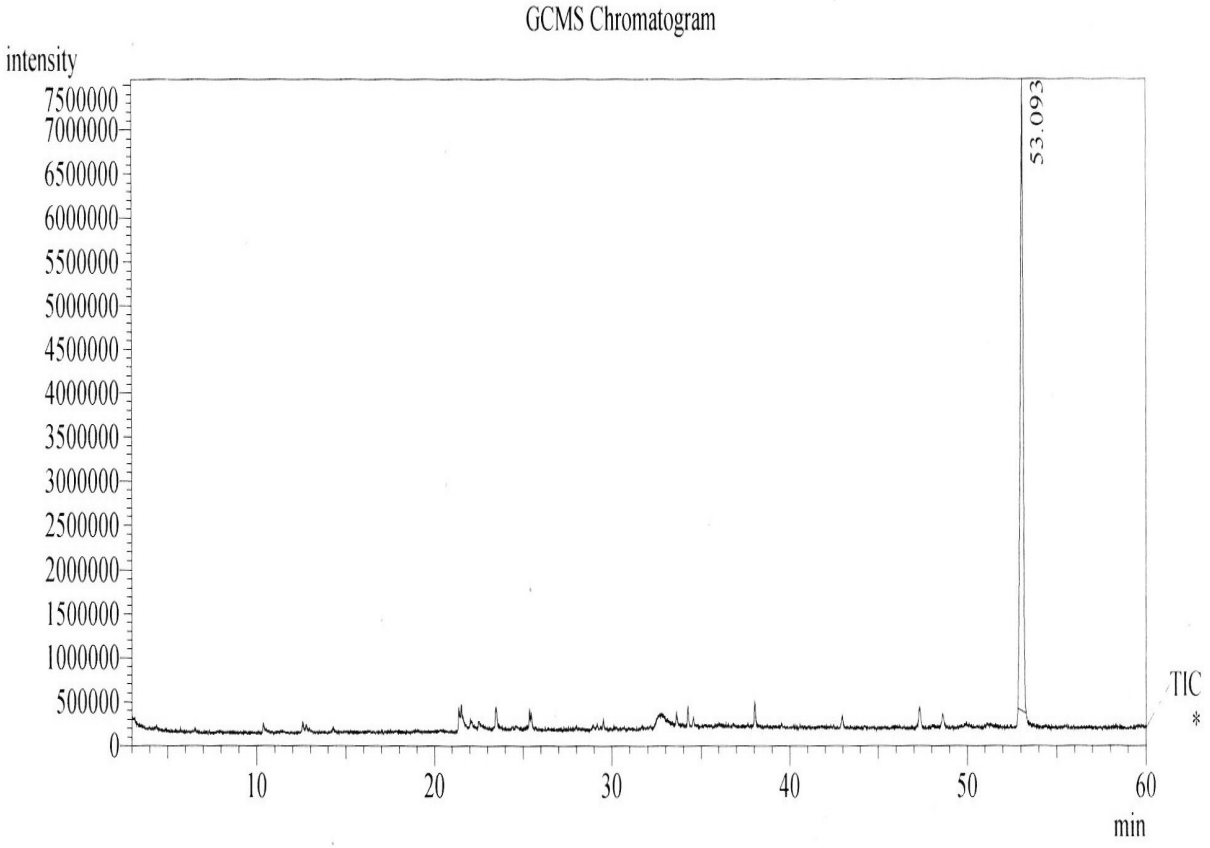

Figure 5. GC–MS chromatogram of fraction 4 from cyclohexanic extract of *L. ferrea* leaves.

4.2 ARTIGO 2 - *IN VITRO* ANTIBIOFILM POTENTIAL OF *Libidibia ferrea* var. *ferrea* AGAINST *Staphylococcus aureus*

In vitro* antibiofilm potential of *Libidibia ferrea* var. *ferrea* against *Staphylococcus aureus

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ABSTRACT

The identification of effective antimicrobial agents active on biofilms is a topic of crucial importance in clinical environments. For that purpose aqueous extracts of leaves (LFAQ) and fruits (LFRAQ) of *L. ferrea* were investigated. The effect of aqueous extracts on biofilm formation by *Staphylococcus aureus* was studied by the crystal violet assay. Chromatographic analyses and fractionation were performed to characterize the most active extract. Leaves extracts did not exhibited significant antibiofilm activity. In contrast, LFRAQ produced a significant biofilm inhibition equal to 71.1% and 55.3% for *S. aureus* at concentrations 2.5mg/mL and 1.25 mg/mL, respectively. Among sub-fractions obtained from LFRAQ, F2 and F4 exhibited antibiofilm effect to 52.7 and 76.3% of inhibition, respectively. Based on comparison with the standards, it was not possible to identify the compounds responsible for biological activity of this extract. The present findings promote the value-adding of *L. ferrea* fruits as natural agent for biofilm control of *S. aureus*, a human pathogenic bacteria.

Keywords: Caatinga, antibiofilm agent, *S. aureus*.

INTRODUCTION

Staphylococcus aureus is an important pathogen worldwide through its versatility, virulence factors and resistance mechanisms, documented as a common cause of nosocomial and community acquired infections ranging from skin infections to myocarditis, endocarditis, osteomyelitis, pneumonia and urinary tract infections (UTI) in human. *S. aureus* infections have a high mortality rate particularly when caused by methicillin-resistant (MRSA) strains (Nguyen et al, 2010). Biofilm formation is a major virulence factor of S. aureus. Moreover, this Gram-positive bacteria is a common cause of infection on implanted catheters and other medical devices, notoriously resistant to antibiotics (Schuenck et al.,2006; Rahimi, Katouli and Karimi, 2016).

Microbial biofilms are a complex matrix of microorganisms in which cells bind together and attach to biotic or abiotic surface that pose a challenge in clinical and industrial settings where the need for sterility is paramount. (Costerton, Stewart, and Greenberg, 1999). Biofilms usually create a sticky gel composed of polysaccharides, proteins and other organic components on a wet surface. Several mechanisms can account for the increased antibiotic resistance in biofilms, including the physical barrier formed by exopolymeric substances, a proportion of dormant bacteria that are inert toward antibiotics and resistance genes that are uniquely expressed in biofilms (Mah and O'Toole, 2001; Patrick et al., 2014; Fuente-Núñes et al, 2013). Together, these bacterial features that create resistance to antibiotics drive the need for novel strategies that will effectively kill bacterial biofilms.

According to the literature data, biological properties as well composition of bioactive compounds of *L. ferrea,* popularly known as ironwood, have been extensively explored. Anti-inflammatory, analgesic and antimicrobial properties have been demonstrated, causing interest in continuing with analysis of its pharmacological and therapeutic characteristics (Carvalho et al., 1996; Silva et al., 2013). Recent work showed that *L.ferrea* fruit aqueous extract was effective in preventing biofilm formation of *Staphylococcus epidermidis* at 4.0 mg/mL concentration (Trentin et al., 2011). Sampaio et al (2009) reported that hydromethanolic fruit extract of ironwood was able to inhibit bacterial growth of oral pathogens (*Candida albicans, Streptococcus mutans, Streptococcus salivarius, Streptococcus oralis* and *Lactobacillus casei*) at MIC concentrations

ranging from 25 to 100µg/mL. Furthermore, the authors indicated the activity of the extract in biofilm formation, however this was less active when compared to the control (Chlorhexidine).

This study aimed to investigate the activity of leaves and fruits extracts of *Libidibia ferrea* var. *ferrea* and their ability to prevent biofilm formation of *S. aureus* using bioguided fractionation. The bioactive fractions were analyzed by HPLC-UV-MS in order to identify the compounds.

MATERIALS AND METHODS

Plant Material

Leaves and fruits of *L. ferrea* var. *ferrea* were collected collected at Parque Nacional do Catimbau (PNC), a preservation area of Conservation Unit of the Caatinga ecosystem, Municipalities of Buíque, Ibimirim and Tupanatinga, Pernambuco State, Brazil., at non-raining season. Botanical identification was made from Herbarium of Instituto de Pesquisa Agronômica de Pernambuco, (Agronomic Institut of Pernambuco State), Brazil. Voucher specimen (IPA 84.035) was deposited at the herbarium IPA.

Extract Preparation

Aqueous extract of leaves and fruits of *Libidibia ferrea var. ferrea* 20% (w/v) were prepared in saline solution by agitation at 300 rpm for 12h at room temperature. After this period, both extracts were filtered and centrifuged (12.000 x g) for 10 min, and the supernatants were collected and lyophilized.

Bacterial strain and culture conditions

Staphylococcus aureus provided by the Culture Collection UFPEDA (Department of Antibiotics, UFPE) was grown overnight on Mueller-Hinton (MH) agar at 37°C. A bacterial suspension of $3x10^8$ colony-forming units (CFU)/mL in 0.9% NaCl was used in the assays.

Antibiofilm formation assay

The effect of *L.ferrea* extracts and fractions on biofilm formation was evaluated as described by Trentin et al (2011). Were added in 96-well microtiter plates, 80 µL of the bacterial suspension (1.0 McFarland), 80 µL of the aqueous extract (2.5, 1.25 and 0.625 mg/mL) or fraction (2.5 mg/mL) and 40 µL of tryptone soya broth (TSB). Following the incubation period (37 ◦C for 24 h) the content of the wells was removed and the wells were washed three times with sterile saline. The remaining attached bacteria were heat-fixed at 60 ◦C for 1 h.

The adherent biofilm layer formed was stained with 0.4% crystal violet for 15 min at room temperature. The stain bound to the cells was solubilized with ethanol and absorbance was measured at 570 nm using a Microplate Reader Epoch®.

The biofilm formation control was considered to represent 100% of biofilm formation, and the extracts were replaced by 80 µL of water in 96-well microtiter plates. Values higher than 100% represent a stimulation of biofilm formation in comparison to the control. The cutoff optical density (ODc) was defined as three standard deviations above the mean OD of the negative control (culture medium), and bacterial strain were classified as nonadherent (OD ≤ ODc), weakly adherent (ODc < OD ≤ 2 x ODc), moderately adherent (2 x ODc < OD ≤ 4 x ODc), or strongly adherent (OD > 4 xODc) (Stepanovic et al., 2000).

HPLC-UV analisys

Fruit extract (10mg) was dissolved in 1mL of methanol:MilliQ water (1:1) and 10µL were analyzed using an analytical HPLC (Agilent) and a C18 Zorbax SB column (4.6 × 250 mm). The solvent system used was a gradient of formic acid (0.3%) (A) and acetonitrile (B), starting with 8% acetonitrile and installing a gradient to obtain 35% B at 15min, at a solvent flow rate of 2.4 ml/min. Detection was achieved with a UV detector (Agilent 1100 series). Spectral data from all peaks were accumulated in the range 200–400 nm, and chromatograms were recorded at 360 nm. The compounds in each sample were identified by comparing their retention times and UV–Vis spectra in the 200–400 nm range with chromatograms relative to Standards (cafeic acid, catechin, chlorogenic acid, coumaric acid, ellagic acid, ferrulic acid, gallic acid, quercetin and rutin).

Fractionation of *L. ferrea* fruit extract

The *L. ferrea* fruit's extract was fractionated on LC–UV–MS autopurification system (Waters Corporation, Milford, MA, USA) consisting of a 9012 quaternary pump, a 9100 autosampler, a 2998 photodiode-array UV detector, and a ACQUITY QDa® single-quadrupole mass detector with electrospray ionization source. LFRAQ (20 mg) were dissolved in water (1 mL) and injected on a preparative C18 column (Xbridge® - 10mm×100mm, 5µm). Solvent A was 0.01% formic acid and solvent B was acetonitrile, starting with

8% acetonitrile and installing a gradient to obtain 35% B at 15min, at a flow rate of 3 ml/min, resulting in 26 fractions (NB1 to NB26). The eluate was directly injected into the electrospray íon source and the mass spectra were acquired and interpreted using the software provided by the manufacturer. According to mass spectra obtained, sub-fractions have been pooled as follow: NB1 to NB4 (F1); NB5 to NB7 (F2); NB8 + NB9 (F3); NB10 to NB12 (F4); NB13 + NB14 (F5); NB15 to NB17 (F6); NB18 to NB20 (F7); NB21 to NB26 (F8).

The sub-fractions F2 and F4 which were the most active against *S. aureus* biofilm formation, were submitted to LC–UV–MS analysis. F2 (1mg) and F4 (1mg) were dissolved in 1mL of MilliQ water and 20µL were injected on C18 column (Xbridge® - 10mm×100mm, 5µm). The solvent system used for sub-fraction F2 was a gradient of formic acid (0.01%) (A) and acetonitrile (B), starting with 8.5% acetonitrile and installing a gradient to obtain 28% B at 11min, at a solvent flow rate of 1 ml/min. For sub-fraction F3 analyses was used the same solvent system with changes in elution gradient, starting with 12% B and installing a gradient to obtain 25% B at 11min, at a solvent flow rate of 1 ml/min.

Statistical analysis

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

RESULTS

Effect of *L. ferrea* extracts on inhibition biofilm formation

Staphylococcus aureus was proven to create strongly biofilm with OD value of 0.18 in extract free conditions, and with the presence of extracts, the possibility of adherence was changed. The biofilm formation and bacterial growth of *S. aureus* in the presence of *L. ferrea* extracts/fractions are demonstrated in **Table 1**. LFAQ at concentrations 2.5 to 0.625 mg/mL did not reveal significant antibiofilm activity. Extract of fruits induced inhibition of biofilm formation (≥ 50%) against examined strain by up to 71.1% and 55.3% at concentrations 2.5mg/mL and 1.25 mg/mL, respectively. Both extracts did not affect planktonic bacterial growth. Sub-fractions obtained from LFRAQ fractionation were tested against antiadhesion of *S. aureus* at concentration 2.5mg/mL, best inhibition concentration for crude extract. In total, two of eight

sub-fractions could inhibit cell attachment of *S. aureus* in 52.7 and 76.3% for F2 and F4, respectively.

Fractionation of *L. ferrea* fruit extract

The crude aqueous extract of *L. ferrea* fruits shoot that showed a high inhibitory activity against *S. aureus* biofilm formation. A chromatographic profile by analitical HPLC corresponding to the fruit extract is presented in Figure 1. Four main peaks were observed. The retention time of the main peaks was as follows: P1 = 1.86 min, P2 = 4.59 min P3 = 5.56 and P4 = 8.50. All peaks were not identified when compared with the standards used.

Subsequently, LFRAQ was fractionated by preparative HPLC, allowed a good separation of a large number of sample components, resulting in eight sub-fractions. Among this sub-fractions, F2 and F4 exhibited better antibiofilm activity and were analyzed by HPLC-DAD-MS to investigate their chemical compounds. The compounds were identified by comparison of retention time and UV spectra with the data obtained for standard solutions analyzed under the same analytical conditions. HPLC–DAD–MS data of the sub-fractions are shown in Table 2. Through the UV absorption spectra could not confirm the identity of peaks obtained in the analysis this fractions.

DISCUSSION

The bacteria ability to form biofilms are involved in major problems associated with infections. The risk becomes even more serious because bacteria within biofilms have been shown to have a decreased susceptibility to antimicrobial agents compared with those in the planktonic form (Babapour et al., 2016).

Inhibition of biofilm growth of bacteria tested was successful for extract and fractions from *L. ferea* fruits. The success of this plant in inhibiting bacterial biofilm formation has been documented. Trentin et al (2011) evaluated the antibiofilm activity against *S. epidermidis* from Brazilian semi-arid plants. Among the species studied, *L. ferrea* was able to inhibit biofilm formation significantly. Inhibition of *L. ferrea* biofilm formation may be related to high content phenolic compounds. Inhibitory effects of polyphenol on biofilm formation has been reported (Matsunaga et al, 2010). Gallic acid is a well-known bioactive compound with several activities, namely, antistaphylococcal and anti-biofilm ((Luís et al., 2014).

This results showed that biofilm formation of *S. aureus* was inhibited but not demonstrated low microbial growth. When bacterial adhesion and biofilm formation occurs by a pathway that does not involve bacterial death is a remarkable characteristic of a new concept in antivirulence therapies. Importantly, it explores new mechanisms of action that may difficult the rapid development of bacterial resistance. This alternative pathway makes pathogen microorganisms more susceptible to other antimicrobials and to the immune system (Martin et al., 2008; Römling and Balsalobre, 2012).

CONCLUSION

In the present study, *L. ferrea* fruit extract showed *in vitro* antibiofilm activity by unknown mechanisms. To our knowledge, this is the first time the antibiofilm activity of *L. ferrea* fruits has been reported against biofilm formed by *S. aureus*.

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Table 1. Effect of *L. ferrea* extracts /fractions on *S.aureus* biofilm formation and bacterial growth.

	2.5 mg/mL		1.25 mg/mL		0.625 mg/mL	
	Biofilm formation (%)	**Bacterial growth (%)**	**Biofilm formation (%)**	**Bacterial growth (%)**	**Biofilm formation (%)**	**Bacterial growth (%)**
LFAQ	53.6 ± 4.0	262.7 ± 16.9	62.2 ± 4.0	190.4 ±24.3	65.6 ± 9.3	229.2 ± 8.4
LFRAQ	28.9 ± 3.4	248.9 ± 19.4	44.7± 7.8	182.5 ±15.1	80.2 ± 1.2	187.9 ± 8.4
F1	58.1 ± 3.3	103.5 ± 16.1	-	-	-	-
F2	47.3 ± 3.6	318.5 ± 11.3	-	-	-	-
F3	85.7 ± 6.9	118.7 ± 6.7	-	-	-	-
F4	23.7 ± 3.7	123.1 ± 8.9	-	-	-	-
F5	61.3 ± 2.0	156.4 ± 7.7	-	-	-	-
F6	60.0 ± 4.1	127.3 ± 6.8	-	-	-	-
F7	48.8 ± 2.2	123.6 ± 13.1	-	-	-	-
F8	88.2 ± 5.1	127.8 ± 11.5	-	-	-	-

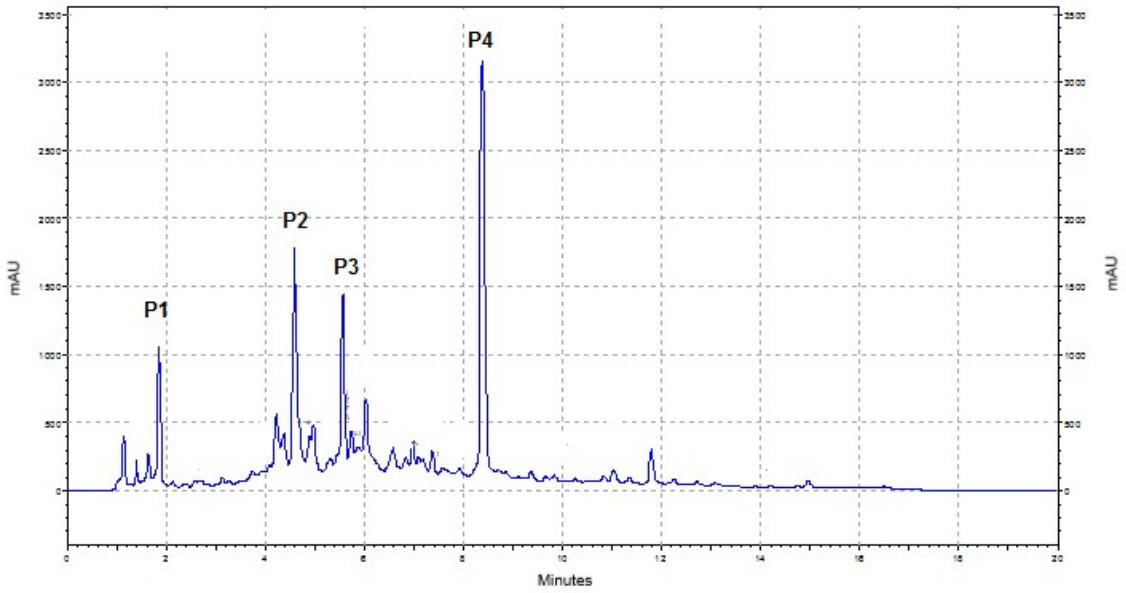
LFAQ: leaves extract of *L. ferrea*; LFRAQ: fruit extract of *Libidibia ferrea var. ferrea*; F1-F8: sub-fractions from fruit extract of *L. ferrea* . Results represent mean ± standard deviation of 3 experiments.

Table 2. Peak identification of sub-fractions F2 and F4 analyzed by HPLC-DAD-MS.

Peak	λ (nm)	RT (min)	$[M-H]^+$ (m/z)	Compound
F2				
P1	225	2.63	633	n.i
P2	225	5.56	465	n.i
F4				
P1	225	2.74	801	n.i
P2	225	3.79	951	n.i
P3	225	6.09	783	n.i

RT: retention time; n.i. not identified.

Figure 1. Chromatographic profiles of the *L.ferrea* fruit extract at 365 nm.



4.3 ARTIGO 3 - ANTIOXIDANT AND PROTECTIVE EFFECTS OF *Libidibia ferrea* var*. ferrea* LEAF

Antioxidant and protective effects of *Libidibia ferrea* var*. ferrea* leaf

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ABSTRACT

Background: Oxidative stress is related to many diseases as a result of accumulation of free radicals in the human body. A lot of researches are going on worldwide directed towards finding natural antioxidants of plants origins including brazilian caatinga plants. The aims of this study were to evaluate antioxidant and biological activities of phytochemical constituents from *Libidibia ferrea* var. *ferrea* leaf's extract**.**

Methods: Aqueous extracts of leaves (LFAQ) was analysed by TLC and high performance liquid chromatography (HPLC). Antioxidant activity was evaluated by DPPH free radical scavenging method, inhibition erythrocyte hemolysis mediated by peroxyl free radicals, radioprotection and DNA protection assays. In addition, toxicity test on *Artemia salina* larvae and *Biomphalaria glabrata* embryos were done.

Results: Phytochemical analysis showed for the first time ellagic acid as leaf's major compoud and the average total phenolic contents (TPC) and Flavonoids of extract were 482 ± 3 mg of GAE/g and 84.2 ± 0.19 mg of QE/g respectively, LFAQ showed significant DPPH radical scavenging (84.1%), and was capable of inhibiting erythrocytes haemolysis up to 88%. The extract was also found to be effective in protecting plasmid DNA against the strand breakage induced by hydroxyl radicals and protective effects against gamma radiation was also observed with low toxicity.

Conclusions: The results suggested that *L. ferrea* leaves could be considered as a new potential source of natural phenolic antioxidants for pharmaceutical, cosmetics or nutraceutical industries.

Introduction

Oxidative stress is defined as lack of balance between the occurrence of reactive oxygen or nitrogen species (ROS/RNS) and the organism's capacity to counteract their action by the antioxidative protection systems, leading to DNA damage and macromolecules associated with the grow and development of many humans diseases like cardiovascular diseases, neurological degenerations (Parkinson, Alzheimer, Huntington's disease and amyotrophic lateral sclerosis), and cancer, as well as the normal ageing processes. (Tiwari et al., 2001; Brown et al., 2010).

ROS are generated by endogenous or exogenous agents. The exogenous source of free radical is from the environment. These include aerobic processes such as cellular respiration, exposure to microbial infections involving phagocyte activation, during intensive physical activity or the action of pollutants/toxins (cigarette smoke, air poluttion), ionizing radiations (from industry, sun exposure, radiotherapy) and chemicals (pesticides) (Kregel and Zhang, 2007). The radiation therapy of cancer generates reactive oxygen species. Cell damage occurs by direct ionization of DNA and other cellular targets and by indirect effects mediated by ROS generated during radiation-induced water hydrolysis. Appropriate antioxidant intervention seems to inhibit or reduce free radical toxicity and thus offers protection against radiation (Yang et al, 2013).

Plants represent one of the richest sources of antioxidant compounds such as phenolics, vitamins, and alkaloids, which may be used as pharmacologically active products to balance the physiological antioxidant/prooxidant status (Jiménez-Zamora et al. , 2016). This compounds play an important role in adsorbing and scavenging free-radicals, quenching oxygen and decomposing peroxides (Port's et al., 2013). They transfer an electron to the free radicals, which thus become stable as their electrons are paired. This prevents damage to cells and tissue caused by oxidant stress (Kaisoon et. al., 2011). Synthetic antioxidants have been suspected of being responsible for liver damage and carcinogenesis. Therefore, there is a growing interest in natural and safer antioxidants.

Although it has already been demonstrated that *Libidibia ferrea* var. *ferrea* fruits contain phenolic compounds (Silva et al., 2011), little is known about the antioxidant potential about other tissues, such as leaves. The present study was designed to investigate the chemical composition of *L. ferrea* leaf and antioxidant

activity. Furthermore, hemolysis assay and radioprotective effect were used as a model of oxidative stress for evaluation of protective effect from free radicals.

Methods

Plant material

Leaves of *L. ferrea* var. *ferrea* were collected collected at Parque Nacional do Catimbau (PNC), a preservation area of Conservation Unit of the Caatinga ecosystem, Municipalities of Buíque, Ibimirim and Tupanatinga, Pernambuco State, Brazil., at non-raining season. Botanical identification was made from Herbarium of Instituto de Pesquisa Agronômica de Pernambuco, (Agronomic Institut of Pernambuco State), Brazil. Voucher specimen (IPA 84.035) was deposited at the herbarium IPA.

Preparation of extract

Aqueous extract of *Libidibia ferrea* var*. ferrea* leaves (LFAQ) 20% (w/v) was prepared in saline solution by agitation at 300 rpm for 12h at room temperature. After this period the mixture was centrifuged (12.000 x g) for 10 min, and the supernatant was collected and lyophilized.

Phytochemical screening

Qualitative determination of the chemical Constituents

LFAQ was subjected to phytochemical screening to determine the presence of different classes of natural products using methods described by Wagner and Bladt (2001). The compounds classes were visualized as aid thin layer chromatography (TLC) on silica gel 60 F254 (Merck, Germany), different systems of development and adequate visualization techniques were used: Dragendorff, NEU-PEG, KOH-Ethanol, Liebermann-Burchard and vanillinsulfuric acid.

Estimation of total phenolic content

Total phenolic content (TPC) was measured by Folin–Ciocalteu assay (McDonald et al., 2001). In brief, Folin reagent in the volume of 1,5 mL (1:10 diluted with H_2O) was mixed with 50 µL (1mg. mL^{-1}) of LFAQ and finally aqueous sodium carbonate (Na_2CO_3,1M) 2,5 mL was added to this reaction mixture. This reaction mixture was incubated at room temperature for 20 min. Absorbance was recorded at 735 nm. Gallic acid was prepared in methanol and used as standard. Total phenolic content was expressed in terms of gallic acid equivalent (mg of GAE/g).

Estimation of total flavonoid content

Flavonoid contents in the extract were determined by a colorimetric method described by Jia, Tang, and Wu (1999) with some modifications. The aqueous extract (250 µl) was mixed with 1.25 ml of distilled water and 75 µl of a 5% $NaNO_2$ solution. After 5 min, 150 µl of 10% $AlCl_3$ H2O solution was added. After 6 min, 500 µl of 1 M NaOH and 275 µl of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. Total flavonoids contents were expressed as mg quercetin equivalents per gram dry weight of extract (mg EQ/g extract).

High performance liquid chromatography (HPLC)

Extract (10mg) was redissolved in 1mL of methanol:MilliQ water (1:1) and 10µL were analyzed using an analytical HPLC (Agilent) and a C18 Zorbax SB column (4.6 × 250 mm). The solvent system used was a gradient of formic acid (0.3%) (A) and acetonitrile (B), starting with 8% acetonitrile and installing a gradient to obtain 35% B at 15min, at a solvent flow rate of 2.4 ml/min. Detection was achieved with a UV detector (Agilent 1100 series). Spectral data from all peaks were accumulated in the range 200–400 nm, and chromatograms were recorded at 360 nm. The compounds in each sample were identified by comparing their retention times and UV–Vis spectra in the 200–400 nm range with chromatograms relative to Standards (cafeic acid, catechin, chlorogenic acid, coumaric acid, ellagic acid, ferrulic acid, gallic acid, quercetin and rutin).

DPPH radical-scavenging activity

The free radical scavenging activity of the extract, and positive control (quercetin) was investigated using 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging method (Vongsak et al., 2013). A total of 25 µl of the *L. ferrea* extract (100µg/mL) or standards gallic acid and quercetin (100µg/mL) was added to 240 µl of DPPH in methanol solution (152 µM). After incubation at 37 ◦C for 20 min, the absorbance of each solution was determined at 517 nm using spectrophotometer for microplates Epoch™. The corresponding blank readings were also taken and percent inhibition was then calculated as follows:

$$Inhibition(\%) = \frac{\mathrm{A1} - \mathrm{A2}}{\mathrm{A1}} \text{ x } 100$$

, where A1 is the absorbance of control, and A2 is the absorbance of samples.

Artemia salina bioassay

Artemia salina eggs (25 mg) were hatched in natural seawater (pH 8.0) at 25-30°C. The eggs were placed in one of two compartments of a container separated by a boundary plate. The compartment with the eggs was covered to maintain a dark ambience. The other compartment was illuminated to attract the phototropic newlyhatched larvae through perforations on the boundary plate. After 24 h, the brine shrimps that had moved to the illuminated compartment were collected using a Pasteur pipette and used in the lethality assay according to procedures described by Meyer et al. (1982). Groups of 12–15 larvae were exposed to extract (12.5 - 400 ppm) diluted in natural seawater and, after 24 h, the survival rates (%) were recorded. In the control group, larvae were incubated in seawater. The assay is considered valid if the mortality percentage of the controls does not exceed 10%.

Embryotoxicity by Biomphalaria glabrata

The embryotoxicity assay was performed on *B. glabrata* according to the method described by Oliveira-Filho and Paumgartten (2000). In each assay, egg masses containing approximately 100 embryos in the blastula stage were placed into Petri dishes (90 x 15 mm) and exposed to aqueous extract (125 – 1000ppm) or filtered tap water (negative control) for 24 h. Next, the egg masses were washed with tap water and placed in Petri plates containing filtered and dechlorinated water. The plates were maintained at 25±2°C for 8 during which mortality was evaluated.

Radioprotective assay

Radioprotection was performed as previously described (Siqueira et al., 2014). *Biomphalaria glabrata* embryos were submitted to doses of 2.5, 4 and 5 Gy of 60Co. After irradiation, the embryos were exposed for 24 hours to aqueous extract of *L. ferrea* (125ppm). After incubation period, embryos were observed for 8 consecutive days and mortality was used to evaluate the damage induced by gamma radiation.

Inhibition of erythrocyte hemolysis mediated by peroxyl free radicals

Fresh blood was obtained from normal volunteers via venipuncture and collected in heparinized tubes. Erythrocytes were isolated by centrifugation at 3000g for 10min and washed four times with PBS, at pH 7.4 (containing 137mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4), and then resuspended using the same buffer to a 10% erythrocytes suspension. Subsequently, 100µL of 10% erythrocytes suspension was preincubated with 50 µL extract (250, 500, 1000 µg/mL) or PBS at 37°C for 30min. Then, 100 µL of 200 mM AAPH [2,2'-Azobis (2-

amidinopropane) dihydrochloride] in PBS was added, and the mixture was incubated at 37°C for 1 h. AAPH-untreated erythrocytes incubated in PBS were used as the control group. Batches of the reaction mixture were incubated at 37°C for 1h. After incubation, 200 µL of the reaction mixture was diluted with 1 ml of PBS and centrifuged at 3000g for 10min. The absorbance at 540 nm of the supernatant (A_{PBS}) was measured by the Epoch Microplate Spectrophotometer. The same volume of the reaction mixture was treated with 1 ml of distilled water to obtain a complete hemolysis. The absorbance of its supernatant (A_{WATER}) was measured under the same condition. The hemolysis percentage was calculated as follows:

DNA nicking assay

The DNA nicking assay was performed using pBR 322 plasmid DNA. The reaction mixture contained 0.3 µL of plasmid DNA, 10 µL of Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid, and 80 mM Fecl3) followed by the addition of aqueous extract (50 and 100 µg/mL). The final volume of the mixture was brought up to 20 µL using distilled water. The mixture was then incubated for 30 min at 37°C and the DNA was analyzed on 1% agarose gel in TBE Buffer (Kaur et al., 2008).

Statistical analysis

Each experiment was performed at least three times and results are presented as the mean ± SD. Statistical analysis was performed by ANOVA. Differences were considered significant at $p < 0.05$.

Results

Phytochemical analysis

The phytochemical analysis conducted on *L. ferrea* extract indicated the existence of cinnamic derivatives, flavonoids and saponins. The total phenolic content (TPC) and total flavonoid content (TFC) of extract were 482 ± 3 mg of GAE/g and 84.2 ± 0.19 mg of QE/g respectively.

The HPLC analysis of extract revealed the presence of four main peaks P1, P2, P3 and P4 which showed good chromatography separation and resolution. The retention time of the main peaks was as follows: P1 = 1.38 min, P2 = 1.83 min P3 = 6.04 and P4 = 8.39 (**Figure 1**). The major compound was ellagic acid, identified according to comparison of retention time with standard.

Antioxidant capacity

In the present study *L. ferrea* extract exhibited high antioxidant activity, compared to standards, shows in Figure 2. DPPH radical scavenging activity of LFAQ at 100µg/mL was found to be 84.1%. On the other hand, gallic acid and quercetin exhibited 89.3% and 88.5% DPPH radical scavenging activity at this same concentration, respectively. These results exhibited that the DPPH radical scavenging activity of extract was similar to standards.

Toxicity to *Artemia salina* and *Biomphalaria glabrata* embryo

The *Artemia salina* mortality results in the presence of *L. ferrea* extract in different concentrations shown in Figure 3. It was observed that the survival rate of larvae was not significantly ($p > 0.05$) affected at the tested concentrations. The Figure 4 shows data from the toxicity assay on *B. glabrata* embryos. LFQA induced a concentration-dependent increase in *Biomphalaria glabrata* embryo deaths. The extract showed low embryotoxicity at concentrations 125, 250 and 500 ppm. In concentration 1000 ppm, extract showed high Embryo-mortality.

Radioprotective effect

The exposure of *B. glabrata* embryos, previously incubated with *L. ferrea* extract (125ppm), to gamma radiation showed radioprotective capacity of the plant investigated. Radioprotective assay at doses of 2.5, 4.0 and 5 Gy showed that the extract compared to controls exhibited a significant radioprotective potential for significantly reduced the number of dead embryos in all tested radiation doses (Figure 5).

Protective effects on AAPH-induced hemolysis in erythrocytes

Normal human erythrocytes were used to investigate the capability of the *L. ferrea* extract to protect erythrocytes against oxidative damage in vitro induced by free radical AAPH. The Figure 6 shows the effects of aqueous extract (250–1000µg/ml) on erythrocytes exposed to the water-soluble radical initiator AAPH. Anti-hemolytic effect of LFAQ was obeserved at concentrations of 250µg/ml, 500µg/ml and 1000µg/ml that were able to inhibit 28%, 85% and 88 % hemolysis, respectively.

DNA Protection assay for oxidative stress

Genoprotective effects of plant extracts were evaluated qualitatively on supercoiled pBR322 DNA plasmid. In this assay the antioxidant activity was measured by the degree of protection of DNA from devastating effects of hydroxyl radicals generated by Fenton's reagent, this damage is related to the conversion of

the supercoiled form of this plasmid DNA to the open circular and further linear forms (Jung and Surh, 2001). Overall electrophoretic pattern was consistent with the protection supercoiled DNA form in the presence of extract at 100 µg/mL but the DNA protection was less clear following treatment at concentration 50 µg/mL (Figure 7).

Discussion

The phytochemical analysis revealed the presence of phenolic compounds in *L. ferrea* leaves. Many reports have indicated that fruit and bark preparations of this plant are rich in phenolic acids and flavonoids (Nakamura et al., 2002; Silva et al., 2011; Vasconcelos et al., 2011). Previous study reported the presence of gallic acid and quercetin in methanollic extract of leaves (Port's et.al., 2013). The first time ellagic acid was reports as major component in leaves of *L. ferrea.* Vasconcelos et al. (2011) observed the presence of ellagic acid in bark extracts of *L. ferrea* with hypoglycaemic activity in wistar rats. Ellagic acid is a phenolic constituent in several plants and it was documented that possesses antibacterial, antioxidant, antiviral, antifibrotic, antiatherogenic, hypoglycemic, antimutagenic and anticancer activities (Borges et al., 2014; Kilic et al.,2014).

Secondary metabolites especially phenolic compounds in plants form the main source of natural antioxidants. The result of DPPH scavenging activity assay in this study indicates that the plant was potently active. The scavenging ability against DPPH radicals was high even at low extract concentrations, this is related with high phenolic content of leaf extract. Silva et al (2011) reported strong antioxidant activity of *L. ferrea* fruits extract evaluated by *in vitro* assays. The possible mechanism of reduction of the DPPH can be correlated to the available hydroxyl groups in the reported phenols (Vijayalaxmi et.al, 2015).

Polyphenols as ellagic acid suggests the capture of free radical preventing oxidative damage of erythrocytes. Several studies with plants showed inhibition of erythrocytes hemolysis induced by reactive oxygen species (Mendes et al, 2011; James and Alewo, 2014; Bouhlali et al, 2016). Erythrocytes are susceptible to oxidative damage due to their high contents of polyunsaturated fatty acids in their membranes, and high concentrations of cellular oxygen and iron-rich hemoglobin (Scott et al., 1993). Although this cells have physiological antioxidant defense system, if reactive oxygen species were not adequately removed or formed additionally in the cells by exogenous sources, oxidative stress may occur.

In addition to erythrocyte hemolysis protection, LFAQ was able to decrease the mortality rate of *B. glabrata* embryos irradiated in non toxic concentrations. Ionizing radiation is known to induce oxidative stress through generation of ROS resulting in an imbalance in prooxidant, antioxidant status in the cells (Jagetia and Reddy, 2005). Therefore, we suggest that antioxidants from *L. ferrrea* extract have an important preventive effect to minimize the toxic effects of radiation-induced oxidative stress. Bhosle et.al. (2005) found that combined treatment of ellagic acid and gamma radiation against tumor cells in rats was effective and also noted that phenolic compound was able to protect normal cells from oxidative stress caused by radiation. Santos et al. (2013) reported that methanolic extract from bark of *Caesalpinia pyramidalis*, plant also found in the Caatinga region, showed radioprotective effect in the concentration of 250 ppm compared to doses of 2.5 and 4 Gy of ^{60}Co.

Our results have also shown that LFAQ was an active scavenger of hydroxyl radicals, such that DNA nicking was significantly prevented by the presence of extract. Hydroxyl radicals generated by the Fenton reaction are known to cause oxidatively induced breaks in DNA strands to yield its fragmented forms (Thomas et.al., 2009). In previous studies *L. ferrea* fruits hydroalcoholic extract had capacity to break the DNA damage induced by hydroxyl radicals (Silva et al., 2011).

Conclusions

In conclusion the results of present study affirms the *in vitro* antioxidant potential of leaf extract of *Libidibia ferrea* var. *ferrea* and also exhibited the protective effects against damage by hydroxyl radicals in erythrocytes, irradiated embryos and DNA. Further studies are needed to clarify the mechanisms of these protective effects.

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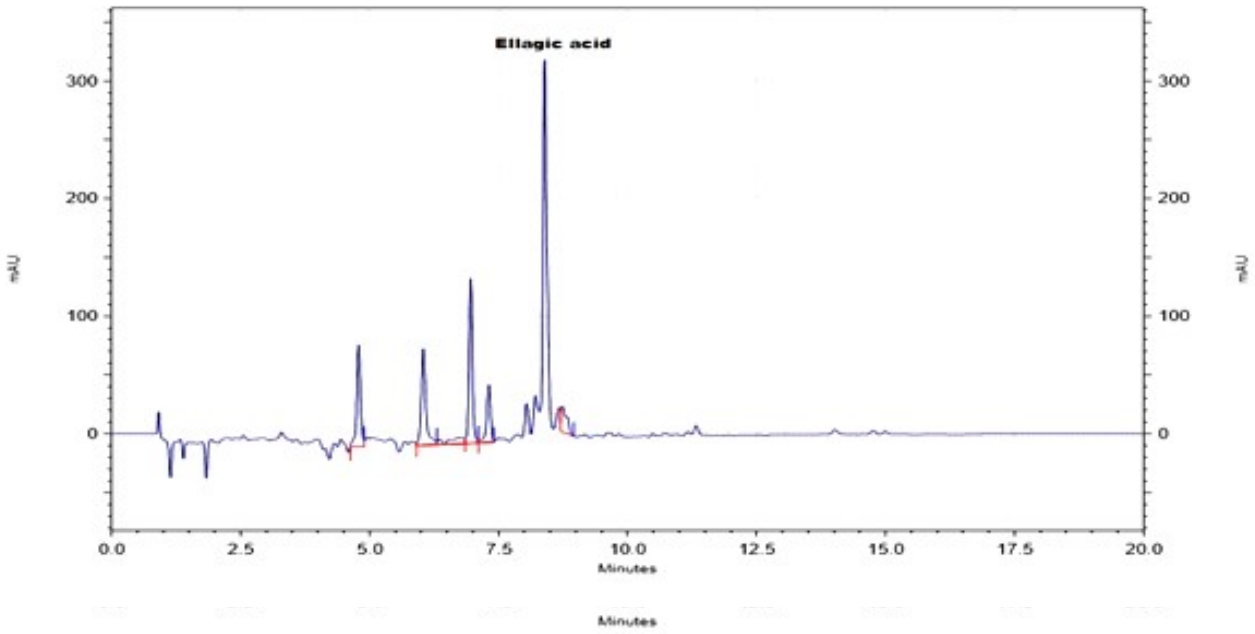

Figure 1. HPLC chromatogram at 365 nm of *Libidibia ferrea* var. *ferrea* leaves extract.

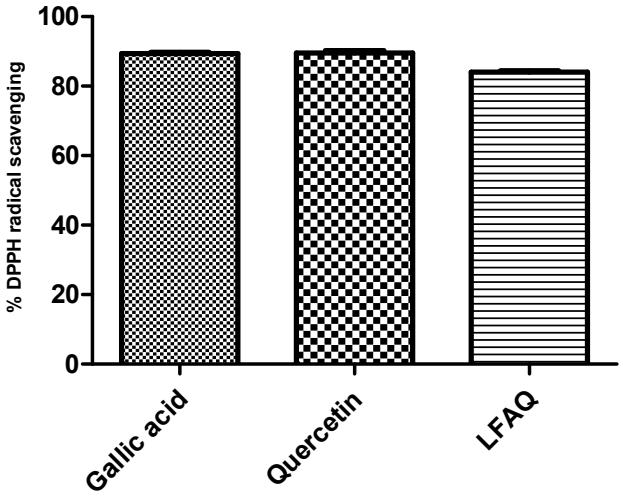

Figure 2. DPPH radical scavenging assay. DPPH radical scavenging activity of leaf extract of *Libidibia ferrea* and the references compounds quercetin and gallic acid. The data represent the percentage of inhibition of 1,1-diphenyl-2-picryl hydrazyl radical (DPPH). The results are mean ± S.D.

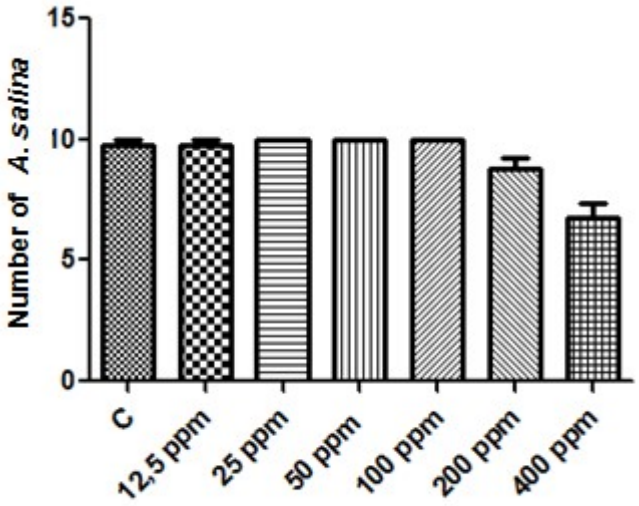

Figure 3. The effects of *Libidibia ferrea* Leaf extract on survival of *Artemia salina* exposed to the aqueous extract at concentrations of 12.5 to 400 ppm. Control(C).

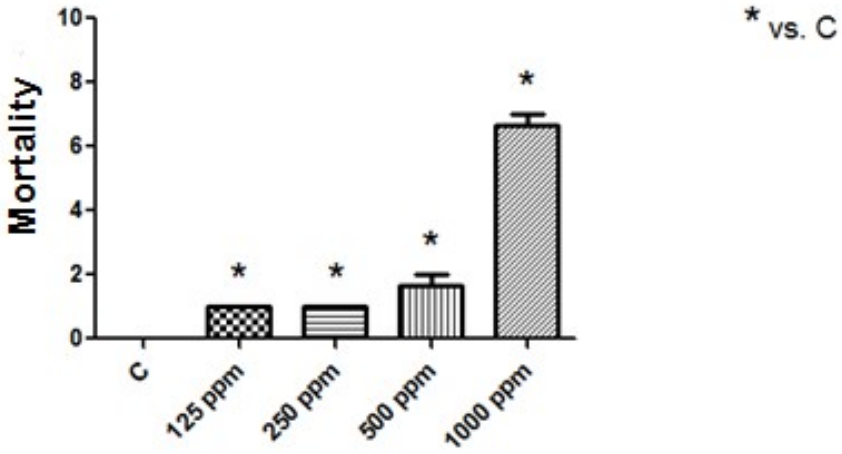

Figure 4. *Biomphalaria glabrata* embryos exposed to the aqueous extract at concentrations of 125, 250, 500 and 1000 ppm. Control(C).

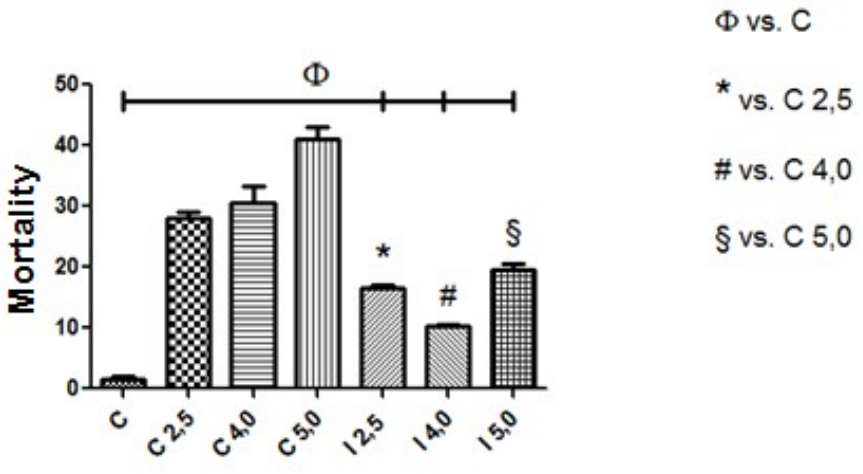

Figure 5. Radioprotective assay of *Libidibia ferrea* leaf aqueous extract. Embryos exposed to ionizing radiation at dose of 2.5, 4 and 5.0 Gy in the presence of *Libidibia ferrea* leaf aqueous extract. Control (C), irradiated control 2.5 Gy (2.5 C), irradiated control to 4.0 Gy (C 4.0), irradiated control to 5.0 Gy (C 5.0). Leaf Extract (I), extract irradiated sheet to 2.5 Gy (I2.5), irradiated leaf extract to 4.0 Gy (f 4.0), irradiated leaf extract to 5.0 Gy (I 5.0).

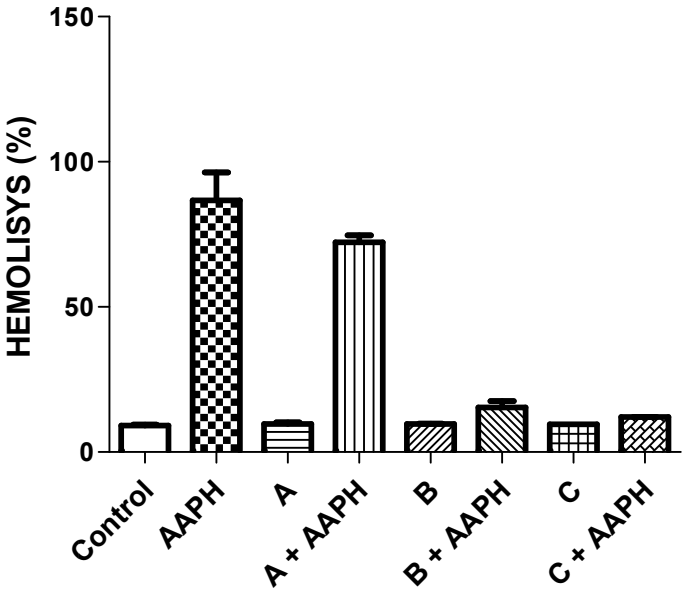

Figure6. Protective effects of *Libidibia ferrea* aqueous extract at different concentrations against AAPH-induced hemolysis in human erythrocytes. Erythrocytes suspension pre-incubated with PBS or extract was incubated with AAPH for 1 h. A:LFAQ (250 µg/mL); B: LFAQ (500 µg/mL); C: LFAQ (1000 µg/mL).

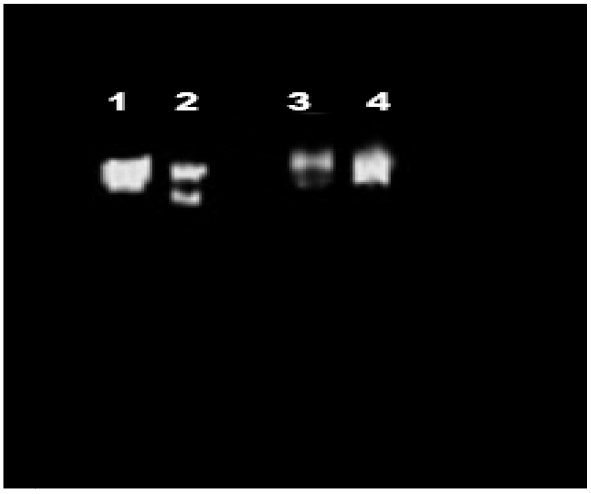

Figure 7. Protective effects of different extracts in DNA nicking assay. (A) Lane 1: negative control (distilled water + DNA), Lane 2: control (DNA + Fenton's reagent), Lane 3: LFAQ (50µg/mL) + Fenton's reagent, Lane 4: LFAQ (100µg /mL) + Fenton's reagent.

5 CONCLUSÕES

Concluímos que os extratos orgânicos de folhas de *Libidibia ferrea* contêm alcalóides, derivados cinâmicos, flavonóides, taninos e terpenos e demosntraram significativa atividade antibacteriana, com efeito bactericida, apresentando maior efeito frente às bactérias gram-positivas.

Entre os exatratos testados, o ciclohexânico de folhas apresentou maior atividade antibacteriana quando comparado com os demais extratos, sendo mais eficaz contra *Staphylococcus aureus*. No entanto, suas frações, constituídas de ácidos graxos, alcanos, esteróides e terpenos, exibiram atividade antibacteriana reduzida, sugerindo sinergismo entre as substâncias presentes.

O extrato bruto aquoso dos frutos de *L. ferrea* mostrou potencial atividade antibiofilme, pois foi capaz de inibir a formação *in vitro* de biofilme de *S. aureus*, importante patógeno humano associado com infecções hospitalares;

Foi observado ainda um alto teor de compostos fenólicos no extrato bruto aquoso das folhas de pau-ferro, tendo como componente majoritário o ácido elágico. O mesmo extrato mostrou ainda significativa atividade antioxidante em baixas concentrações, não foi tóxico para *Artemia salina* e *Biomphalaria glabrata*, e foi ainda capaz de proteger eritrócitos e embriões de B. *glabrata* do estresse oxidativo induzido pelo AAPH e radiação gama, respectivamente.

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ANEXO A - SIMPLE, MULTICOMPONENT, ECOFRIENDLY, MICROWAVE-MEDIATED ROUTE FOR THE SYNTHESIS OF ANTIMICROBIAL 2-AMINO-6-ARYL-4-(3H)-PYRIMIDONES

Original Articles

Simple, Multicomponent, Ecofriendly, Microwave-Mediated Route for the Synthesis of Antimicrobial 2-Amino-6-aryl-4-(3*H*)-pyrimidinones

DOI: 10.1080/00397911.2013.807517

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pages 374-380

Publishing models and article dates explained

Received: 15 Apr 2013
Published online: 20 Nov 2013

Abstract

In this work, we describe a multicomponent microwave-mediated synthesis of 10 2-amino-6-aryl-4-oxo-1,6-dihydro-pyrimidine-5-carbonitriles in good chemical yields (44–67%), four of them not related in earlier literature. All pyrimidinones synthesized herein had their antimicrobial activity evaluated against the following microorganisms: *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Two of the synthesized substances displayed good antimicrobial activity against *P. aeruginosa* and *S. aureus*, two bacteria responsible for nosocomial infections.