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ALINE DE PAULA CAETANO PEREIRA

CARACTERIZAÇÃO QUÍMICA E POTENCIAL BIOLÓGICO DE METABOLITOS SECUNDÁRIOS DE *Commiphora leptophloeos* (Mart.) J.B.Gillett

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como pré-requisito para obtenção do título de Doutor em Ciências Biológicas, área de concentração em Biotecnologia.

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RESUMO

Commiphora leptophloeos, popularmente conhecidas como Imburana de Sertão pertence à família *Burseraceae* e é tradicionalmente usada por tribos indígenas brasileiras para o tratamento de suas enfermidades. O presente trabalho objetivou analisar o perfil fitoquímico,isolamento de substância e as atividades: antioxidante e antimicrobiana dos extratos com diferentes polaridades de cascas de *C. leptophloeos*. Os compostos fenólicos foram identificados por HPLC e encontrados: os ácidos gálico, clorogênicos e protocatecóico. O H1NMR revelou a presença de hinoquinina, uma lignana bioativa caracterizada pela primeira vez nesta especie. No que se refere a atividade antimicrobiana, os valores de MIC de hinoquinina variou 0,0485-3,125 mg / mL em diferentes isolados clínicos de *S. aureus*, e mostraram uma atividade bactericida contra o MRSA isolados a partir de sangue (MMC 0,40 mg / mL) e secreção de pós-operatório (MMC = 3,125 mg / mL). Os extratos mostraram atividade antimicrobiana contra espécies *de Mycobacterium*, tais como *M. smegmat*is (CIM = 12,5 mg / mL) e de *M. tuberculosis* MIC (= 52 mg / mL). A toxicidade dos extratos também foi avaliada por testes in vitro com HC50 de 313 ± 0,5 ug / ml., Foi realizada a caracterização fitoquímica, mostrando compostos fenólicos dotados de alta atividade antioxidante em cinco extratos, como aquosa (CLAQE), metanol (CLMEE) clorofórmio (CLCLE), ciclo-hexano (CLCHE) e acetato de etilo (CLAEE). revelando a presença de taninos, cumarinas, flavonóides e açúcares redutores. CLAQE apresentou os maiores teores de compostos fenólicos com 33,64 ± 0,5 mg GAE / g e flavonóides com 19,3 ± 0,70 mg QE / g para os teores de taninos CLCLE mostrou 9,70 ± 0,2 mg TAE / g, por cumarinas CLCHE conteúdo mostrou 1,14 ± 0,81 mg BZE / g e de açúcares solúveis CLMEE apresentada 53,30 g.100g-1mg. A capacidade antioxidante total (TAC%) todos os extratos mostraram uma actividade antioxidante significativa para ambas as concentrações de 1-10 mg / ml (≥100%, valor de p <0,001). Todos os extratos induziram baixos níveis de morte celular (viabilidade ≥ 80%) por período de 24 horas. Além disso, estes valores citotóxicos não foram correlacionadas com a fenóis (r = -0,15) ou flavonóides (r = -0,20). Portanto presentes resultados mostraram que *C. leptophloeos* possui alto potencial antioxidante bem como propriedades inibitórias contra a MRSA, bem como vários Gram-positivos, Gram-negativos e microorganismos fungos, Mycobacterium.

Palavras-chave: Imburana, produtos naturais, estresse oxidativo, fitoquímica

ABSTRACT

Commiphora leptophloeos, usually known as Imburana of Sertão belongs to the Burseraceae family and is traditionally used by Brazilian indigenous tribes for the treatment of their infirmities. Herein we performed full phytochemical characterization, showing its notable amounts of phenolics compounds endowed with high antioxidant activity in five extracts such as aqueous (CLAQE), methanolic (CLMEE) chloroform (CLCLE), cyclohexane (CLCHE) and ethyl acetate (CLAEE). *C. leptophloeos* phytochemical prospection revealed the presence of phenolic contents, tannins, coumarins, flavonoids and reducing sugars, secondary metabolites used by the plants for their defense. The qualitative phytochemical analysis by TLC of *C. leptophloeos* extracts detected the presence of phenolic compounds, flavonoids and reducing sugars. H^1NMR analysis revealed the presence of hinokinin, a bioactive lignan characterized in this article. The phenolic compounds were identified by HPLC and three were described: Gallic, Chlorogenic and Protocatechuic acids. CLAQE presented the major contents of phenolic compounds with 33.64 ± 0.5 mg GAE/g and flavonoids with 19.3 ± 0.70 mg QE/g, for tannins contents CLCLE showed 9.70 ± 0.2 mg TAE/g, for coumarins content CLCHE showed 1.14 ± 0.81 mg BZE/g and for soluble sugars CLMEE presented 53.30 $g.100g^{-1mg}$. In Total antioxidant capacity (%TAC) all extracts showed significant antioxidant activity for both concentrations of 1-10 mg/mL (≥100%, *p*-value <0.001). All the extracts induced low levels of cell death (viability ≥ 80%) within 24-hour period. In addition, cytotoxic results were not correlated with either phenols (r = -0.15) or flavonoids (r = -0.20). Altogether, we showed that the barks extracts from *C. leptophloeos* are antioxidants source being of potential use in preventing oxidative conditions. CLAQE presented the major contents of phenolic compounds with 33.64 ± 0.5 mg GAE/g and flavonoids with 19.3 ± 0.70 mg QE/g, for tannins contents CLCLE showed 9.70 ± 0.2 mg TAE/g, for coumarins content CLCHE showed 1.14 ± 0.81 mg BZE/g and for soluble sugars CLMEE presented 53.30 $g.100g^{-1mg}$. In Total antioxidant capacity (%TAC) all extracts showed a significant antioxidant activity for both concentrations of 1-10 mg/mL (≥100%, *p*-value <0.001). The present findings showed that *C. leptophloeos* possesses inhibitory properties against MRSA as well as various Gram-positives, Gram-negatives and Fungi microorganisms, and should also be investigated for its potential against Mycobacterium. Furthermore, we have identified for the first time the presence of hinokinin in Commiphora genus. The MIC values of hinokinin ranged from 0.0485 to 3.125 mg/mL in different *S. aureus* clinical isolates, and showed a bactericidal activity against MRSA isolated from blood (MMC 0.40 mg/mL) and postoperative secretion (MMC = 3.125 mg/mL). *C. leptophloeos* extracts showed antimicrobial activity against Mycobacterium species such as *M. smegmatis* (MIC = 12.5 mg/mL) and *M. tuberculosis* (MIC = 52 mg/mL). Herein we also determined *C. leptophloeos* toxicity by HC_{50} tests *in vitro* with hemolytic activity detected of 313±0.5 µg/mL. All the extracts induced low levels of cell death (viability ≥ 80%) by 24-hour period. In addition, these cytotoxic values were not correlated with either phenols (r = -0.15) or flavonoids (r = -0.20).

Key-words: imburana, natural products, oxidative stress, phytochemical

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1. INTRODUÇÃO

Os produtos naturais vêm recuperando espaço e importância na indústria farmacêutica, tanto na sua utilização direta quanto para uso como fonte inspiradora de novos padrões moleculares. Por isso, diversos estudos têm investigado a presença de compostos bioativos com potencial biológico em extratos vegetais, principalmente em plantas utilizadas pelas comunidades no combate a diversas patologias.

O bioma Caatinga possui grande variedade de espécies vegetais, sendo muitas delas endêmicas e adaptadas às condições de estresse ambiental característicos das regiões semiáridas. O estresse hídrico o qual essas espécies são submetidas levam a produção de uma série de metabolitos vegetais que culminam em moléculas de estruturas complexas e com grande diversidade de esqueletos e grupos químicos funcionais. Assim, estudos recentes apontam espécies vegetais da Caatinga como fontes promissoras de biomoléculas de que apresentam atividades biológicas importantes, e as atividades antioxidante e antimicrobiana podem ser destacadas entre elas

Dentre esses metabolitos destacam-se os compostos fenólicos, bastante presentes no nosso dia a dia, embora nem sempre nos demos conta disso. Muito do sabor, odor e coloração de diversos vegetais que apreciamos são gerados por esses compostos. Eles não são apenas atrativos para nós, mas também para outros animais, os quais são atraídos para polinização ou dispersão de sementes. Além disso, esse grupo de compostos é importante para proteger as plantas contra os raios UV, insetos e microorganismos como fungos e bactérias. Há inclusive certas espécies vegetais que desenvolveram compostos fenólicos para inibir o crescimento de outras plantas competidoras (ação alelopática). Além de sua importância na proteção das plantas contra fatores ambientais e bióticos adversos, esses compostos agem como aceptores de radicais livres, interrompendo a reação em cadeia provocada por estes, além de atuarem também nos processos oxidativos catalizados por metais, tanto *in vitro*, como *in vivo*

Os mecanismos de oxidação e redução são fundamentais para a sobrevivência das células, principalmente no que diz respeito à produção de energia e síntese de moléculas biológicas importantes. Porém, esses mecanismos geram radicais livres que, quando em excesso, podem acarretar sérios danos às células e o surgimento de diversas doenças, como câncer, envelhecimento precoce, doenças cardiovasculares e

neurodegenerativas, disfunções cognitivas, dentre outras. Diversos estudos têm investigado a presença de compostos químicos com potencial biológico em extratos vegetais, que podem ser responsáveis pelos efeitos preventivos apresentados pelas plantas contra várias doenças. Desse modo, os estudos envolvendo a propriedade antioxidante de compostos vegetais apresentam diversas perspectivas.

Os vegetais também desempenham um papel importante como fonte de compostos com atividade antimicrobianas, pois o uso irracional de antibióticos levou o o surgimento de microrganismos multirresistentes o que contribuiu para o aumento nas buscas e desenvolvimento de novos medicamentos antimicrobianos, encorajando os pesquisadores a procurar novas substâncias a partir de várias fontes, incluindo plantas medicinais.

Nesse contexto, *Commiphora leptophloeos* uma espécie exclusiva da caatinga, usada popularmente no tratamento de doenças do estômago, enjoo, tônico,cicatrizante, gastrite e úlcera, bronquites e inflamações do trato urinário, representa um excelente alvo para a busca de novas substâncias ativas, não havendo relatos de isolamentos e aplicações desses compostos nesta espécie.

Portanto este trabalho objetivou isolar, purificar, identificar, e aplicar biologicamente o metabólito secundário do extrato de casca de *Commiphora leptophloeos.*

2. REVISÃO DE LITERATURA

2.1 Potencial Biotecnológico de Plantas da caatinga

As plantas exercem um papel fundamental na sobrevivência das comunidades, desde os seus primórdios, sendo utilizadas para diversos fins, como: alimentícios, medicinais, ornamentais, místico/religiosas, madeireiras e para confecção de artesanatos. Conhecer as possibilidades de usos, locais de aquisição e as partes das plantas utilizadas, é muito importante para a conservação das espécies nativas popularmente utilizadas como medicinais. A forma de uso dos vegetais difere de acordo com as tradições ou região e o conhecimento sobre a utilização dos recursos vegetais vem sendo transmitido, de pai para filho, desde as antigas civilizações, demonstrando assim, a importância que cada comunidade dedica às espécies vegetais. Muitas vezes, essa comunidade só dispõe desses vegetais para sua alimentação como também para tratamentos de suas enfermidades. (Gomes et al. 2008, Diegues 1996; Guarim-Neto et al. 2000; Torres et al. 2009).

A busca por alívio e cura de doenças pela ingestão de ervas, talvez, tenha sido uma das primeiras formas de utilização dos produtos naturais (Veiga-Junior et al., 2005). Diversas plantas foram utilizadas pelos indígenas como remédio para suas doenças e como veneno em suas guerras e caças (Carvalho, 2004). Ainda hoje em muitas regiões do país e até mesmo nas grandes cidades brasileiras, plantas medicinais são comercializadas em feiras livres, mercados populares e encontradas em quintais residenciais.

O Brasil possui aproximadamente 35 mil espécies de vegetais, distribuídas nos diferentes tipos de biomas (Albernaz, 2010; Vieira et al.,2010), porém apenas 17% delas são exploradas para pesquisas de compostos biologicamente ativos (Moreira, 2013; Rodrigues et al., 2013). Essa grande biodiversidade da flora brasileira com potencial terapêutico leva a um significativo consumo de fitoterápicos e preparações extraídas das plantas.

Os principais passos para a utilização de um composto bioativo a partir de recursos vegetais são: a extração, a triagem farmacológica, o isolamento e caracterização desses compostos bem como a avaliação toxicológica e clínica. Esta última é essencial para garantir a eficácia de um composto bioativo, podendo fornecer

também, sua farmacocinética, biodisponibilidade, segurança e interações medicamentosas (Sasidharan et al., 2011).

Um levantamento bibliográfico considerando apenas o mecanismo de ação dos princípios ativos envolvidos, mostra a eficácia das plantas medicinais no tratamento da doença de Alzheimer (Mahdy et al., 2012; Orhan et al., 2012), de doenças cardíacas (Lang et al., 2012; Xing et al., 2012), Malária (Sisodia, et al., 2012; Mbeunkui et al., 2012), Leishmania (Hazra et al., 2012), Esquistossomose (Oliveira et al., 2012), Câncer (Malhotra et al., 2012; Park et al., 2012), Herpes (Astani et al., 2012), como antibiótico (MulaudzI et al., 2012), anti-inflamatório (Niu et al., 2012), , antioxidante (konrath et al., 2012), antinociceptivo (LIMA et al., 2012), antidiabético (Souza et al., 2012; Lima et al., 2012) e antidiurético (Gasparotto Junior et al., 2012).

A Organização Mundial da Saúde (OMS) já reconhece o uso da medicina popular e fitoterápica como pratica paralela a "medicina oficial " dominante sugerindo ser uma alternativa viável e importante também às populações dos países em desenvolvimento, devido ao baixo custo (Santos et al., 2012). Porém uma das grandes preocupações com o uso de plantas medicinais como forma de tratamento ou cura é o seu uso indiscriminado e sem comprovação científica. Conforme Vieira et al. (2010) o aumento do consumo de plantas medicinais nas últimas décadas foi devido a muitas pessoas acreditarem que esses produtos seriam "naturais", ou seja, não apresentariam "produtos químicos" passando a ser sinônimos de produtos saudáveis, seguros e benéficos à saúde. Mas grande parte das interpretações distorcidas sobre os efeitos deste tipo de medicamentos ocorre devido à difusão de informações errôneas por parte da população e, além disso, sem qualquer controle na maioria dos países. O aproveitamento adequado dos princípios ativos de uma planta exige o preparo correto, ou seja, para cada parte a ser usada, para cada grupo de princípio ativo a ser extraído e para cada doença a ser tratada, existe forma de preparo e uso adequados (Arnous; Santos; Beinner, 2005).

O Governo Federal aprovou a Política Nacional de Plantas Medicinais através do Decreto Presidencial Nº. 5.813, de 22 de junho de 2006, onde as ações decorrentes desta política são manifestadas no Programa Nacional de Plantas Medicinais e Fitoterápicos – PNPMF e uma de suas propostas é inserir plantas medicinais, fitoterápicos e serviços relacionados à fitoterapia no SUS, com segurança, eficácia e qualidade, em conformidade com as diretrizes da Política Nacional de Práticas Integrativas e

Complementares no SUS (BRASIL, 2006). Esses medicamentos devem ser extraídos de espécies cultivadas no Brasil não ameaçadas de extinção seguindo a recomendação da Organização Mundial de Saúde (OMS) de que os países devem usar os recursos naturais disponíveis no próprio território para promover a atenção primária à saúde e contribuir para o uso sustentável da biodiversidade nacional (BRASIL, 2013). Ratificando assim, a importância desses vegetais para fins terapêuticos.

Dessa forma, a caatinga configura uma importante região pois além de ser o único bioma exclusivamente brasileiro, localizada predominantemente na região Nordeste, abrangendo nove estados (Alagoas, Bahia, Ceará, Maranhão, Paraíba, Piauí, Pernambuco, Rio Grande do Norte e Sergipe) e pequena parte da região Sudeste (porção Norte do Estado de Minas Gerais) as espécies vegetais representantes desse ecossistema são amplamente utilizadas na medicina popular pela comunidade local.

Os estudos já realizados sobre a caatinga revelam que, além da importância biológica, este bioma encerra um considerável potencial econômico, com espécies de excelente uso como forragens, frutíferas e medicinais (ALBUQUERQUE, 2000). A variedade de plantas medicinais utilizadas para as mais diversas enfermidades torna a Caatinga ímpar no cenário dos fitoterápicos.

Dentre as Unidades de Conservação implementadas na Caatinga, há o Parque Nacional da Serra do Catimbau (PARNA Catimbau), localizado no Sertão Pernambucano, distribuído entre os municípios de Buíque, Ibimirim e Tupanatinga, com área de 607 km2. Criado em 13 de dezembro de 2002, o PARNA Catimbau está inserido em uma região definida como área prioritária para pesquisa científica (MMA, 2015).

Albuquerque e Oliveira (2007) observaram que apesar das espécies exóticas serem uma fração significativa da flora medicinal local, as espécies nativas apresentam um maior percentual de uso e indicações, destacando-se as espécies *Amburana cearensis*, *Myracrodruon urundeuva*, *Anadenanthera colubrina*, *Obtusifolium sideroxylon*, e *Ziziphus joazeiro*.

Frasso et al. (2012), realizou o primeiro relato de atividade contra *Tricomonas vaginalis* dos extratos de *Polygala decumbens* (planta da Caatinga), sendo este estudo baseado no uso e conhecimento popular da planta pelas comunidades. Nos estudos de Araújo et al. (2014), os extratos de *Parapiptadenia rígida*, coletada nos estados de Pernambuco, Paraíba e Rio Grande do Norte, apresentaram promissora atividade

antimicrobiana contra *Staphylococcus epidermidis* e *Shigella flexneri*, além de significativa atividade anti-inflamatória (Albuquerque et al., 2007; Araújo et al., 2014). Trentin et al. (2011) realizaram a primeira triagem antibacteriana e antibiofilme de plantas da Caatinga contra *Staphylococcus epiermidis*, o que, segundo os autores, confirma que os relatos etnofarmacológicos podem ser indicadores de novos produtos antibacterianos e antibiofilmes.

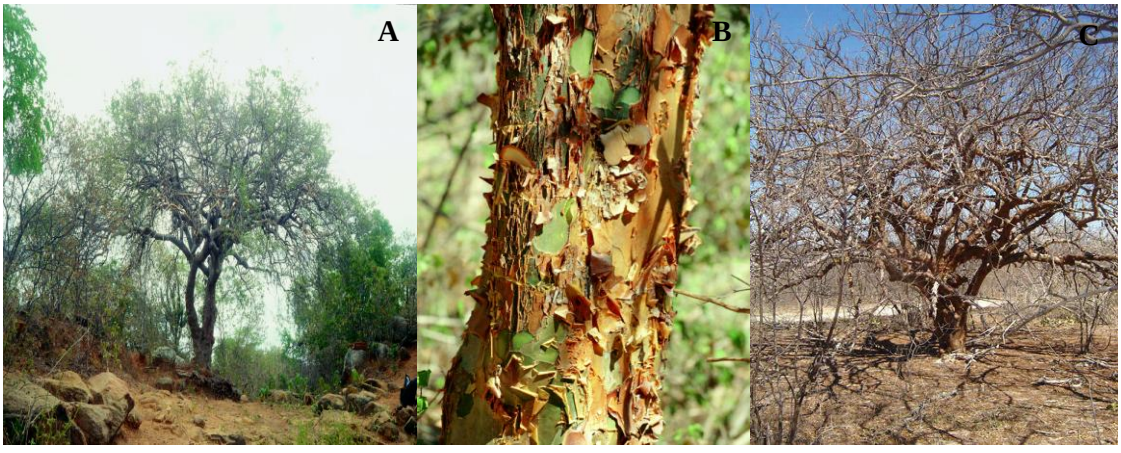
Embora venha crescendo o número de estudos em relação ao potencial biológico de plantas da Caatinga, muitas plantas que são utilizadas pelas comunidades para fins medicinais ainda não foram submetidas a estudos científicos para confirmar sua eficácia no tratamento de algumas doenças (Silva, et al., 2015) Por isso, a expansão de ações voltadas para a bioprospecção bioquímica de plantas da Caatinga é de extrema importância, uma vez que suas espécies vegetais podem ser fonte de biomoléculas que podem se tornar novas alternativas para a indústria bem como a regulamentação e uso de fitoterápicos no Sistema Único de Saúde tornam as espécies da caatinga uma importante fonte de busca, uma vez que, o estresse hídrico o qual essas plantas são submetidas contribui para que o vegetal produza uma variedade de metabolitos secundários (Arcoverde et al., 2014).

2.2 Gênero *Commiphora*

Conhecida popularmente como imburana, imburana-de-cambão e umburana (Braga, 1996), *C. leptophloeos* é uma arvore de pequeno porte, de comportamento decíduo. Sua casca mede até 0,63 cm de espessura, a casca externa ou ritidoma é lisa, lustrosa, desprendendo-se em lâminas delgadas, revolutas, muito irregulares e características(Figura 1).

Figura 1 (A,B,C): *Comminphora leptophloeos* (Mart.) J. B. Gillett.

Fonte: http://www.cnip.org.br/bdpn/ficha.php?cookieBD=cnip7&taxon=1182



Conforme a idade da casca, a cor varia do verde, quando jovem, a laranja-avermelhada quando envelhecida, e plúmbea nos momentos de maior rigor das secas ou em árvores tendentes a morrer. Suas folhas são forrageiras, tanto verdes como secas. O pólen e néctar são aproveitados pelas abelhas e, nos troncos ocos, abriga abelhas nativas selvagens, os frutos são comestíveis quando bem maduros, com uma polpa agridoce; a casca e a semente dessa espécie são usadas na forma de garrafadas e de xaropes no tratamento de doenças do estômago, enjoo e tosse (Agra, et al 2007). O infuso, o decocto e o xarope da casca do caule são usados como tônico e cicatrizante no tratamento de feridas, gastrite e úlcera, bronquites e inflamações do trato urinário. Das sementes se extrai um óleo medicinal. Em Alagoas e em Sergipe, os índios das tribos *kariri-shokó* e *shokó* usam a casca e a madeira como incenso para combater diabete, diarreia ou hipertermia (Carvalho, 2008).

C. leptophloeos representa uma espécie típica da caatinga e sem relatos do potencial biológico dos seus extratos, havendo apenas dois estudos de isolamento e aplicação dos óleos essenciais e atividade antimicrobiana do extrato aquoso para avaliar o potencial de biofilme (Agra, 2007). Porém, seu gênero *Commiphora (Burseraceae*) compreende mais de 150 espécies, distribuídas nas regiões tropicais e subtropicais, ocorrendo especialmente no nordeste da África, sul da Arábia e Índia (Langenheim, 2003; Vollesen, 1989).

As espécies deste gênero são caracterizadas como pequenas árvores ou arbustos com pequenos ramos, casca cinza-claro e castanho-avermelhado e exudatos resinoso. Seus exudatos são comumente usados como perfume, incenso, embalsamamento e pomadas, e seus valores medicinais são registrados na literatura antiga do Egito, Roma, Grécia e China tendo sido gradualmente reconhecido pela humanidade (Langenheim, 2003; Nanjing , 2006).

C. myrrha, C. opobalsamum, C. mukul e C. molmol são as mais utilizadas e reconhecidas. O reconhecimento do valor terapêutico e medicinal de *myrrha* (conhecido como *Guggul* na Índia) e os exudatos resinosos de *C. mukul* data de 3.000 anos atrás no sistema médico ayurvédico. *Guggul* é considerada como a mais importante erva na monografia autorizada Charaka Samhitâ para o tratamento de obesidade, e é usada como um agente hiperlipidêmico para o tratamento de dislipidemia (Kuppurajan *et al*, 1978;. Singh *et al*, 1994;. Khanna *et al*, 2010). A resina de *C. molmol* é vendida como

um agente antiparasitário no mercado com o nome comercial de Mirazid no Egit,,mo (Abdul-Ghani *et al*., 2009).

Com relação aos estudos fitoquímicos deste gênero, mais do que 300 moléculas foram identificadas. El Ashry et al. (2003) e Hanus et al. (2005), relatam a presença de Terpenos especialmente o sesqui e triterpenos como os mais abundantes constituintes neste gênero. Muitos trabalhos têm descrito a análise de Cromatografia Gasosa de óleo volátil de *Commiphora* de diferentes espécies, abrangendo *C. myrrha* (Dekebo et al, 2002a;. MortezaSemnani e Saeedi, 2003), *C. guidottii* (Craveiro et al., 1983), *C. quadricincta* (Assad et al., 1997*), C. holtziana* (Provan et al., 1987; Dekebo *et al*., 2002a), e *C. sphaerocarpa Kataf* (Dekeboet al., 2002a). Monoterpenos incluindo a-pineno, canfeno, b-pineno, mirceno e limoneno, furanosesquiterpenos e Diterpenos também estão e presentes neste gênero. Os esteróides com onze esteróides pregnane e nove esteróides colestano são encontrados apenas nas espécies de *C. mukul* e têm atraído interesse por sua ação anti-tumoral, anti-inflamatória e propriedades hipolipemiantes (Ramawat e Merillon, 2008; Shishodia et a*l*., 2008).

Muitos outros metabólitos secundários foram encontrados, tais como: flavonoides e lignanas longa derivados de cadeia alifáticos (Hough et al, 1952;. e Jones Nunn, de 1955; Bose e Gupta, 1964). Os flavonóides desse gênero são encontrados na flor, caule e casca (Patil et al., 1973, Zhu et al, 2001;. Shen et al, 2007).

Com relação aos aspectos farmacológicos e toxicológicos, o extrato metanólico da resina de *C. mukul* demonstrou significativa inibição da formação de NO estimulados com lipopolissacarideos (LPS) -macrófagos de camundongos com um valor de IC50 de cerca de 15 mg / mL (Meselhy, 2003; Matsuda et al, 2004a.). Mecanismos Anti-inflamatórios deste extrato contra a inflamação induzida por LPS tem sido relatado recentemente (Cheng et al., 2011). Cembrane, diterpenos, triterpenos polypodane, esteróides e lignanas isolado desta espécie foram testados quanto à sua produção de NO e atividades inibitórias da COX, impedido produção de Oxido Nítrico com valores de IC50 de 1,1, 3,3, 21,1 e 42,3 mM,respectivamente (Meselhy, 2003). Em um estudo in vivo utilizando ratos Lewis, os resultados mostraram que diminuiu o nível de marcadores inflamatórios, tais como MMP-2, NO e PGE2, e impediu a expressão da proteína relacionada com a inflamação, em tecidos oculares (Kalariya et al., 2010).

Extratos etanólicos de *C. myrrha* e frações de acetato de etila assim como suspensão de resina *C. molmol*, possuem atividade analgésica comprovada através do

ensaio da Placa Quente, comprovando assim o uso popular destas espécies para tratamento de feridas,dor óssea e fraturas em medicamentos indígenas (Su et al. 2011, Dolara et ai.1996, Annu et al, 2010).

Os extratos de *C. myrrha, C. molmol, e C. mukul*, foram investigados para as suas propriedades antiproliferativas contra as células tumorais. Shoemaker et al. (2005) relataram o efeito inibitório do extrato aquoso de resina *C. myrrha* contra oito linhas de células de tumorais. Dez espécies *Commiphora* recolhidos na África do Sul foram avaliadas para atividades antiproliferativas contra colônia humana HT-29, adenocarcinoma, adenocarcinoma da mama humano MCF-7 e linhagens de células de glioblastoma humano SF-268 (Paraskeva et al., 2008). Guggulsteronas inibiu a proliferação de uma ampla variedade de linhas celulares de tumores humanos, incluindo leucemia, mieloma múltiplo, carcinoma de cabeça e pescoço, carcinoma do pulmão,melanoma, carcinoma da mama, carcinoma do ovário e rim câncer. Eles também foram ativos contra a leucemia Gleevec-resistente, mieloma múltiplo e doxorubicinresistant dexametasona-resistentes células de câncer de mama (Shishodia et al., 2007).

Quanto as atividades antiparasitárias e antimicrobianas, um medicamento que contém 300 mg extrato purificado de resina *C. molmol*, é vendido no mercado como um antiparasitário (Mirazid) (Abdul-Ghani et al., 2009). Subsequentemente, verificou-se que a resina de *C. molmol* exibia efeito terapêutico sobre coccidiose hepática induzida pelo parasita *Eimeria stiedae* em coelhos domésticos (Baghdadi e Al-Mathal, 2010). Além, Mirazid mostrou efeito terapêutico para *Giardia lamblia* (Fathy, 2011).

Segundo Termentzi et al.(2011), não só resina deste gênero exibe ação antimicrobiana mas também a folha, caule, casca são ativos contra diversos microorganismos como: *Pseudomonas aeruginosa* (Kumari et al., 2011a), *Fusarium culmorum, Phytophtora cryptogea e Alternaria solani* (Fraternale et al., 2011).

Espécies deste gênero também possuem atividade antioxidante. C. *myrrha* , através dos óleos essenciais e três furanosesquiterpenos mostrou atividade de eliminação de radicais DPPH com os valores de EC 50 de 1,08, 4,29 e 2,56 mg / ml (Fraternale et al.,2011). Os extratos de caule de *C. tenuipetiolata, C. neglecta e C. mollis* mostrou atividade antioxidante no ensaio de ABTS com valores de IC50 de 5,10, 7,28 e 8,82 mg / ml (Paraskeva et al., 2008).

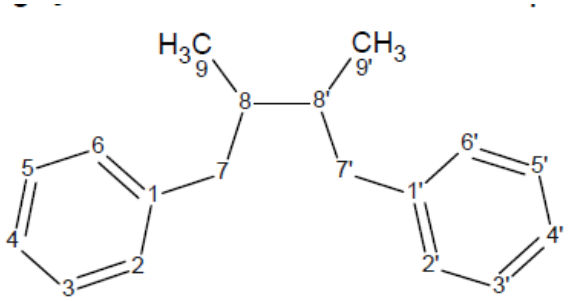
Outros ensaios como: atividade hipopilidemia, hipotensoras e proteção cardíaca, antidiabética, antiulcera e toxicidade também foram realizados, demonstrando um amplo aspecto de atividades relacioadas as espécies deste gênero (Khanna et al., 2010, Nohr et al, 2009, Ojha et al., 2008, Bellamkonda et al. 2011, Al-Howiriny et al., 2005; Gowrishankar et al., 2004; Li et al, 2008;.. Rao et al, 2001).

2.3 *Lignana* (Hinoquinina)

O termo *lignana* foi introduzido por Haworth em 1936, com o intuito de designar e agrupar uma classe de produtos naturais, cujas evidências estruturais mostravam-se relacionadas ao acoplamento de duas unidades C6C3 (propilbenzeno) através dos carbonos centrais de suas cadeias laterais propílicas.

Em 2000 a IUPAC propôs uma definição análoga à inicialmente proposta por Haworth, na qual denomina-se como *lignana* toda substância oriunda do acoplamento entre duas unidades C6C3 (propilbenzeno), particularmente caracterizada por uma ligação carbono-carbono entre as posições 8 e 8' (Moss,2000) (Figura 2)

Figura 2: Padrão de conectividade e numeração para *lignanas,* segundo a IUPAC.



Um estudo sobre a distribuição filogenética de plantas produtoras de lignanas mostrou que, levando-se em conta somente as 66 lignanas mais frequentes na literatura, a produção destas relaciona-se a um vasto grupo constituído por 126 diferentes famílias vegetais, entre elas a *Burseraceae* (Umezawa,2003)

Desde que a humanidade incorporou os vegetais à sua dieta, ela tem consumido, sem conhecimento, plantas ricas em lignanas. Muitas destas plantas são largamente

utilizadas, há milênios, nas práticas da medicina tradicional oriental. Delas podemos destacar a planta chinesa *Kadsura coccinea*, utilizada no tratamento de artrite reumatóide e úlceras gástricas e duodenais, rica nas lignanas (-)-wuweizisu e (-)-gomisina (Wescott,2003). Também podemos destacar a planta japonesa *Fraxinus japonica*, largamente utilizada como diurético, antipirético, analgésico e anti-reumático, que contém as lignanas (+)-pinoresinol e (-)-olivil (Adlercreutz,2002)

Dos hábitos ocidentais, podemos destacar o consumo dietário das sementes da linhaça (*Linus usitatissimum*), ricas em SDG , o diglicosídeo da lignana (-)-*seco*-isolariciresinol . Seu consumo está frequentemente associado a efeitos benéficos no tratamento e prevenção dos cânceres de mama, intestino, próstata e tireóide, à redução do risco de doenças cardíacas, bem como a efeitos benéficos no tratamento da diabetes (Wescott,2003)..

Apesar da vasta ocorrência de diferentes atividades biológicas entre as lignanas, as quais tem sido intensamente estudadas nas últimas décadas num esforço para a obtenção de possíveis novos protótipos de fármacos, podemos destacar a atividade anti-HIV das dibenzilbutirolactonas, das ariltetralinas e da gomisina G cujos mecanismos de ação atuam através da inibição da enzima transcriptase reversa viral (Trazii, 2008)

A hinoquinina é uma lignana pertencente à classe dibenzilbutirolactona, que já foi descrita na família *Burseraceae*, no gênero *Bursera* (Maldini et al., 2009), Estudos mostram que esta lignana apresenta efeitos antiespasmódicos (Zhang *et al*., 2008), genotóxicos e eficaz redução dos danos induzidos pelo cromossomo DXR(doxorubicina), fármaco utilizado na quimioterapia contra o câncer (Medola et al.,2007). Portanto, a hinoquinina, isolada pela primeira vez na espécie *Commiphora leptophloeos* representa um potencial fármaco com promissoras atividades biológicas.

2.4 Estresse Oxidativo e Ensaio Antioxidante

O oxigênio é vital para a maioria dos organismos, mas, paradoxalmente pode ser fonte de moléculas capazes de danificar sítios biológicos essenciais. Espécies reativas de oxigênio (ROS), de carbono e nitrogênio são continuamente produzidas por meio da geração de energia através da cadeia transportadora de elétrons microssomal e mitocondrial. Fontes exógenas de formação de ROS são representadas pelo tabagismo, poluição, exercício físico intenso, consumo excessivo de álcool e agrotóxicos, dentre

outros (Frankel, 2005). Os radicais livres são espécies químicas que contém um ou mais elétrons desemparelhados, são altamente instáveis e causam danos a outras moléculas através da captação de elétrons, a fim de alcançar a estabilidade. Células vivas geram (ROS), como resultado das alterações fisiológicas e processos bioquímicos (Deepa et al., 2012).

Os compostos antioxidantes podem ser definidos como substâncias que, quando presentes em pequenas concentrações em relação ao substrato oxidável, são capazes de retardar ou mesmo inibir substancialmente a oxidação do substrato (Niki, 2010)

Segundo Sun et al. (2011), a produção excessiva de ROS e reações que levam a produção de radicais livres podem levar ao estresse oxidativo celular, podendo causar doenças degenerativas e diversas patologias como Alzheimer e Parkinson (Markesbery, Lovell, 2006; Gomez-Pinilla; Nguyen, 2012), câncer (Bianchi; Antunes, 1999, Barnett et al., 2006), doenças cardiovasculares como a aterosclerose (Vokurkova et al., 2007; Bhattacharya et al., 2011), sendo também a causa principal da morte celular e lesões tecidulares resultantes de infartes do miocárdio (Ide et al., 2001), processos inflamatórios e envelhecimento (Wang et al., 2007). Mais recentemente, foi demonstrado que o estresse oxidativo também pode ser a causa da diabetes tipo II (Dogru et al., 2012).

A formação de radicais livres pode ser ocasionada por fatores endógenos e exógenos, alguns exemplos estão descritos na Tabela 2.

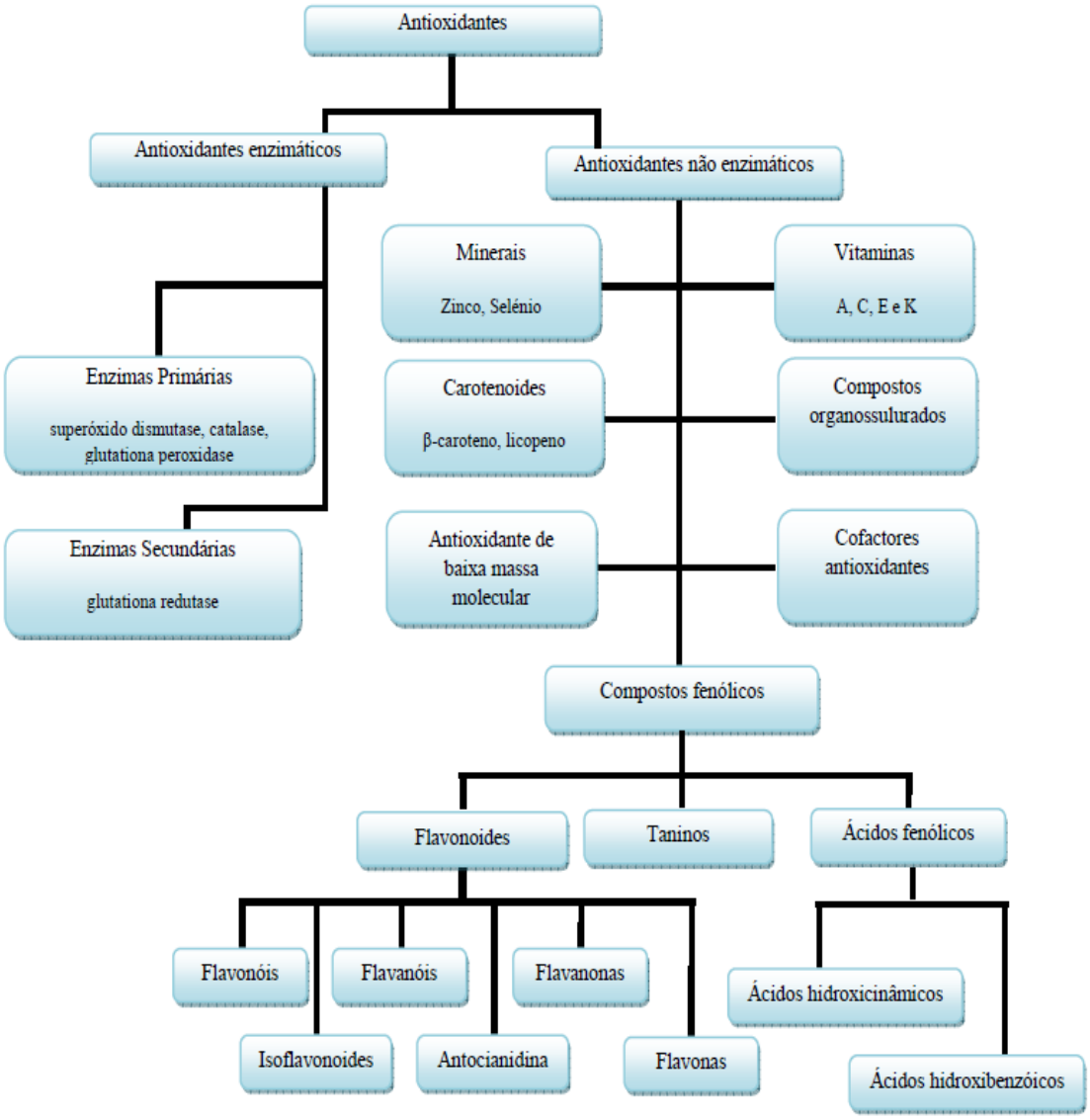
Tabela 1. Fontes endógenas e exógenas de formação de radicais livres

Endógenas	Exógenas
Respiração aeróbica	Ozônio
Inflamações	Radiações gama e ultravioleta
Peroxissomos	Medicamentos
Enzimas do citocromo P450	Dieta
	Cigarro

Fonte: Moreira,2013

Os compostos antioxidantes podem ser de origem natural, os quais dividem-se em dois grandes grupos, os enzimáticos e os não enzimáticos (Figura 3). e os de origem sintéticos (Cheung et al., 2003). Os antioxidantes enzimáticos incluem as principais enzimas antioxidantes, como a superóxido dismutase, catalase e glutationa peroxidase. Alguns exemplos de antioxidantes não enzimáticos incluem a vitamina C, compostos fenólicos hidrossolúveis e compostos lipossolúveis (vitamina E e carotenoides) (Ndhlala et al., 2010).

Figura 3. Classificação dos antioxidantes



Fonte: Ratnam, 2006

De acordo com Sun et al. (2011), atualmente existem disponíveis muitos antioxidantes sintéticos como butilhidroxianisol e butil-hidroxitolueno, entretanto, estes podem se acumular no corpo resultando em lesão hepática e carcinogênese. Por esta razão, como um esforço para proteção do ser humano aos radicais livres e retardamento do progresso de muitas doenças crônicas tem se dado grande atenção aos antioxidantes naturais.

Antioxidantes naturais são obtidas a partir de recursos vegetais incluindo ervas aromáticas, especiarias, frutas, legumes, oleaginosas e produtos (Shahidi; Zhong, 2010). Alguns estudos mostraram que a ingestão de frutas e verduras reduz a taxa de doenças cardíacas, câncer, e outras doenças degenerativas, e que este fato pode está atribuído a presença de antioxidantes naturais nestes alimentos, uma vez que, os vegetais são capazes de combater esses radicais livres (Motamed; Naghibi, 2010).

Nesse contexto, os vegetais constituem uma importante fonte de produtos naturais ativos que diferem em estruturas e propriedades biológicas. A determinação da atividade antioxidante de produtos naturais teve início com Chipault et al. (1952) em especiarias, como o alecrim e a salvia ingredientes utilizados em alimentos desde os primórdios da história.

Diversos autores (Deepa et al, 2009, SA et al., 2009, Sousa et al., 2007, Bernardes et al, 2011)constataram que espécies vegetais são ricas em compostos antioxidantes. Todavia, a quantidade destas substâncias em vegetais é amplamente influenciada por diversos fatores: genéticos, condições ambientais, grau de maturação e variedade da planta, entre outros. O substrato utilizado no ensaio, o solvente e técnica de extração utilizada, também, são fatores que influenciam a capacidade antioxidante (Moure et al, 2001; Naczk E Shahidi, 2004).

No que se referem aos solventes, frequentemente são usadas soluções aquosas de etanol, metanol e acetona, entre outras, em diferentes concentrações, cuja eficácia depende da polaridade dos polifenóis presentes na amostra, bem como, do grau de polimerização e da sua interação com os outros constituintes (Naczk E Shahidi, 2004). Alem disso, recomenda-se a combinação de pelo menos dois ciclos de extração, de modo a garantir a eficácia do processo, permitindo extrair compostos com diferentes estruturas químicas (Perez-Jimenez et al., 2008).

A diversidade química existente entre os compostos antioxidantes, em especial entre os compostos fenólicos, impõe a necessidade de avaliar a capacidade antioxidante

por diversos ensaios, com mecanismo de ação diferente. Neste sentido, vários ensaios têm sido desenvolvidos, os quais diferem tanto em relação ao mecanismo de reação, como no que se referem às espécies-alvo, as condições em que ocorre a reação e na forma de expressar os resultados.

Dentre os métodos que determinam a habilidade dos antioxidantes em sequestrar radicais, destacam-se: Determinação de fenólicos totais, Método de Complexação do Fosfomolibdênio, DPPH (2,2-difenil-1-picrilhidrazila), ABTS (2,2'-azino-bis (3-etilbenzotiazolin) 6-ácido sulfônico) e β-caroteno, conforme descrito na Tabela 2.

Tabela 2: Ensaioa Antioxidantes

Ensaio	Principio/Mecanismo	Vantagem	Desvantagem
DPPH (2,2-difenil-1-picrilhidrazila	Este método baseia-se na transferência de elétrons de um composto antioxidante para um oxidante DPPH é reduzido formando difenil-picril-hidrazina, de coloração amarela com consequente desaparecimento da absorção. Ele é um radical livre estável que, na presença de um antioxidante doador de hidrogênio, pode ser reduzido em meio alcoólico, formando difenil picrilhidrazina Esta redução pode ser verificada mediante espectrofotometria 515–528 nm, pela diminuição da absorbância, com simultânea mudança de coloração violeta escura original, para amarela clara \ • Ensaio Rápido • Compatível com exibições de alto rendimento		• A interação entre a concentração de DPPH • e antioxidantes não é linear • DPPH não é solúvel em meio aquoso, apenas em solventes orgânicos • O resultado pode ser influenciado pela: luz, oxigênio, tipo de solvente e alguns compostos (como o • carotenóides e eugenol) .

Ensaio	Principio/Mecanismo	Vantagem	Desvantagem
ABTS (2,2'-azino-bis (3-etilbenzotiazolin) 6-ácido sulfônico)	O método consiste em monitorar o decaimento do cátion-radical ABTS*+, produzido pela oxidação do ABTS Radical de ABTS quando oxidado tem intensa cor, que deve ser diminuiu uma composto antioxidante. O resultado é medido espectrofotometria de 600- 750 nm e expressa em relação a Trolox (Trolox antioxidante equivalente Capacidade - TEAC).	• Método Simples • Adequado para antioxidantes hidrofílicos e lipofílicos; • Reação pode ser realizada numa vasta gama de pH	• Ausência de significado biológico; • Geração de radicais por processos enzimáticos ou reações químicas; • Reações com alguns composos fitoquímicos levando a falso valorea TEAC; • O valor de TEAC não correlacionado com o número de eletrod doados por um antioxidante.
β-caroteno	Ensaio beaseado na capacidade de uma amostra contendo substâncias antioxidantes em retardar a oxidação do β-caroteno na presença de luz. O resultado é medido espectrofotometria de 443 nm	• Adequado para compostos lipofílicos e / ou termossensível; • Compatível com exibições de alto rendimento	• A reprodutibilidade pode ser afetada pelo pH, temperatura e metais • Pigmentos de alimentos, como os carotenóides, pode interferir nesta reação

Ensaio	Principio/Mecanismo	Vantagem	Desvantagem
Folin-Ciocalteu	Este ensaio mede a capacidade de redução da amostra e baseia-se na transferência de eletrons a partir de compostos fenólicos (ou outras espécies redutoras) de molibdénio (presente em FCR), em meio alcalino. Os complexos formados tem uma cor azul que é monitorizada espectrofotometricamente a 750-765 nm. Os resultados são normalmente expressos em equivalentes de ácido gálico.	• Simplicidade; • Instrumentação prontamente disponíveis; • Compatível com alto rendimento; • Correlação linear entre o teor de fenólicos e atividade antioxidante	• A falta de significado biológico; • A falta de padronização dos métodos podem levar a várias ordens de magnitude diferença; • Como FCR também pode ser reduzida por diversos compostos não fenólicos, neste ensaio não fornece a concentração fenólico direita, que pode ser determinar por análise de HPLC (High Performance Cromatografia Liquide).
Redução do Ferro (Ferro FRAP)	Na presença de um composto antioxidante o tripyridyltriazinecomplex férrico (Fe (III) - TPTZ) é reduzido resultando numa mudança de cor de amarelo para azul (medido a 593 nm).	Simplicidade; • Instrumentação prontamente disponíveis; • Compatível com alto rendimento; • Correlação linear entre o teor de fenólicos e atividade antioxidante	Pode ser influenciado por: (i) um complexo formado por Fe (III) e qualquer espécies quelante na amostra também pode reagir com o e antioxidante alterar o resultado; (Ii) do solvente utilizado; (Iii) condição de pH; •Alguns antioxidantes (tais como glutationa) são não é capazes de reduzir o Fe (III);

SILVA et al,2016 (ADAPTADO)

2.5 Atividade antimicrobiana

O surgimento da terapêutica antimicrobiana representou um marco para a humanidade, a medicina passa a ter esperança na possibilidade da cura de problemas de saúde que levavam a óbito inúmeras pessoas (Oliveira, 2006). Define se antibióticos como sendo substâncias produzidas, pelo menos em parte, por processos biológicos, e que apresentam capacidade de impedir a sobrevivência ou mesmo o crescimento de microrganismos (Murray et al., 2000).

O uso de antimicrobianos com espectro maior do que necessário, o esquema posológico inadequado e o tempo prolongado de antibioticoterapia são fatores que facilitam a seleção de cepas resistentes (Hoefel *et al*. 2004; Hoefel, Lau Tert , 2006). A resistência aos antimicrobianos tem seu desenvolvimento como consequência natural da habilidade das populações bacterianas de se adaptar às pressões seletivas a que são expostas, devido a sua estrutura genômica peculiar (Santos, 2004).

Trabulsi e Alterthum (2008) explicitam que a resistência bacteriana pode ocorrer naturalmente, sendo comum a todas as bactérias de uma espécie, ou adquirida. Quando adquirida, ocorre uma modificação no perfil de sensibilidade a determinado(s) agente(s) antibiótico(s) por parte de determinadas cepas bacterianas ocorrendo alterações da informação genética, tanto cromossômicas quanto extracromossômicas, sendo responsáveis pelo processo de aquisição de resistência (Strol et al., 2004).

Podem ser citados vários mecanismos de resistência das bactérias aos antibióticos, como a destruição ou inativação da droga, pela destruição do anel beta - lactâmico, pela enzima beta-lactamase ou penicilinase produzida pelo microrganismo; incapacidade do antibiótico de penetrar na superfície das células bacterianas; alteração dos sítios -alvo das drogas, como a troca de um aminoácido. A bactéria pode possuir uma via bioquímica alternativa que desvia a reação particular que é inibida pelo antibiótico da célula e efluxo rápido onde a droga é ejetada para fora antes que possa se tornar efetiva (Gonçalves, 2010).

De acordo com dados do Ministério da Saúde, no Brasil, cerca de 70% das bactérias que causam infecções hospitalares são resistentes a pelo menos um dos antimicrobianos comumente utilizados para o tratamento dos pacientes. Apesar do hospital tradicionalmente ser um lugar de reabilitação e restabelecimento da saúde, nota- se que o mesmo está se tornando cada vez mais um reservatório de bactérias resistentes e também uma via de transmissão de graves doenças infecciosas, muitas

vezes por práticas inadequadas dos profissionais de saúde (Oliveira, 2006). As Unidades de Terapia Intensiva (UTI's), berçários de alto risco, unidades de oncologia e queimados são os locais onde mais frequentemente ocorrem infecções, sendo considerados ambientes reservatórios de bactérias multirresistentes (ANVISA, 2007; Machado, 2009). O grau de comprometimento imunológico dos pacientes atendidos nestes setores, procedimentos invasivos utilizados na rotina ambulatorial e o elevado uso de antimicrobianos agravam esta situação (Martins *et al*., 2004). Ainda, a menor adesão às práticas de higienização das mãos devido ao excesso de trabalho intensifica a transmissão de bactérias multirresistentes interpacientes (Machado, 2009).

Os Bacilos Gram-negativos fermentadores de glicose estão envolvidos em muitas das infecções adquiridas em UTI's (ANVISA, 2007). Para os microrganismos deste grupo são relatadas elevadas taxas de resistência aos antimicrobianos do grupo das quinolonas, aos beta-lactâmicos e aminoglicosídeos, principalmente devido à produção de beta-lactamases.

Klebsiella spp. e Escherichia coli apresentam um risco ainda maior, por produzirem as beta-lactamases de espectro ampliado (ESBL), o que restringe as alternativas terapêuticas eficazes (ANVISA, 2007).

Tem sido relatados casos de resistência aos carbapenens no âmbito hospitalar ocasionados em bactérias Gram-negativas por meio da expressão enzimática de PC (carbapenemase de *Klebsiella pneumonia*) (Dienstmann et al., 2010). Esta enzima confere também resistência às penicilinas, cefalosporinas e monobactâmicos (YIgit *et al*., 2001). Logo após a introdução da penicilina G (benzilpenicilina) na terapêutica antimicrobiana, principalmente contra Gram-positivos, evidenciou - se o surgimento de cepas de *S. aureus* resistentes, e surgimento posterior de elevada resistência também à penicilina V, ampicilina, amoxicilina e carbenicilina (Moreira e Daum, 1995). Meticilina e Oxacilina, betalactâmicos penicilinase -resistentes, foram introduzidos como esperança de maior eficácia no tratamento das infecções envolvendo *Staphylococcus* multirresistentes.

Hospitais de grande porte com serviço de emergência aberto ao público e centros de referência para pacientes infectados, evidencia-se um grande índice de cepas já resistentes a estes fármacos, conhecidas pelas siglas MRSA ou ORSA (*Staphylococcus aureus* meticilina ou oxacilina resistentes) (Rohrer , Maki E Berger-Bächi, 2003; Mark *Et al*., 2002).

Em virtude dessas resistências busca por novos agentes antimicrobianos a partir de plantas superiores tem sido de grande interesse. Desde a década de 90, a busca por formas alternativas de tratamentos vem demonstrando grande popularidade o que tem levado as indústrias a se concentrarem em programas de triagem a fim de identificar novos Princípios ativos a partir de fontes naturais (kumar et al., 2010).

Conforme Haida et al. (2007), as plantas constituem-se importantes fontes de substâncias biologicamente ativas, servindo para o desenvolvimento e a síntese de um grande número de fármacos. A pesquisa com Briófitas até Angiospermas e suas diversas atividades farmacológicas têm se tornado uma alternativa promissora no combate às infecções causadas por bactérias e fungos (MoranteS et al., 2007; Peres et al., 2009; Lee et al., 2008; Santos et al., 2010; Morais-Braga et al., 2013). A Tabela 3 descreve algumas plantas da Caatinga Pernambucana que tiveram sua atividade antimicrobiana avaliada e comprovada.

Tabela 3 – Atividade antimicrobiana testada para espécies da Caatinga.

Família/Nome científico	Parte vegetal	Microorganismos sensíveis	Referência
Anacardiaceae			
Myracrodruon urundeuva	Casca de caule	Sa/Bs, Sa/Bs/Ml/Ec/Kp/Ca/Na, Sm/Smi/Ss/Ssa/Lc/Ca/ Ct/Ck, Pa	ALMEIDA et al., 2012, JANDÚ et al., 2013, ALVES et al., 2009 FERNANDES, 2011
	Sementes	Bs	FARIAS et al., 2013
	Caule	Bs/Cc/Sa/Sf/Ec/Kp/Pa/ Fs/Fo/Fm/Fd/Fl	SÁ et al., 2009a e b
	Folha	Kp/Ml/Sc/Pa/Sa	OLIVEIRA et al., 2012
***Schinopsis brasiliensis* Engl.**	Sementes	Sch	FARIAS et al., 2013
	Folha	Ec/Kp/Sa/Ef/Ssp/Pa	
	Casca de caule	Kp/Ssp/Pa	
	Casca da Raíz	Ec/Kp/Sa/Ef/Ssp/Pa	
	Endocarpo	Ec/Kp/Sa/Ef/Ssp/Pa	SARAIVA, 2007
	Exocarpo	Ec/Kp/Sa/Ef/Ssp/Pa	
	Flor	Ec/Kp/Sa/Ef/Ssp/Pa	

Tabela 3 (continuação)

Família/Nome científico	Parte vegetal	Microorganismos sensíveis	Referência
Schinopsis brasiliensis Engl.	Casca de caule	Sa/Pa/Kp/Ec, Pa/Ef/Sa/So	MACHADO, 2012, SILVA et al., 2012a
	Folha	Sa/Pa/Kp/Ec /Ca/Ck/Ct/Sa/Kp/Ssp/Pa/Ec	SARAIVA et al., 2013, MACHADO, 2012, GUIMARÃES, 2010
Spondias tuberosa Arruda	Casca de caule	Sa/Ef/Ecl, Sm/So/Ssal/Sp/Ca/Ck/ Cg/Cp	ROCHA et al., 2013 CARVALHO, 2012
	Folha	Kp/Sm/Pa/Pm/Mm/ Sl/Ec	SILVA et al., 2012b
Burseraceae			
Commiphora leptophloeos (Mart.) J. B. Gillett	Casca de caule	Ms	ALMEIDA et al., 2012
Cactaceae			
Cereus jamacaru DC.	Folhas frescas	Ec/Bc/Sa	SILVA, 2012

Tabela 3 (continuação)

Família/Nome científico	Parte vegetal	Microorganismos sensíveis	Referência
Caesalpiniaceae			
Hymenaea courbaril L	Casca de caule	Ec/Ef/Ssp/Sa/Pa/Cspp/Aspp/Se	SÁ et al., 2011, GONÇALVES et al., 2013
	Entrecasca	Ml/Sa/Ec	FERNANDES et al., 2005
	Resina	St/Sa/Ec/Pa/Sh	PEREIRA et al., 2007
	Polpa farinácea	Sa/Ef/Sf	MARTINS et al., 2010
Libidibia ferrea	Frutos	Ap, Ca/Sm/Ssal/So/Lc, Sm/So	MARTINS et al., 2014 SAMPAIO et al., 2009 MARREIRO et al., 2014
Bauhinia cheilantha (Bong.) Steud.	Folha	Ef	CAFFARO, 2014
	Casca de caule	Sa/Se/Ef	
	Caule	Sa/Se/Ef/Pa/Ec/Kp	
Capparaceae			
Crateva tapia L.	Galho	Pa	SALVAT et al., 2001

Tabela 3 (continuação)

Família/Nome científico	Parte vegetal	Microorganismos sensíveis	Referência
Celastraceae			
Maytenus rigida Mart.	Casca de caule	Sa/Ms, Ca/Ck/Cg/Cp,	ALMEIDA et al., 2012 FERNANDES, 2014
	Folha	Sa/Pm	ROCHA, 2003
	Entrecasca de caule	Sa	SANTOS et al., 2011
Euphorbiaceae			
Croton blanchetianus Baill.	Folha	Sa/Ec/Bc, Ah/Lm/Sent	ANGÉLICO et al., 2014 MELO et al., 2013
Jatropha mollissima (Pohl) Baill.	Látex	Lm/Sa/St/Sty	ROCHA; DANTAS, 2009
Fabaceae			
Amburana cearensis (Allemão) A. C. Sm	Semente	Fo/Fs/Cm/Sc/Ca	SANTOS et al., 2010
	Casca de caule	Ec/Ef/Ksp/Ssp/Sa/Lspp/Cspp/Aspp/Vspp, Ms	SÁ et al., 2011 , ALMEIDA et al., 2012

Tabela 3 (continuação)

Família/Nome científico	Parte vegetal	Microorganismos sensíveis	Referência
Mimosaceae			
Anadenanthera colubrina (Vell.) Brenan	Casca de caule	Ca, Sa	LIMA et al., 2014 PALMEIRA et al., 2010
	Casca de caule	Sm/Ec/Pa/Sa	SILVA, 2011
	Galho	Sm/Ssa/Ef/Ec/Sa	
	Folha	Sm/Ssa/Pa/Sa/Sm/Ec	
	Casca de caule	Sa/Pa/Sso/Sen/Ca	WEBER SOBRINHO, 2010
	Casca de caule	Sa/Bs/Ms	ALMEIDA et al., 2012
	Casca de caule	Sm/So/Sp/Ca/Ck/Cg/Cp	CARVALHO, 2012
	Casca de caule	Sa/Ef/Ecl	ROCHA et al., 2013
	Casca de caule	Ec/Sa	FERNANDES, 2014
Mimosa tenuiflora (Willd) Poir.	Casca de caule	Sa/Ms, Sso/Ssp	ALMEIDA et al., 2012 GONÇALVES et al., 2013

Tabela 3 (continuação)

Família/Nome científico	Parte vegetal	Microorganismos sensíveis	Referência
Olacaceae			
Ximenia americana L.	Casca de caule	Ec/Pa/Pv/Sa/Bs/Ca	MAIKAI et al., 2009
	Casca de caule	Sa/Pa	ALLA et al., 2013
	Raiz	Ef	KONÉ et al., 2004
	Folha	Pa/Bs/Ca	OGUNLEYE; IBITOYE, 2003

Legenda: Aa = *Alternaria alternata*; Ac = *Aeromonas caviae*; Ah = *Aeromonas hydrophila*; An = *Aspergillus niger*; Ap = *Aspergillus parasiticus*; Asp = *Aeromonas spp*.; Bc = *Bacillus cereus*; Bs = *Bacillus subtilis*; Ca = *Candida albicans*; Cc = *Corynebacterium callunae*; Cg = *Candida guilliermondii*; Ck = *Candida krusei*; Cm = *Colletotrichum musae*; Cn = *Cryptococcus neoformans*; Cp = *Candida parapsilosis*; Csp = *Corynebacterium spp*.; Ct = *Candida tropicalis*; Ec = *Escherichia coli*; Ecl = *Enterobacter cloacae*; Ef = *Enterococcus faecalis*; Fd = *Fusarium decemcellulare*; Fl = *Fusarium lateritium*; Fm = *Fusarium moniliforme*; Fn = *Fusobacterium nucleatum*; Fo = *Fusarium oxysporum*; Fp = *Fonsecaea pedrosoi*; Fs = *Fusarium solani*; Hp = *Helicobacter pylori*; Kp = *Klebsiella pneumonie*; Ksp = *Klebsiella spp*.; Lc = *Lactobacillus casei*; Lm = *Listeria monocytogenes*; Lsp = *Listeria spp.*; Ml = *Micrococcus luteus*; Mm = *Morganella morganii*; Ms = *Micobacterium smegmatis*; Pa = *Pseudomonas aeruginosa*; Pg = *Porphyromonas gingivalis*; Pi = *Prevotella intermedia*; Pm = *Proteus mirabilis*; Psp = *Proteus spp*.; Pv = *Proteus vulgaris*; Sa = *Staphylococcus aureus*; Sc = *Saccharomyces cerevisiae*; Sch = *Salmonella choleraesuis*; Se = *Staphylococcus epidermidis*; Sen = *Salmonella enterica*; Sent = *Salmonella enteritidis*; Sf = *Streptococcus faecalis*; Sl = *Serratia liquefaciens*; Sm = *Streptococcus mutans*; Sma = *Serratia marcescens*; Smi = *Streptococcus mitis*; So = *Streptococcus oralis*; Sp = *Streptococcus pyogenes*; Spa = *Streptococcus parasanguis*; Ss = *Streptococcus sobrinus*; Ssa = *Streptococcus sanguinis*; Ssal = *Streptococcus salivarius*; Sso = *Shigella sonnei*; Ssp = *Salmonella spp*.; St = *Salmonella thiphimurium*; Sty = *Salmonella typhi*; Tr = *Trichophyton rubrum*; Vsp = *Vibrio spp*.;

FONTE: Cabral, 2014 (ADAPTADO)

Nesse contexto os vegetais podem ser uma boa fonte para direcionar a busca por compostos promissores por possuirem várias vias metabólicas secundárias (Bertucci et al., 2009). As plantas com maior atividade antimicrobiana são aquelas ricas em polifenóis, flavonóides e taninos (Doughari; El-Mahmood; Tyouina, 2008; Rodrigues et al., 2013), terpenóides, alcalóides, lectinas, polipeptídeos, e cumarinas (Reschke; Marques, 2007; Haida et al., 2007).

A determinação do composto biologicamente ativo a partir de um material vegetal é dependente do tipo de solvente usado no processo de extração (Das et al., 2010). Na extração de compostos hidrofílicos, por exemplo, são usados solventes polares tais como metanol, etanol ou acetato de etila. Já para compostos mais lipofílicos, são usados diclorometano ou uma mistura de diclorometano/metanol na proporção de 1:1 (Sasidharan et al., 2011).

De acordo com González-Lamothe et al. (2009), os produtos do metabolismo secundário acumulado pelas plantas podem atuar de duas formas: como "potencializadores de atividade antibacteriana", favorecendo a atividade de antibióticos cuja ação encontra-se limitada por mecanismos de multirresistência desenvolvidos pelos micro-organismos; ou como "atenuantes de virulência", adequando a resposta do sistema imune do hospedeiro à infecção.

O estudo de agentes antimicrobianos de origem vegetal inicia com uma avaliação biológica completa dos extratos para garantir a eficácia e segurança, seguido pela identificação de princípios ativos, formulações de dosagem e perfil farmacocinético do novo fármaco (Das et al., 2010).

De acordo com Silva Jr. et al. (2009), o aumento de estudos de atividade antimicrobiana com plantas inclui diminuição de novas substâncias antimicrobianas sintéticas, além de muitas serem potencialmente tóxicas e apresentarem efeitos colaterais no paciente

Devido ao aumento alarmante na incidência de novas doenças infecciosas e resistência aos fármacos atuais, existe uma grande necessidade em descobrir novos compostos com atividade antimicrobiana e, possivelmente, com novos mecanismos de ação (Adwan et al., 2010).

A busca de novos agentes antimicrobianos é importante, uma vez que o elevado potencial de recombinação genética das bactérias tem provocado o aumento de cepas multirresistentes e, consequentemente, tornado ineficazes muitos fármacos

antimicrobianos disponíveis no mercado (Trias and Gordon, 1997; Labarca, 2002; Alvarez; Labarca; Salles, 2010).

3. OBJETIVOS

3.1. Geral

O objetivo deste trabalho foi isolar, identificar, purificar e aplicar biologicamente os metabólitos secundários do extrato de casca de *Commiphora leptophloeos.*

3.2 Específicos

- Isolar e identificar metabolito secundário do extrato clorofórmio
- Avaliar a atividade antimicrobiana de extrato clorofórmio e do metabólito isolado
- Avaliar a atividade antioxidante dos extratos Cloroformio, Metanólico, Ciclo Hexano e Acetato de Etila

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Capítulo 01

Artigo a ser submetido a Oxidative Medicine and Cellular Longevity

THE MEDICINAL PLANT *Commiphora leptophloeos* IS AN IMPORTANT ANTIOXIDANT AND NONCYTOTOXIC CAATINGA SPECIE

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Abstract

Commiphora leptophloeos, usually known as Imburana of Sertão, belongs to the Burseraceae family, and is traditionally used by Brazilian indigenous tribes for the treatment of their infirmities. Herein we performed a thorough your phytochemical characterization, showing its notable amounts of phenolics compounds endowed with high antioxidant activity in five extracts such as aqueous (CLAQE), methanolic (CLMEE) chloroform (CLCLE), cyclohexane (CLCHE) and ethyl acetate (CLAEE). *C. leptophloeos* phytochemical prospection revealed the presence of phenolic contents, tannins, coumarins, flavonoids and reducing sugars, secondary metabolites used by the plants for their defense. CLAQE presented the major contents of phenolic compounds with 33.64 ± 0.5 mg GAE/g and flavonoids with 19.3 ± 0.70 mg QE/g, for tannins contents CLCLE showed 9.70 ± 0.2 mg TAE/g, for coumarins content CLCHE showed 1.14 ± 0.81 mg BZE/g and for soluble sugars CLMEE presented 53.30 $g.100g^{-1mg}$. In Total antioxidant capacity (%TAC) all extracts showed a significant antioxidant activity for both concentrations of 1-10 mg/mL (≥100%, *p*-value <0.001). All the extracts induced low levels of cell death (viability ≥ 80%) by 24-hour period. In addition, these cytotoxic values were not correlated with either phenols (r = -0.15) or flavonoids (r = -0.20). Altogether, herein we show that the barks extracts from *C. leptophloeos* are antioxidants source that being of potential use in preventing oxidative conditions.

1. Key-words: Imburana; Natural Products; Oxidative stress; Phytochemical**Introduction**

Commiphora leptophloeos, usually known as Imburana of Sertão, belongs to the *Burseraceae* family including trees and shrubs from tropical and subtropical regions, and is traditionally used by indigenous tribes for the treatment of their infirmities [1,2]. The Commiphora genus comprises over 150 species most of which are confined to Eastern Africa, and in Brazil is found where the vegetation is exposed to adverse climate and soil conditions, typical of the Sertão physiognomy. Sertão is a semi-arid region in Northeast Brazil characterized by a very dry and extremely hot weather throughout the year and with low rainfall rates [3]. One of the plants strategies in harsh environments is the increasing synthesis of secondary metabolism products (polyphenols, lignans, flavonoids), the ones aiding in growth and development but not strictly required for plant survival, involved in plant advantage and usually conferring a positive effect for its use as medicinal purposes [4,5].
Polyphenols have been found to exhibit various bioactivities molecules, based on the number of phenol rings and the binding structural properties, polyphenols are divided into several subclasses such as phenolic acids, flavonoids, stilbenes, lignans and tannins [6]. Flavonoids, quercetin and rutin are most commonly studied for their cardiovascular effects [7]. Protocatechuic acid, gallic acid, caffeic acid are natural phenolic acids abundantly found and well known for their antioxidants properties [8]. Antioxidants are able to remove or avoid the development of free radicals and reactive oxygen species (ROS) inhibiting cell deterioration. Therefore, there is an increasing interest in identifying substances able to inhibit DNA bases oxidation by ROS, especially the ones preventing DNA damage [9].
Traditionally, *C. leptophloeos* is used as an infusion, tea or syrup for the treatment of flu, cough, bronchitis, urinary and liver disease. Being aware that plant species from Sertão usually present advantageous survival features due the presence of several compounds as above mentioned and since *C. leptophloeos* is one of the plants used by local individuals for its benefits in several disorders, herein we focused on its antioxidant characterization. To our knowledge this is a novel report in Commiphora genus.

2. Materials and Methods

2.1 Biological material (Plant)

The stem bark of *C. leptophloeos* was collected at *Parque Nacional do Catimbau*, Pernambuco – Brazil. The authors confirm that the named authority *Instituto Chico Mendes de Conservação da Biodiversidade* granted permission (SISBIO 16806) for our described field searches. The botanical identification and the deposition of plant specimens were performed at the Herbarium of the Institute of Agricultural Research of Pernambuco (IPA-PE) (IPA nº 84037).

2.2 Preparation of the extracts

The dried bark (25 g) of *C. leptophloeos* was obtained by saturation in order of increasing polarity: submitted to Cyclohexane (CLCHE), Chloroform (CLECL), Ethyl Acetate (CLAEE), Methanolic (CLMEE) and Aqueous (CLAQE) (250 mL) by agitation at 180 rotations per minute (rpm). After 24 hours, the extract was filtered (Whatman® number 2) and concentrated at 45ºC under vacuum in a rotary evaporator (Concentrator

5301, Eppendorf®). The powder produced was kept at -20°C for future use. For phytochemical and antioxidant analysis, the extracts were dissolved in your respective solvents at the concentration of 1-100 µg/mL and 1-10 mg/mL for all biological assays.

2.3 Quantitative Phytochemical Screening

2.3.1 Determination of total phenol contents

The amount of phenolic total from the extracts was determined according to the Folin-Ciocalteu procedure with a few *in house* modifications [10]. Samples (200 uL) were introduced into test tubes with 1.0 mL of Folin-Ciocalteu reagent (1:1 v/v) and 2.5 mL of sodium carbonate (20%). The mixture was incubated for 30 minutes at room temperature and allowed to stand for 30 minutes. The absorbance from the blue colored mixture was measured at 765 nm (Gene Quant 1300, GE Healthcare). The amount of total phenol was calculated as milligrams (mg) of Gallic Acid Equivalents (GAE)/g of dry mass from calibration curve of Gallic acid standard solution. For the Gallic acid, the curve absorbance *versus* concentration is described by the equation $y= 1.5221x + 0.0081$ ($r^2= 0.9712$).

2.3.2 Determination of total flavonoids contents

The flavonoid content was measured by the aluminum method using chloride quercetin as reference compound [11,12]. This method is based on the formation of a flavonoid-aluminum complex with maximum absorbance at 415 nm. Approximately 100 µl of the methanol extract (10 mg/ml) was mixed with 100 µl of Aluminum trichloride ($AlCl_3$) at 20% in methanol and a drop of acetic acid, and then diluted in 5 mL of methanol. A reading was held at the spectrophotometer at an absorbance of 415 nm after 40 minutes. The blank samples were prepared from 100 µl of plant extracts and a drop of acetic acid and then diluted to 5 ml in methanol. The absorbance of the standard solution of quercetin (0.5 mg/ml) in methanol was measured under the same conditions. The amount of flavonoids from plant extract in equivalent of quercetin (EQ) was calculated by the following formula: $\mathbf{X = (A.m_0)/ (A_0.m),}$ where, **X** is the flavonoid content, mg/mg of the plant extract in EQ, **A** is the absorbance of the plant extract solution, $\mathbf{A_0}$ is the absorbance of the standard solution of quercetin, **m** is the weight of plant extract (mg) and $\mathbf{m_o}$ is the weight of the quercetin solution (mg) [13].

2.3.3 Determination of total tannins contents

The analyses of tannins content in fruits and vegetables were performed according to The International Pharmacopoeia and AOAC method [14,15].

2.3.4 Determination of total coumarins contents

Coumarins contents were evaluated by Borntrager reaction, a spectrophotometric method for the determination of the coumarins, based on the solubility of free coumarins derivatives in polar organic solvents and the solubility of their soluble alkali phenolates. Coumarin absorbs at 280 nm, however, ionization of phenolic hydroxyls in the molecule by alkaline hydroxide causes a bathochromic deviation to 320 nm, which is proportional to the coumarins concentration [16].

2.3.5 Determination of total sugars

The procedure to determinate the contents of soluble sugars in the extract of *C. leptophloeos* was based in proposed methodology [17]. Five standard glucose solutions were prepared, the concentration of each solution were obtained by dissolving the glucose (20, 40, 60, 80 and 100 mg) in flasks of 1 L each, then the final volume were obtained with distilled water. For the preparation of phenol solution was added 50 mL of distilled water in a glass Becker of 100 mL containing 5 g of phenol (PA). Then the solution was transferred to a volumetric flask 1 L and the final volume was completed with distilled water. For each extract sample (10 mg/mL), a 0.5 mL aliquot of each standard solution and 0.5mL of each sample was mixed in two test tubes, then 0.5mL of phenol solution 5% was added into the tubes and manually vigorously shook. Subsequently, 2.5 mL of concentrated sulfuric acid was added rapidly to the mixture, the tubes remained at rest until they come into equilibrium with the room temperature. Later, the samples absorbance was recorded at 490 nm spectrophotometrically; all determinations were performed in duplicate.

2.4 Antioxidant Screening

2.4.1 Total antioxidant capacity by phosphomolybdenum assay (P-Mo)

The total antioxidant capacity (%TAC) was evaluated by the method of Prieto *et al* 1999 [18]. An aliquot of 0.1 mL from the sample solution was combined with 1 mL of reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95 ºC for 90 minutes. After samples cooled down to room temperature, the absorbance was measured at 695 nm and compared to a blank template (1 mL of reagent and 0.1 mL of the solvent). Total antioxidant activity was expressed in relation of ascorbic acid and calculated by following formula: $\mathbf{\% TAC = (A_s - A_c) \times 100 / (A_{aa} - A_c)}$, where A_c stands for the absorbance of the control (blank template, without extract), A_s stands for the absorbance in the presence of the extract and A_{aa} was the absorbance of ascorbic acid.

2.4.2 β-carotene activity

Antioxidant activity of the *C. leptophloeos* compounds was determined according to modified version of the β-carotene method by Mueller and Boehm, 2011 [19]. β-carotene (0.1 mg) was added to a volumetric flask together with linoleic acid (20 mg) and Tween 40 (100 mg), all chloroform dissolved. After evaporation with an oxygenator, to this mixture free of chloroform were added 25 ml of the water saturated with oxygen for 30 minutes and stirred vigorously. The solution presented clear absorbance 0.7 at 470 nm, the solution displayed an orange color, was protected from light and used promptly. For each test was added 250 uL of this solution and 10 uL of methanol (control) or the same volume for the standard solutions and sample extracts. Readings of all samples were taken immediately (as time zero) and at 15 minutes' intervals for 120 minutes. The cuvettes were thermostated at 50°C between measurements. Absorbance of the sample was recorded at 470 nm spectrophotometrically; all determinations were performed in triplicate. Antioxidant activity (%AA) of the extracts was calculated using following formula: **%AA = [100**

(DR_c -DRs)/ DR_c], where: DR (Degradation rate); (a/b)/60 a = Absorbance of sample at 470 nm before incubation, b = Absorbance of sample at 470 nm after incubation, DR_c (Degra Degradation rate of the control sample), DR_s (Degradation rate of tested sample) and expressed as mg α-tocopherol Equivalent (mg ATE/g extract).

2.4.3 DPPH radical scavenging activity

This assay was executed using the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich)[20]. The DPPH radical scavenging was calculated using the following formula: **% scavenged [DPPH] = (A_c – A_s)/ A_c × 100,** where A_c: control absorbance and A_s: sample absorbance.

2.4.4 ABTS radical scavenging activity

To prepare the ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); Sigma-Aldrich) stock solution we used potassium persulfate solution and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) stock solution weighed at 192mg of ABTS, 378.4g of potassium persulfate and 25mg of trolox using analytical scale. The ABTS standard solution was dissolved in 50mL of water to a 7mM concentration, the potassium persulfate solution and trolox standard solution was dissolved in 10mL and 50mL of distilled water, respectively. ABTS radical cation ($ABTS^{++}$) was produced by reacting ABTS stock solution (5mL) with 88mL of potassium persulfate solution and allowing the mixture to stand in the dark at room temperature for 16 hours before use. From the standard solution of trolox (2.000µM), solutions were prepared in 10mL flasks, ranging the concentrations from 100µM to 3.000µM. To determine the standard-curve, 30µL of each trolox solution were transferred to a test tube then 3.0mL of ABTS radical solution was added, the tubes with the mixes were homogenized in an orbital shaker at 25°C temperature and 1300 rpm rotation. The reading was held after 6 minutes, and the ethyl alcohol was used as blank template to calibrate the spectrophotometer. To determine of total oxidant activity (AAT), 30µL of each extract dilution was transferred to test tubes containing 3.0mL of $ABTS^{++}$ and mixed in a tube shaker at 25°C temperature and 500 rpm rotation. Then the absorbance was measured at 734nm using the spectrophotometer and ethyl alcohol as blank control. The ABTS radical reaction with the antioxidant occurs over a period span from 1 to 6 minutes.

2.4.5 (TPTZ) 2,4,6- Tripyridyl-S-Triazine assays

The principle of this method is based on the reduction of a Ferric-Tripyridyltriazine complex to its ferrous, with color formation in the presence of antioxidants compounds. The FRAP reagent contained HCl solution in 40 mM/L, TPTZ solution (2,4,6-tripyridyl-s-triazine; Sigma Aldrich) in concentration of 10 mM/L, 20 mM/L of $FeCl_3$ and 0.3 mM/L of Acetate buffer in pH 3.6. For the determination of the standard curve in a dark environment was transferred an aliquot of 90 µL of each solution of ferrous sulfate (500µM, 1000µM, 1500µM and 2000µM) for test tubes, adding 270 µL of distilled water and 2,7 mL of the FRAP reagent. The solution was homogenized and kept in a water bath at 37° C until use. For determination of antioxidant activity was used 90 µL of plant extract instead of ferrous sulphate. The absorbance was measured at 595 nm and the FRAP reagent was used as blank template [21].

2.4.6 Superoxide radical scavenging assay

The SOD activity (Cu, Zn-SOD + Mn-SOD) was measured on the basis of the ability of these enzymes to inhibit the reduction of blue of nitroblue tetrazolium (NBT) by superoxide anion generated from the self-oxidation of hydroxylamine in alkaline pH [22]. For the tests, 20 μL of supernatant samples and 35 μL of solution containing chloride NBT at 572 μM and EDTA at 100 μM were added to a 96 well microplate. The reaction was initiated by adding 145 μL of sodium carbonate solution at 500 mM (pH 10,2) containing 51 mM of sodium hydroxylamine chloride. The NBT reduction by superoxide anion to blue formazan product was measured spectrophotometrically as a constant increase of absorbance at 560 nm ($r^2 > 0.98$) within 30 minutes in intervals of 5 minutes each. The rate of NBT reduction in the absence of the extract was used as a reference. One unit of SOD was defined as the enzyme activity able to inhibit NBT reduction by 50% of the reference value.

2.4.7 Hydrogen peroxide radical scavenging assay

Different concentrations of each extracts were dissolved in 3.4 mL of phosphate buffer (pH 7.4; 0.1 M) and mixed with 600 mL of hydrogen peroxide (43 mM). The absorbance value (at 230 nm) of the reaction mixture was recorded after 10 min. For each concentration, a separate blank sample was used for background subtraction [21]. The scavenging activity was measured by the following formula: **% scavenged [H_2O_2] = (A_c - A_s)/ A_c x 100**, where A_c stands for the absorbance of the control (blank, without extract) and A_s stands for the absorbance in the presence of the extract.

2.4.8 Nitric oxide radical scavenging assay

Nitric oxide (NO) was generated from sodium nitroprusside and measured by the Greiss reaction [23]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitric ions that can be estimated by using Greiss reagent. Scavengers of NO compete with oxygen, leading to a reduction in the production of NO. Various concentrations of the tested extracts were mixed with sodium nitroprusside 10 mM in phosphate buffer saline (PBS) pH 7.4, in final volume of 200 uL and incubated at 25 ºC for 150 minutes. Afterwards, the samples were added to Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride) and the absorbance was measured at 540 nm. Ascorbic acid was used as a positive control. The percentage of inhibition was measured by the following formula: % inhibition = **(A_c – A_s) /A_c x 100**, where A_c stands for the absorbance of the control (blank, without extract) and A_s stands for the absorbance in the presence of the extract, expressed in percentage of Ascorbic Acid equivalent (%AA).

2.4 Cell cytotoxicity assay

For macrophages *in vitro* assay, whole blood (5 mL) was obtained from healthy, non-smoking volunteers by venipuncture, after obtaining written informed (National Ethics Committee reference number: 30667014.5.0000.5208). The Peripheral Blood Mononuclear Cells (PBMC) was obtained from whole blood using the Ficoll-quantum method [24,25]. Macrophage colony-stimulating factor (M-CSF) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) were used for differentiation of

primary human monocytes to macrophages cells in 6 days and applied in subsequent trials.

Human macrophages cells were seeded at a density of 5×10^6 cells/well into a 96-well plate in DMEM culture medium supplemented with 10% FBS and incubated at 37°C, in a humidified 5% CO_2 atmosphere overnight. After incubation, DMEM was replaced by DMEM 10% FBS containing 0.5, 1, 5, 10, 25, 50, 100, and 200 µg/mL of *C. leptophloeos* extracts, except in control, where the culture medium was replaced by fresh DMEM. Cells were incubated for 24 h and 48 h, at 37°C, in a humidified 5% CO_2 atmosphere. Afterwards, the culture medium was replaced by 100 µL of fresh DMEM along with 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) per well and incubated in the dark, for 4 h, at 37°C, in a humidified 5% CO2 incubator. Negative control consisted of untreated cells, MTT and solubilizing buffer, positive control consisted in medium without cell, MTT and solubilizing buffer and blank consisted in dead cells (Triton-X 0.1- 1%), MTT and solubilizing buffer. Finally, recorded absorbance at 570 nm by ELISA microplate reader.

2.7 Statistical analysis

Each experiment was performed in biological duplicates and technical triplicates and results are presented as means and ± standard deviation (SD). Statistical analysis of all data were performed using GraphPad Prism version 5.0 with $p<0.05$ and 95% CI considered significant. The results were analyzed by analysis of variance (ANOVA) followed Bonferroni and Student's t-test. The concentration needed for 50% inhibition (IC50) was estimated graphically by linear regression analysis and the correlation indices were calculated using the Pearson coefficient (r).

3. Results

3.1 Quantitative Phytochemical Screening

C. leptophloeos quantitative phytochemical prospection revealed the presence of secondary metabolites such as phenolic contents, tannins, coumarins, flavonoids and reducing sugars (Table 1). CLAQE presented the major contents of phenolic compounds CLAQE (33.64 ± 0.5 mg GAE/g), followed by CLMEE (20.3 ± 0.78 mg GAE/g), CLCHE (15.58 ± 0.53 mg GAE/g), CLCLE (12.54 ± 0.55 mg GAE/g), and CLAEE (11.37 ± 0.04 mg GAE/g). On the other hand, for tannins contents showed: CLCLE (9.70 ± 0.2 mg TAE/g), CLMEE (7.68 ± 0.15 mg TAE/g), followed by CLAQE (4.12 ± 0.1 mg TAE/g). Only three *C. leptophloeos* extracts showed some coumarins content: CLCHE (1.14 ± 0.81 mg BZE/g), CLCLE (1.17 ± 0.07 mg BZE/g) and CLMEE (2.30 ± 0.10 mg BZE/g). Our study indicated the presence of high content of flavonoid compounds, being that: CLAQE (19.3 ± 0.70 mg QE/g), CLCLE (17.9 ± 0.08 mg QE/g), CLCHE (15.7 ± 0.2 mg QE/g), CLAEE (14.2 ± 0.13 mg QE/g) and CLMEE (12.1 ± 0.45 mg QE/g). All extracts analyzed showed relevant amounts of soluble sugars, the results showed that CLMEE presented 53.30 $g.100g^{-1mg}$, followed by CLAQE with 50.20 $g.100g^{-1mg}$, 40.13 $g.100g^{-1mg}$ for CLAEE, CLCLE with 30.22 $g.100g^{-1mg}$, and the CLCHE presenting 32.00 $g.100g^{-1mg}$, data showed in table 1.

3.2 Antioxidant Screening

From the %TAC of *C. leptophloeos* it can be observed that all extracts showed a significant antioxidant activity for both concentrations of 1-10 mg/mL (≥100% of phosphomolybdenum inhibition, *p*-value <0.001), and not significant difference between the extracts with high similarity to the control. However, there is a significant difference between the CLAQE and CLCLE (Figure 1B), with concentration ranging from 70.30 ± 0.01 to 77.10 ± 0.03 mg ATE/g, respectively. In DPPH radical scavenging assay, all extracts showed high percentage of capture compared to standard at concentrations ranging from 10-100 µg/mL, there is not statistically significant difference in radical sequestration among the evaluated extracts. At the concentration of 25 µg/mL, CLMEE showed greater effect when compared with others. Already CLAQE showed higher antioxidant action and DPPH radical capture in concentrations of 50 µg/mL and 75 µg/mL when compared with other extracts evaluated (Figure 1C). ABTS data shown in figure 1D at a concentration of 1 µg/mL, the best antioxidant activity was observed in 120 minutes, where there is no statistical difference among all extracts tested. However, at all times analyzed, CLMEE, CLCLE and CLAEE showed significant antioxidant activity when compared to other tests. Between 45-60 minutes, the CLAQE showed the major potential, with a significant difference compared to the CLMEE (Figure 1D). In the concentration of 10 µg/mL all extracts, except CLMEE showed high antioxidant potential for all times (Figure 1E). In table 2, FRAP assays showed that CLMEE had the highest antioxidant potential with 224.36 ± 0.12 µg/mL TEAC, followed by CLAQE (86.335 ± 0.54 µg/mL TEAC), CLCHE (69.840 ± 0.24 µg/mL TEAC), CLAEE (63.940 ± 0.12 µg/mL TEAC) and CLCLE (57.850 ± 0.15 µg/mL TEAC). The inhibition SOD radical was achieved in the following order: CLCLE (45.7 ± 0.55 µg/mL SOD), CLAQE (35.32 ± 0.33 µg/mL SOD) CLAEE (28.63 ± 1.33 µg/mL SOD), CLCHE (21.41 ± 2.3 µg/mL SOD) and CLMEE (10.87 ± 1.22 µg/mL SOD). Our results showed that % H_2O_2 scavenging activity from concentration of 100 µg were higher for CLCHE (126.71 ± 1.7 %), followed by CLAQE (27.02 ± 0.05 %), CLAEE (25.22 ± 0.28 %), CLCLE (24.98 ± 0.2 %) and CLMEE (15.53 ± 4.18 %). In NO radical scavenging assay CLAEE (111.88 ± 2.4 %AA) demonstrated higher antioxidant capacity, followed by CLCHE (99.46 ± 1.2 %AA), CLAQE (85.12 ± 0.02 %AA), CLMEE (56.39 ± 0.08 %AA) and CLCLE (50.22 ± 1.56 %AA), when compared with the standard used (166.4 ± 10.2 %AA), all data showed in table 2.

3.3 Cell Cytotoxic and Viability

The cytotoxic activity of each extract was performed using human macrophages and they showed low cytotoxicity. Even at the highest tested concentration (1, 10 and 100 µg/mL), all the extracts induced low levels of cell death (feasibility of more than 80%) by 24-hour period, showed in figure 2. In addition, these cytotoxic values were not correlated with either phenolic content (r = -0.15, *p*-value < 0.05) or flavonoids (r = -0.20, *p*-value < 0.05), these phytochemicals contents are shown in Table 1.

4. Discussion

4.1 Phytochemical profile

The Commiphora genus, natural order Burseraceae, currently lacks body of scientific literature, even though the presence of bioactive compounds in medicinal Caatinga

plants have already been reported. Quantitative phytochemical analysis clearly demonstrated the presence of number important active constituents from C. leptophloeos with different phenolic and flavonoid contents which can be correlated with each biological activity, as well as tannins, coumarins and reducing sugars, secondary metabolites used by the plants for their defense. Tannins are natural polyphenols found in plants, responsible for prevention of microbial infections [26] and provides protection against ionizing radiation, avoiding the formation of free radicals from the action of ionizing radiation [27]. In general, coumarins present important anti-inflammatory and its antioxidant effects are due to the fact that they are phenolic acids derived from cinnamic acid, [28,29] still have antibacterial potential [30], and are associated with tannins content that may influence upon antimicrobial activity. Soluble and reducing sugars are substances that have aldehyde organic function, being important reducing agents when reacting with alkaline metals. Therefore, they were quantified to determine its relationship with the antioxidant activity of *C. leptophloeos* extracts. Its reducing capacity is somehow due to a free hydroxyl in its composition. All extracts analyzed showed relevant amounts of soluble and reducing sugars, which implies to direct correlation with the great antioxidant property shown in previous assays. The measurement of total soluble sugars is important because it can predict the potential of reducing sugars of our extract.

4.2 Antioxidant Screening

TAC shows the potential of total inhibition of extracts front the formation of various free radicals [31,32]. Biologically, this activity reflects the combined action of ascorbic acid, β-carotene, α-tocopherol, bilirubin and other plasma antioxidants such as polyphenols and flavonoids - present in large amounts in our extracts and shown in table 1. The extracts do not have a concentration dependency to present their actions (Figure 1A). From our findings, our %TAC described in figure 1A shown to be correlated with the total phenols and flavonoids, and although not concentration dependent which explains much of our potential antioxidant described in other tests ($r = 0.12$, p-value < 0.05). Here, a positive correlation is observed between the results obtained for the CLCHE and CLMEE against lipid peroxidation and β-carotene autoxidation (Figure 1B) and CLMEE in FRAP assay with substantial interference in the ion reduction (Table 2). The protection percentage by the β-carotene assays reveled that all extracts showed similar antioxidant effect to control, ≥100%, data shown in figure 1B. The values observed for the DPPH assay can measure the antioxidant capacity of our extracts based on the action power of different substances in *C. leptophloeos* in the kidnapping of $^{\bullet}OH$ radicals (Figure 1C). This antioxidant potential is very well characterized allied to measure the ability of the extracts to donate electrons and stabilize whole radical, and this is seen in our results from ABTS radical scavenging assay. Note be compared between the two assays (Figures 1D-E), the effect of *C. leptophloeos* extracts is not dependent on the concentration, for the 10 µg/mL observed effect is statistically lower than 1 µg/mL. Thus, we can understand that the action of the extracts is dependent on the time of action, and not just of the concentration tested, once that, when the concentration is tenfold increased, the effect is not enhanced at a significant difference over time. In FRAP assays, our tests showed a high potential for reducing $Fe^{+}3$-TPTZ complex reflecting various reaction mechanisms related to the iron synthesis, highlights for CLMEE (224.36 ± 0.12 µg/mL TEAC). These data reflect the potential of our extracts in stabilizing highly reactive radicals,

offering a prospection of this capacity *in vivo* [33,34]. This potential can be attributed to the action of phenols and flavonoids which provide greater reducing power in our extracts (Table 2). The high activity of the SOD radical is associated with increased production of ROS, for example, during the process of ischemia and reperfusion, represented by lipids against oxidative damage [35]. Our results show not only a high potential for reducing SOD radicals, but also shows a moderate correlation between the rate of decline of these radicals and capture of H_2O_2 radicals ($r = 0.72$), featuring a potential antioxidant activity of the compounds of *C. leptophloeos*, data shown in table 2. Elevated SOD activities result in increased production of H_2O_2 which can stimulate catalase activity, supporting and increasing oxidative stress [36]. In H_2O_2 scavenging radical, the high percentage of capture is seen to CLCHE and CLAQE and be correlated with the phytochemical compounds present in these plant (Table 2). H_2O_2 in µM amounts has a low oxidation potential, however, in states of great oxidative stress, H_2O_2 is likely to generate the $^{\bullet}OH$ radical, highly reactive. Besides being a oxidant radical, H_2O_2 has a cytotoxic effect on cells, particularly deregulating energy and cellular respiration pathways for the generation of hydroxyl radicals in the cell interior [37]. However, phenolics compounds accelerate the process of converting H_2O_2 to H_2O [38,39]. In our studies, a large percentage of elimination of the peroxide is directly correlated with phenols contents from CLAQE (33.64 ± 0.5 mg GAE/g) and CLCHE (15.58 ± 0.53 mg GAE/g) (p-value < 0.01), as shown in Table 1-2. During hypoxia, mitochondrial respiratory chain produces other main type of oxidant radical: Nitric Oxide (NO), reactive nitrogen radicals that leads to lipid peroxidation [40,41]. In the hydrophobic core of the cell membrane, flavonoids prevent access of oxygen radicals inside the cell, protecting not only the structure more membrane function. By acting on basic mechanisms such as signal transduction and cell interaction, such polyphenols are capable of interacting with several enzymes, including xanthine oxidase (XAO) and nitric oxide synthases (NOS); and flavonoids have been reported as inhibitors of the action of these enzymes [42,43]. Preliminary studies shown the potential and as the flavonoids signaling pathways such as MAPK and acting with specific interaction on the path of NADPH oxidase [40,44]. Our results showed a moderate catch rate of NO radical by the extracts (Table 2). The best result obtained by CLAEE (111.88 ± 2.4 %AA) with a value close to that obtained by the standard of ascorbic acid (166.4 ± 10.2 %AA), and a positive correlation ($r = 0.24$, p-value < 0.05) was observed between these tests and the flavonoids content of our extracts, data shown in table 1-2.

4.3 Cell Cytotoxic and Viability

The equilibrium between the therapeutic and toxicological actions of a natural compound is of paramount importance to measure its use as security as a natural drug. Previous studies showed that plants of Commiphora genus not only had no cytotoxic effect, as presented as large margin between the dose required for its pharmacological effect and that sufficient to cause damage [45,46]. Based on our results and in order to characterize the cytotoxic activity of these compounds, we assessed the toxicological studies of the CLCLE and CLMEE in an *in vitro* assay system using human macrophages cells and were found to be nontoxic. Three concentrations (1-100 µg/mL) were tested in this assay at 24hours of treatment, where all concentrations not present significant toxic effect or wide effect on cell viability in the first 24 hours of exposure to the extracts. This data is very important because we can see that the doses that confer

antioxidant activity tested here do not have cytotoxic potential, confirming its insurance use.

5. Conclusion

In this study we identified that the barks extracts from *C. leptophloeos* contain an important amount of phenolic compounds, exhibit high antioxidant activity, free radical scavenging and *in vitro* lipid peroxidation inhibition activities. We also identified a direct correlation between the total phenolic content and the antioxidant activities using different *in vitro* antioxidant models. Altogether, we show for the first time that the bark extracts from *C. leptophloeos* are relevant sources of antioxidants, which might be useful in preventing the progress of various oxidative processes.

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TABLE 1: Phytochemical analyses of extracts from barks of *Commiphora leptophloeos*.

Commiphora leptophloeos **Extract**	**Phenolic contents**	**Tannins**	**Coumarins**	**Flavonoids**	**Reducing sugars**
CLAQE	33.64 ± 0.5	4.12 ± 0.1	-	19.3 ± 0.70	50.20
CLMEE	20.3 ± 0.78	7.68 ± 0.15	1.17 ± 0.07	12.1 ± 0.45	53.30
CLCLE	12.54 ± 0.55	9.70 ± 0.2	2.30 ± 0.10	17.9 ± 0.08	30.22
CLCHE	15.58 ± 0.53	-	1.14 ± 0.81	15.7 ± 0.2	32.00
CLAEE	11.37 ± 0.04	-	-	14.2 ± 0.13	40.13

CLAQE, *C. leptophloeos* aqueous extract; CLMEE, Methanolic extract; CLCLE, Cloroform extract; CLCHE, Cyclohexane extract and CLAEE, Ethyl acetate extract. Phenolic contents are expressed in mg/g of Gallic Acid Equivalent (GAE). Tannins are expressed in mg/g of Tannic Acid Equivalent (TAE). Coumarins are expressed in mg/g of Benzopyrone Equivalent (BZE). Flavonoids are expressed in mg/mL of Quercetin Equivalent (QE). Reducing sugars are expressed in $g.100g^{-1mg}$. Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD and p-value < 0.05.

TABLE 2: Antioxidant values of *Commiphora leptophloeos*.

Commiphora leptophloeos **extracts**	**FRAP assays** (TEAC)	**Superoxide Dismutase assays**	**H_2O_2 assay**	**Nitric oxide assays** (% AA**)**
CLAQE	86.335 ± 0.54	35.32 ± 0.33	27.02 ± 0.05	85.12 ± 0.02
CLMEE	224.36 ± 0.12	10.87 ± 1.22	15.53 ± 4.18	56.39 ± 0.08
CLCLE	57.850 ± 0.15	45.70 ± 0.55	24.98 ± 0.2	50.22 ± 1.56
CLCHE	69.840 ± 0.24	21.41 ± 2.3	126.71 ± 1.7	99.46 ± 1.2
CLAEE	63.940 ± 0.12	28.63 ± 1.33	25.22 ± 0.28	111.88 ± 2.4
TRO	80 ± 0.01	-	23.29 ± 1.22	-
AA	90.50 ± 0.04	-	-	166.4 ± 10.2
QC	-	-	-	-
SOD	-	1.65 ± 0.05	-	

CLAQE, *C. leptophloeos* aqueous extract; CLMEE, Methanolic extract; CLCLE, Cloroform extract; CLCHE, Cyclohexane extract and CLAEE, Ethyl acetate extract. TRO, Trolox. AA, Ascorbic acid. QC, Quecertin. SOD, Superoxide dismutase enzyme. TEAC, Trolox Equivalent Antioxidant Capacity. % GAE, Gallic Acid Equivalent. % AA, Ascorbic Acid Equivalent. ABTS assays contents are expressed in µg/mL of TEAC. FRAP assays are expressed in µg/mL of TEAC equivalents. β-carotene bleaching assay are expressed in µg/mL of QC. Superoxide dismutase assays are expressed in µg/mL of SOD. DPPH assays are expressed in µg/mL of TEAC. P-Mo are expressed in % GAE. H_2O_2 assay are expressed in % H_2O_2 scavenging. Nitric oxide assays are expressed in % AA. Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD and p-value < 0.05.

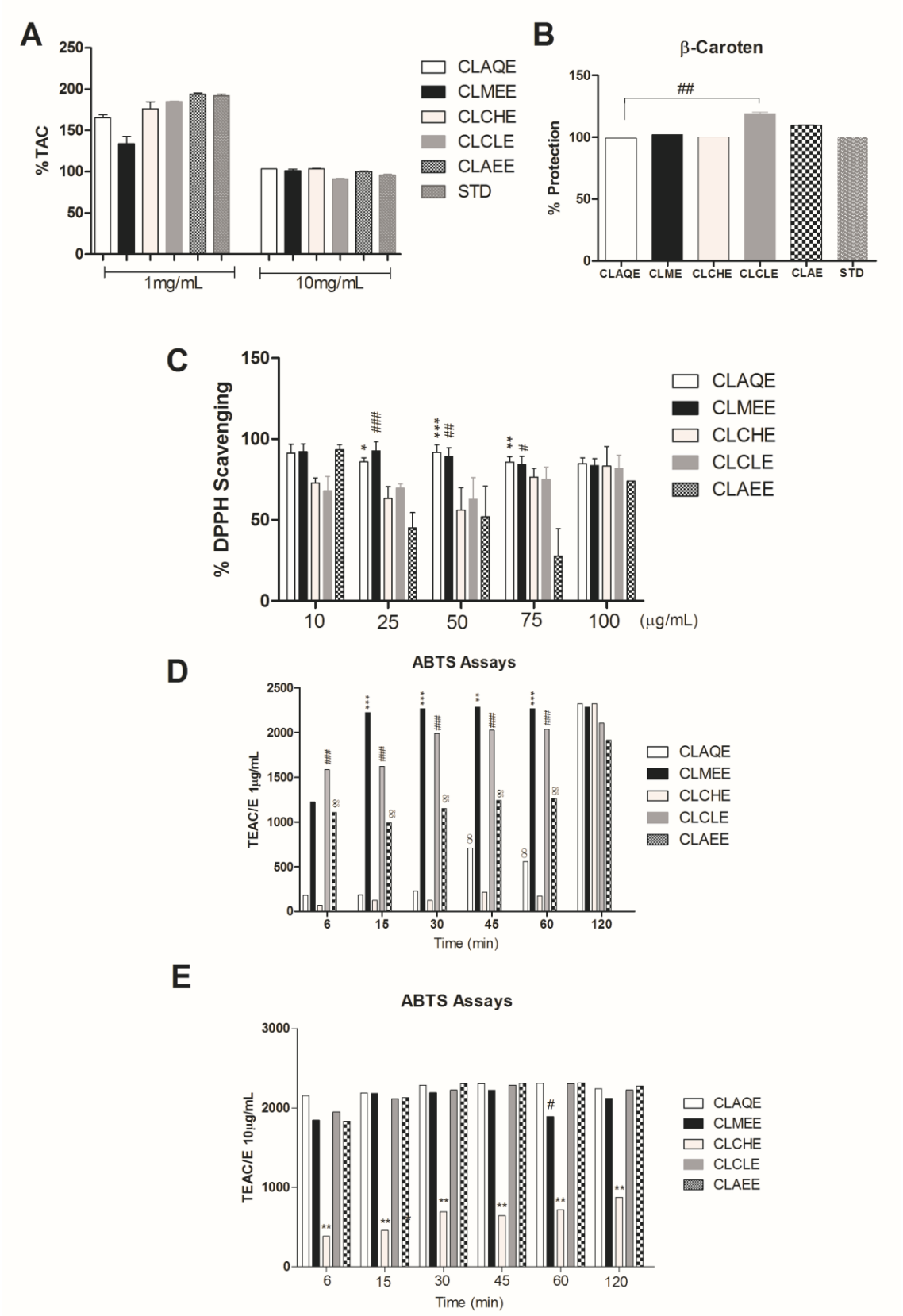

FIGURE 1: Antioxidant values of *Commiphora leptophloeos*. CLAQE, *C. leptophloeos* aqueous extract; CLMEE, Methanolic extract; CLCLE, Cloroform extract; CLCHE, Cyclohexane extract and CLAEE, Ethyl acetate extract. TRO, Trolox. AA, Ascorbic

acid. QC, Quercetin. TAC, Trolox Equivalent Antioxidant Capacity, in 1mg and 10mg % GAE, Gallic Acid Equivalent. % AA, Ascorbic Acid Equivalent. ABTS assays contents are expressed in µg/mL of TEAC. **A**. Results of total antioxidant (TAC) evaluation by P-Mo and expressed in % GAE; **B.** Protection percentage by the β-carotene assays β-carotene bleaching; **C.** DPPH radical scavenging assay, all extracts showed high percentage of capture compared to standard at concentrations of 10 µg/mL and 100 µg/mL there is no statistically significant difference in radical sequestration among the evaluated extracts, DPPH assays are expressed in µg/mL of TAC; **D-E.** ABTS values in this study, shown in figure 1D at a concentration of 1 µg/mL, the best antioxidant activity was observed in 120 minutes, where there is no statistical difference among all extracts tested. Data were obtained from three independent experiments; each performed in triplicates (n = 9) and represented as mean ± SD and p-value < 0.05.

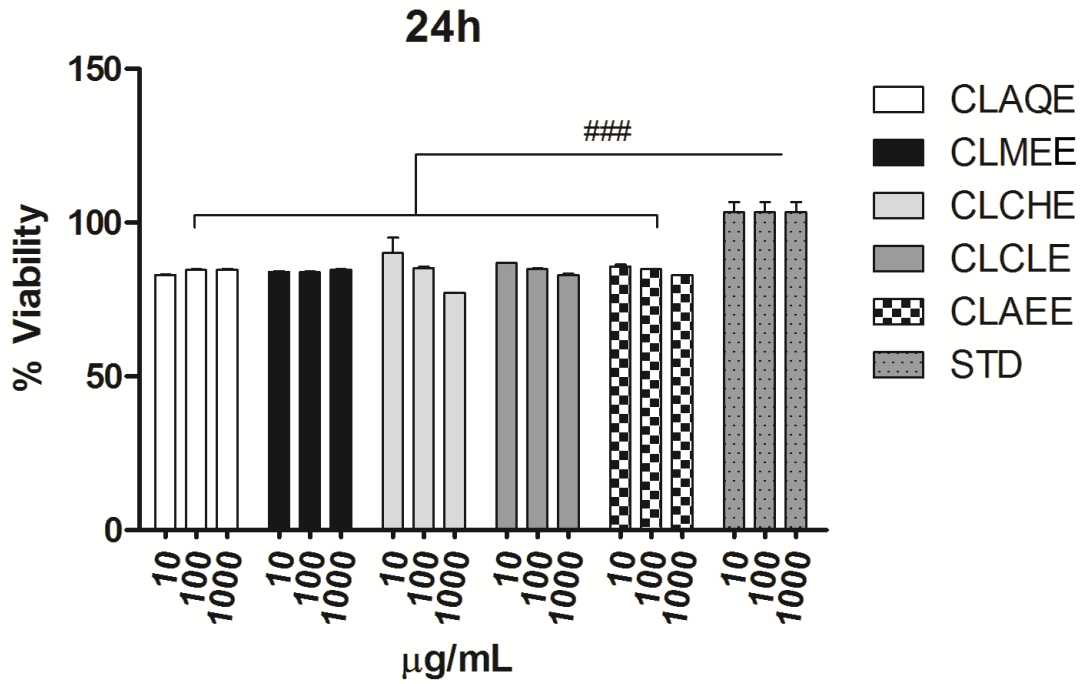

FIGURE 2: Effects of *Commiphora leptophloeos* extracts on viability of human macrophages cells according to MTT assay. Viability was assessed at three concentrations for all extracts (1, 10 and 100 µg/mL) were tested in this assay at 24hours of treatment, where all concentrations not present significant toxic effect or wide effect on cell viability in the first 24 hours of exposure to the extracts. The results for the control group cultured without extracts were shown on the right (STD). CLAQE, *C. leptophloeos* aqueous extract; CLMEE, Methanolic extract; CLCLE, Cloroform extract; CLCHE, Cyclohexane extract and CLAEE, Ethyl acetate extract. All experimental data were presented as average values ± SD, $n = 4$ and p-value < 0.05.

Capítulo 02

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***Commiphora leptophloeos* Phytochemical and Antimicrobial Characterization**

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Abstract

Commiphora leptophloeos is usually known for its medicinal purposes from local communities in Northeast Brazil. The qualitative phytochemical analysis by TLC of *C. leptophloeos* extracts detected the presence of phenolic compounds, flavonoids and reducing sugars. H^1NMR analysis revealed the presence of hinokinin, a bioactive lignan characterized in this article. The phenolic compounds were identified by HPLC and three were described: Gallic, Chlorogenic and Protocatechuic acids. The MIC values of hinokinin ranged from 0.0485 to 3.125 mg/mL in different *S. aureus* clinical isolates, and showed a bactericidal activity against MRSA isolated from blood (MMC 0.40 mg/mL) and postoperative secretion (MMC = 3.125 mg/mL). *C. leptophloeos* extracts showed antimicrobial activity against Mycobacterium species such as *M. smegmatis* (MIC = 12.5 mg/mL) and *M. tuberculosis* (MIC = 52 mg/mL). Herein we also determined *C. leptophloeos* toxicity by HC_{50} tests *in vitro* with hemolytic activity detected of 313±0.5 µg/mL. The present findings showed that *C. leptophloeos* possesses inhibitory properties against MRSA as well as various Gram-positives, Gram-negatives and Fungi microorganisms, and should also be investiagated for its potential against Mycobacterium. Furthermore, we have identified for the first time the presence of hinokinin in Commiphora genus.

Key-words: Imburana; Multidrug resistance; Hinokinin; Natural Products.

1. Introduction

The discovery of antibacterial agents was a monumental event in medicine and represented a historic moment in human health. However, the widespread misuse of these drugs has brought to light two problems involving treatment of bacterial infections: the emergence of Multidrug-resistance Organisms (MDROs) and the existence of bacteria that are innately resistant to most antibiotics. The rising number of MDROs is an imminent threat worldwide with Methicillin-resistant *Staphylococcus aureus* (MRSA), who acquired a gene that makes them resistant to all β-lactam antibiotics, a recurrent example but not the only one. Tuberculosis (TB) has been accompanying human history for a quite long time now and still remains as a public health issue. In 2015, TB became the deadliest infectious disease in the world (WHO, 2015), 1 million children under the age of 14 years old fell ill with TB, 140.000 children died and over 53 million otherwise healthy children carry the TB bacillus. TB is a leading killer of HIV-positive people, in 2015, 1 in 3 HIV deaths was due to TB infection, and globally in 2014, an estimated 480 000 people developed multidrug-resistant TB (MDR-TB) (Venturini et al., 2014; WHO, 2015). The primary cause of MDROs is inappropriate treatment, beyond that, misuse of drugs or use of poor quality medicines, can also cause drug resistance (WHO, 2014).

A promising source for new antibacterial compounds is the natural products produced mainly by medicinal plants (Lawn et al., 2013; Zumla et al., 2013). Plant-derived antimicrobial compounds belong to an exceptionally wide diversity of classes, including terpenoids (Bhalodia et al., 2011), lignans (Teponno et al., 2016), alkaloids and peptides (Bhatter et al., 2016), phenolic compounds (Heleno et al., 2015) and coumarins (Bhatter et al., 2015, 2016). All of the aforementioned compounds are gathered as secondary metabolism products in plants, being the ones aiding in growth and development but not strictly required for plant survival. Secondary metabolism products are involved in plant advantage and usually confers a positive effect for its use as medicinal purposes (Harborne, 1997; Kroymann, 2011).

Commiphora leptophloeos, usually known as Imburana of Sertão, belongs to the *Burseraceae* family including trees and shrubs from tropical and subtropical regions, and is traditionally used by indigenous tribes as an infusion, tea or syrup for the treatment of their infirmities such as infectious and inflammatory ones (Bennett and Prance, 2000; Silva et al., 2011). The aim of the present study included characterization of *C. leptophloeos* extracts, isolation of biomolecules and fractions with antimicrobial activity, and analysis of possible toxic effect in human blood cells.

2. Materials and Methods

2.1 Biological material (Plant)

The stem bark of *C. leptophloeos* was collected at *Parque Nacional do Catimbau*, Pernambuco – Brazil. The authors confirm that the named authority *Instituto Chico Mendes de Conservação da Biodiversidade* granted permission (SISBIO 16806) for our described field searches. The botanical identification and the deposition of plant specimens were performed at the Herbarium of the Institute of Agricultural Research of Pernambuco (IPA-PE) (IPA nº 84037).

2.2 Preparation of the extracts

The dried bark (25 g) of *C. leptophloeos* was obtained by saturation in order of increasing polarity: submitted to Cyclohexane (CLCHE), Chloroform (CLECL), Ethyl Acetate (CLAEE), Methanolic (CLMEE) and Aqueous (CLAQE) (250 mL) by agitation at 180 rotations per minute (rpm). After 24 hours, the extract was filtered (Whatman® number 2) and concentrated at 45ºC under vacuum in a rotary evaporator (Concentrator 5301, Eppendorf®). The powder produced was kept at -20°C for future use. For phytochemical and antimicrobial analysis, the extracts were dissolved in your respective solvents at the concentration of 100 mg/mL for all biological assays.

2.3 Phytochemical analysis

2.3.1 Determination of phenolic acid compounds by HPLC

For the determination of phenolic acids, the extract powder (0.5 g) was diluted in methanol: water (20%, v/v) at ultrasonic bath sonicator for 30 minutes. Then the extracts were filtered and passed through a SPE C18 cartridge with the following solvents: acetone, trichloroacetic acid, water (4%, v/v) and methanol. After evaporation in a rotary evaporator (Concentrator 5301, Eppendorf®), the samples were re-suspended in methanol. The qualitative analysis of phenolic content of each extract was performed by UFLC (Ultra-Fast Liquid Chromatographic - LC-20AD, Shimadzu). Separations were conducted on a XR ODS, 50 X 3.0 X 2.2 µm column. The elution was performed with water: acetonitrile: methanol: ethyl acetate: glacial acetic acid (86:6:1:3:1, respectively). The column temperature was set at 40°C and the flow rate was 0.4 mL/minutes for 5 minutes. Prior to injection, sample extracts (200 µL) were filtered with PTFE syringe 0.22 µm filters (Phenomenex, UK). Phenolics in each bark extract were identified by comparison of their retention times with corresponding standards and by their UV spectra obtained with the diode array detector - DAD (SPD-M20A). Gallic acid, vanillic acid, protocatechuic acid, chlorogenic acid, coumaric acid, ferulic acid, quercetin and rutin were used as standard compounds. The linear regression equation for each standard curve was obtained by plotting the amount of standard compound injected against the peak area.

2.3.2 Qualitative Phytochemical analysis by TLC

An aliquot of 100 μL of each *C. leptophloeos* extracts was subjected to qualitative phytochemical analysis to ascertain the presence of secondary metabolites such as: coumarins (Gocan and Cimpan, 2007), flavonoids (Garcia et al., 1993), tannins and phytosteroids (Pascual et al., 2008), reducing sugars (Krishnamurthy et al., 2012) and saponins (Ng et al., 1994) respectively. 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 as Dragendorff test, NEU-PEG, KOH-Ethanol, Acetic Anhydride test, Vanillin-sulfuric acid, Quercetin, Tannic acid, Benzopyrone equivalent and others reagents, according to the respective method of elucidation.

2.3.3 Determination of total phenol content

The amount of phenolic total from the extracts was determined according to the Folin-Ciocalteu procedure with a few *in house* modifications (Singleton et al., 1965). Samples (200 uL) were introduced into test tubes with 1.0 mL of Folin-Ciocalteu reagent (1:1 v/v) and 2.5 mL of sodium carbonate (20%). The mixture was incubated for 30 minutes at room temperature and allowed to stand still for 30 minutes. The absorbance from the blue colored mixture was measured at 765 nm (Gene Quant 1300, GE Healthcare). The amount of total phenol was calculated as milligrams (mg) of Gallic Acid Equivalents (GAE)/g of dry mass from calibration curve of Gallic acid standard solution. For the Gallic acid, the curve absorbance *versus* concentration is described by the equation $y= 1.5221x + 0.0081$ ($r^2= 0.9712$).

2.3.4 Chromatographic analysis by Flash and CCD chromatography

Based on the initial phytochemical results and visualization of the presence of phytosteroids and lignans in qualitative tests, the CLCLE with 0.8g yield was then submitted to procedure of Flash Chromatography (Clark et al., 1978; Still, 2002) using silica gel 60 F254 (Merck, Germany) as the stationary phase and $CHCl_3$/MeoH (99.1) as the mobile phase. From this experiment, 45 fractions with 15 mL each were obtained. The fractions were submitted to Thin Layer Chromatography (TLC) in different groups depending on their chromatography patterns and similarity as follows: F1-F8, F9, F10-F14, F15-19, F20-26, F27-F28, F29-F38, F39-F42 e F44-F45. The fraction F9, that yielded 0.06 g was once submitted to silica gel column chromatography and 100 new fractions were obtained and analyzed following the data from Cambridge Crystallographic Data Centre (CCDC); among all fractions, F16 was the purest one and was the submitted to ^{1}H RMN.

2.3.5 Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy

The ^{1}H NMR spectra of F16 fraction were obtained in Mercury-Varian spectrometer using 200 MHz – ^{1}H. The solvent used was deuterated chloroform ($CDCl_3$) whose respective peaks in RMN ^{1}H were used to adjust the frequency scale.

2.4 Antimicrobial activities

2.4.1 Microorganisms and Inocula preparation

Fifteen microorganisms strains from eleven species (*Aspergillus sp*., *Bacillus subtilis*, *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, including clinical isolates of *Staphylococcus aureus*), obtained from microorganism collection of the Department of Antibiotics of Federal University of Pernambuco (UFPEDA), were used for the antimicrobial tests, according to table 1. The bacterial strains were cultured at 37°C for 18 hours in Mueller-Hinton Broth and the fungal cultures maintained in Sabouraud Dextrose Agar medium and incubated at 35°C for 24 hours. As to the preparation of the inoculum, the pricked out strains were transferred to the sterile saline solution (0.9% NaCl), composed of a bacterial/fungal suspension (inoculum) until obtaining the concentration of 10^6 UFC/mL according to the scale of McFarland. The susceptibility tests were performed by Mueller Hinton agar-well diffusion method (Davis and Stout, 1971; Hombach et al., 2013).

2.4.2 Minimum Inhibitory Concentration (CMI) and Minimal Microbicidal Concentration (MMC) tests

The CMI and MMC were determined on plant extracts that showed antimicrobial activity, by a broth microdilution method (Hombach et al., 2013; M. P. Weinstein, 2012). Briefly, 100 µL of Mueller-Hinton Broth, plus different concentrations of plant extracts were prepared and transferred to each microplate well to obtain dilutions of the active extract, ranging from 0.001 to 100 mg/mL. Then, 10 µL of a fresh culture (final concentration of 1×10^6 CFU/mL) of test organisms was added. Microplates were incubated at 37°C for 24 h and CMI was defined as the lowest concentration of the extract that restricted the visible growth of microorganism tested. To determine MMC, 100 µL from each well that showed no visible growth was reinoculated on MH agar plates; then the plates were incubated at 37°C for 24 h. MMC was defined as the lowest extract concentration showing no bacterial growth, DMSO was used as blank.

2.4.3 Antimycobacterial culture

The *M. smegmatis* and *M. tuberculosis* was maintained on Middlebrook 7H9 broth containing 0.05% Tween 80 and 10% (v/v) OADC (Oleic Acid, Albumin, Dextrose and Catalase) supplement. The culture screening was performed by Ziehl-Neelsen staining before used in the antimicrobial assays. Two-fold serial dilutions of CLCLE extract and Rifampicin were made with 100 µl of each; sterile distilled water and 7H9 Middlebrook culture medium for *M. tuberculosis* and *M. smegmatis* in plates of 96 well microplates. The plates were incubated at 37°C for 24 days. The developer (40µL) used was iodonitrotetrazolium (INT) of Sigma-Aldrich. Minimum Inhibitory Concentration (MIC) values were recorded as the lowest concentrations of extracts showing no growth, and bacterial growth in the wells was indicated by color change (Damato and Collins, 1990; Sadaphal et al., 2008).

2.4.4 Antimicrobial Activity of *C. leptophloeos* Hinokinin against selected MRSA clinical isolates

The study included four *S. aureus* strains obtained from Department of Antibiotics of Federal University of Pernambuco (UFPEDA). Colonies with macroscopic characteristics of antimicrobial susceptibility patterns from isolates were determined according to Kirby Bauer disc diffusion technique as described by CLSI (Hombach et al., 2013) and Minimum Inhibitory Concentration (CMI). The following three antibiotics were used to determine the antibiogram of the isolates: Tobramycin (10µg), Vancomycin (30 µg) and Cefoxitin (30 µg). Detection of methicillin resistant *S. aureus* (MRSA) was carried out using cefoxitin (30µg), an inhibition zone diameter of ≤ 21mm was reported as methicillin/oxacillin resistant and ≥ 22mm was considered as methicillin/oxacillin sensitive (CLSI, 2014; Hombach et al., 2013; Rabelo et al., 2014).

2.5 *In Vitro* Hemolytic Assays

For hemolytic *in vitro* assay, whole blood (5 ml) was obtained from healthy, non-smoking volunteers by venipuncture, after obtaining written informed consent (National Ethics Committee reference number 30667014.5.0000.5208). Human erythrocytes from citrated blood were immediately isolated by centrifugation at 1500 rpm for 10 min at 4 ºC. After plasma removal, the erythrocytes were washed three times with phosphate-buffered saline (PBS; pH 7.4) and then re-suspended using the same buffer, and a 1% erythrocyte suspension was prepared. The hemolytic activity of *C. leptophloeos* extracts was tested under *in vitro* conditions. Each tube received 1.1 mL of erythrocyte suspension and 0.4 mL of extract with different concentrations ranging from 50 to 500 µg/mL. The negative control was solvent only and the positive control received 0.4 mL of Quillaja saponin (0.0025%). After 60 min of incubation at room temperature, cells were centrifuged at 1500 rpm for 10 min and the

supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm length. The average value was calculated from triplicate assays. The hemolytic activity was calculated by the following formula: **Hemolytic activity (%) = (A_s- A_b) × 100/(A_c-A_b);** where A_b is the absorbance of the control (blank, without extract), A_s is the absorbance in the presence of the extract and A_c is the absorbance of saponin solution (Sulaiman and Gopalakrishnan, 2013).

2.6 Statistical analysis

Each experiment was performed in biological duplicates and technical triplicates and results are presented as means and ± standard deviation (SD). Statistical analysis was performed by Student's t-test and ANOVA tests. Differences were considered significant at $p < 0.05$. The concentration needed for 50% inhibition (IC_{50}) was estimated graphically by linear regression analysis.

3. Results

3.1. Phytochemical Analyses of *C. leptophloeos*

The qualitative phytochemical analysis of *C. leptophloeos* by HPLC detected the presence of three particular compounds: Gallic acid (GA), Chlorogenic acid (CGA) and Protocatechuic acid (PCA), as showed in Figure 1. The qualitative phytochemical analysis by TLC of *C. leptophloeos extracts* detected the presence of Phenolic compounds, Flavonoids and Reducing sugars in all extracts. Additionally, CLAQ presented Tannins, CLMEE presented Tannins, Coumarins and Saponin, CLCLE presented Tannins, Coumarins, Phytosteroids and Lignans, and CLCHE presented coumarins, as shown in table 2. The estimation of total phenolic content revealed that CLAQE (33.64 ± 0.5 mg of GAE/g) and CLMEE (20.3 ± 0.78 mg of GAE/g) exhibited the highest phenolic content ($p < 0.05$). The other extracts showed phenolic content values ranging from 13.8 ± 0.53 to 12.54 ± 0.55 mg of GAE/g, showed in Table 2. The fraction F16 from CLCLE studied shown the presence of hinokinin in our analysis and the spectrum ^{1}H NMR ($CDCl_3$, 200 MHz) of F16 from CLCLE showed two multiplets (δH 2.45 e 2.48) in high field, being referent to sp^3 hydrogens connected to neighboring carbons of chiral carbon (C-7 and C-7`). In δH 2.85 was observed a doublet of doublet (dd, J=4.7, 14.5 Hz,1H) of 1 H connected directly to chiral carbon C-8. In δH 3.00 the doublet of doublet (dd, J=4.7, 14.5 Hz,1H) of hydrogen 1 connected directly to the chiral carbon (C-8`). In δH 3.85 (dd, J= 7.0, 9.2 Hz,1H) and 4.15 (dd, J=6.2, 9.0 Hz,1H) two doublets of doublets regarding the hydrogens linked to the carbon C-9. In δH 5.9471 a multiplet in 4 H linked to the carbon C-10 and C-10', simultaneously. Finally, a 6H a multiplet in δH 6.5 assigned to aromatic hydrogens were identified. The spectrum elucidation is show in Figure 2. Hinokinin is one of the constituents of secondary metabolites of *C. leptophloeos* described for the first time in this species.

3.2 Antimicrobial Screening

The antibacterial activity of *C. leptophloeos* extracts was recorded against various microorganisms and is presented in Tables 3-5. Overall, all plant extracts exhibit a range of inhibitory potentials with broad spectrum, as they inhibited all bacteria and yeasts species tested. The better antimicrobial results observed were provided by CLMEE, in which MIC ranged from 0.097 mg/mL to 50.0 mg/mL (Tables 3 and 4). CLMEE presented better antimicrobial activities against Gram-positive bacteria with best results for *Bacillus subtilis* (MIC = 3.125 mg/mL), *E. faecalis* (MIC = 25 mg/mL) and *M. luteus* (MIC = 0.097 mg/mL and MMC = 12.5 mg/mL). Against *S. aureus* strain, the best bacteriostatic action was the CLCLE (MIC = 1.125 mg/mL) and, for this reason, we use F16 fraction purified *C. leptophloeos* hinokinin to evaluate the action of this lignan against MRSA strains (Table 5). *C. leptophloeos* also showed antifungal activity, such as *Aspergillus sp.* (MIC values ranged from 3.125 to 6.25 mg/mL) and *C. albicans* (MIC values ranged from 6.25 to 12.5 mg/mL) showed in Table 3. The antimicrobial activities against Gram-negative bacteria were showed in Table 4. CLCHE showed better bacteriostatic effect against *P. aeruginosa* (MIC = 6.25 mg/mL) and *K. pneumoniae* (MIC and MMC = MMC 12.5 mg/mL), CLAEE showed the better activity against *E. coli* (MIC and MMC = 12.5 mg/mL), and CLCLE was tested against *Mycobacterium*, indicating *M. smegmatis* (MIC = 12.5 mg/mL) to be more susceptible to CLCLE than *M. tuberculosis* (MIC = 52 mg/mL), Table 4. The MIC values of hinokinin (obtained from the purification F16 from CLCLE) ranged from 0.0485 to 3.125 mg/mL for the different *S. aureus* clinical isolates tested, and showed a bactericidal activity against MRSA isolated from blood (MMC 0.40 mg/mL) and postoperative secretion (MMC = 3.125 mg/mL) showed in Table 5.

3.3 *In vitro* Hemolytic assays

The concentration from CLAQE, CLMEE, CLAEE, CLCLEE and CLCHE extracts demonstrated a HC_{50} (concentration required for 50% of hemolysis) of 313 ± 0.5 µg/mL; 304.9 µg/mL ± 0.8; 287.49 µg/mL ± 3.0; 239.5 µg/mL ± 1.4 and 177.21 µg/mL ± 0.45, respectively.

4. Discussions

4.1 Phytochemical Analyses of *C. leptophloeos*

The Commiphora genus present bioactive compounds known and used as therapy for several pathologies in folk culture. Herein we performed a thorough phytochemical characterization from *C. leptophloeos*, showing its notable amounts of phenolic namely Gallic acid (GA),

Chlorogenic acid (CGA) and Protocatechuic acid (PCA). GA is endowed with pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial and antiproliferative activity (Kroes et al., 1992; Vishnu Prasad et al., 2010). The CGA has been recently claimed to modulate glucose and lipid metabolism *in vivo*, upon unbalanced metabolic conditions such as diabetes (Hwang et al., 2014; Lou et al., 2011; Sato et al., 2011; Zhao et al., 2012). PCA, major metabolite of anthocyanin, shows beneficial activities to human's health such as reduced risk of cardiovascular diseases (Wang et al., 2010), anti-inflammatory, antioxidant and free radical scavenging activities (Li, 2011), as well as estrogenic and antiestrogenic activity (Kakkar and Bais, 2014).

Our phytochemical characterization showed the presence of other bioactive compounds in *C. leptophloeos*, among them, an important class of lignans, named hinokinin, that has been recently investigated in order to establish its biological activities. Lignans are important components of foods and medicines biosynthetically deriving from the radical coupling of two molecules of coniferyl alcohol at C-8/C-8′ positions. In the past years, the biological activities of several lignans have been studied in depth (Aehle et al., 2011; Zhang et al., 2014) and among them, the hinokinin (Figure 2) is emerging as a new interesting compound with pharmacological potential. Hinokinin was isolated for the first time in 1933 by Yoshiki and Ishiguro from an ether extract of *Hinoki* wood - *Chamaecyparis obtuse* - as a colorless crystalline compound and over the years it was being gradually characterized and described by other researchers (Desai et al., 2014; Timple et al., 2013; Vanoeveren et al., 1994).

4.2 Antimicrobial Screening

C. leptophloeos shows an antibacterial activity against the human Gram-positive pathogens such as *B. subtilis*, *E. faecalis*, *M. luteus* and *S. aureus*, and all the extracts of Commiphora barks have shown the activity (Table 3). The inhibition of bacterial growth *in vitro* by the extracts of *C. leptophloeos* could be due to the presence of some active compounds including flavonoids, phenolic acids and tannins, described in table 2, known to be effective antimicrobial agents against a wide array of microorganisms. These active compounds may act alone or in combination to inhibit bacterial growth.

Our results against *M. luteus* strain evaluated in MIC ranged from 0.097 to 12.5 mg/mL, and the CLMEE which best showed bacteriostatic and bactericidal action (MMC = 12.5 mg/mL), particularly when compared to other studies with Commiphora genus (Latha et al., 2005) and when compared with the value MIC obtained by the aminoglycoside Kanamycin (MIC = 6.8 mg/mL). *M. luteus* is a naturally constituent of mammalian skin microflora, considered a nosocomial contaminant, mainly in immunodeficient individuals and cause meningitis (Fosse et al., 1985) and endocarditis (Miltiadous and Elisaf, 2011) in severe infections. This is a risk factor able to intensify selection and dissemination of multidrug resistance strains.

Regarding antifungal action of *C. leptophloeos*, we included *Aspergillus sp.* and *Candida albicans*, the most prevalent fungal species of the human microbiota associated to several complications in immunosuppressed individuals (Nobile and Johnson, 2015). *C. leptophloeos* extracts indicated efficient action against both tested species.

Gram-negative pathogens are particularly worrisome, due to their resistance to nearly all drugs firstly considered for treatment. The reason to their wide action relies on their several pathways to β-lactam resistance through β-lactamases enzymes production, therefore interfering with the mechanism of action of β-lactam antibiotics. This same premise has become more frequent in Gram-positive infections (e.g. Staphylococcus). *P. aeruginosa* is a ubiquitous opportunistic pathogen, having outer membrane cell structure conferring pronounced resistance to xenobiotics (Gaspar et al., 2013). Previous studies had already reported the action of the aqueous extract of *C. leptophloeos* inhibition of biofilms of *P. aeruginosa* (Trentin et al., 2013) and *S. epidermidis* (Trentin et al., 2011). According to these aforementioned studies, this greater potential of action is due the presence of tannins in CLCHE, as noted in our phytochemical profile (Table 2).

E. coli is a bacterium usually found in intestinal microflora, nevertheless, some can cause debilitating and sometimes fatal human diseases (Riley, 2014). Pathogenic strains are divided into intestinal pathogens causing diarrhea and extraintestinal mainly related to urinary tract infections pyelonephritis, cystitis, and urosepsis (Croxen et al., 2013). Only two fractions, the above-mentioned and the CLMEE, showed bactericidal action (MMC ranging 12.5 to 25 mg/mL), suggesting that this action may be due to the presence of high amounts of phenolic acids (Figure 1), such as GA, which might contribute to the inhibition *E. coli* strain.

K. pneumoniae is a type of Gram-negative bacteria that may cause different types of infections including pneumonia, meningitis, bloodstream infections. Generation of extended- spectrum β-lactamases is one of the major mechanisms by which clinical *K. pneumoniae* develop resistance to antibiotics (Cai et al., 2016). Herein we identified that the CLCHE and CLAEE fractions effectively act as antibacterial agent in the Gram-negative tested strains (Table 4). The antibacterial activities of *C. leptophloeos* extracts were also detected against *M. smegmatis* and *M. tuberculosis* strains as showed in Table 4. A bacteriostatic effect against Mycobacterium species by plants secondary metabolites may be due to pathogen's thick outer membrane that is highly hydrophobic and possibly provided a permeability to the extract (Firmani and Riley, 2002; Yamori et al., 1992).

Oxacillin and methicillin resistant *S. aureus* (MRSA) are resistant to all β-lactam agents including cephalosporins and carbapenems, causing global commitment in stopping its spread responsible for approximately 40% of *S. aureus* infections in global Intensive Care Units (ICU) (Cuny et al., 2015; Frieden, 2013). In this study, four MRSA clinical isolated strains reported as Methicillin Resistance (Oxacillin Resistance) according CLSI, 2014 confirmed with CFX inhibition disc tests (cefoxitin is used as a surrogate for mecA-mediated oxacillin resistance) (CLSI, 2014) *C. leptophloeos* hinokinin showed the highest antibacterial activity against MRSA isolated of blood, with a bacteriostatic activity (MIC) of 0.39 mg/mL and bactericidal (MMC) of 0.40 mg/mL, both with very similar values (Table 5). Our results point towards a promising antimicrobial potential against *S. aureus* resistant, especially when compared to others studies (Abdallah et al., 2009; Abdulgader et al., 2015; Saeed and Sabir, 2004).

Regarding hemolytic activity of *C. leptophloeos* extracts, the values obtained for hemolysis were superior to the ones regarding its antimicrobial activity, showing its safety. Even though a phytochemical study on another Commiphora genus (Hanus et al., 2005) has shown the presence of possibly toxic compounds, the toxicity assay performed *in vitro* for Amburana hemolytic properties did not show the same results. Regardless, we recognize the importance of applying other methods to assess toxicity from these extracts over other cellular components.

Hemolytic assays were performed to assess cell safety in future pharmacological preparations without causing any harm. In this study our trials with Commiphora showed lower hemolytic activity when comparing to other species from the Caatinga biome (Silva et al., 2011; Tam et al., 2015).

5. Conclusion

In this study we identified that the barks extracts from *C. leptophloeos* contain an important amount of phenolic compounds, such as GA, PCA and CGA. Furthermore, we have identified for the first time the presence of hinokinin in Commiphora genus. The present findings showed that *C. leptophloeos* possesses interesting inhibitory properties against *S. aureus* multi-drugs resistance species, action against various Gram-positives, Gram-negatives and Fungi microorganisms, and should also be studied for their potential against *Mycobacterium*.

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TABLE 1: Microorganisms used in this study distributed according to groups: bacteria and fungi

Bacteria	UFPEDA	**Bacteria**	UFPEDA	**Fungi**	UFPEDA
Mycobacterium smegmatis	71	*Staphylococcus aureus*	02	*Aspergillus sp.*	807
Mycobacterium tuberculosis	82	*Staphylococcus aureus*	672[1]	*Candida albicans*	1007
Bacillus subtilis	86	*Staphylococcus aureus*	677[1]		
Micrococcus luteus	100	*Staphylococcus aureus*	682[1]		
Enterococcus faecalis	138	*Staphylococcus aureus*	728[1]		
Escherichia coli	224				
Klebsiella pneumoniae	396				
Pseudomonas aeruginosa	416				

ATCC, American Type Culture Collection. [1] Infection site of *Staphylococcus aureus* strains; 672, blood; 677, postoperative secretion; 682, ocular discharge; 728, oropharynx.

TABLE 2: Phytochemical analyses of extracts from barks of *Commiphora leptophloeos*.

Commiphora leptophloeos **Extract**	**Phenolic contents**	**Phytochemical screen**	
		Positive tests for	**Negative tests for**
CLAQE	33.64 ± 0.5	Phenolic compounds, Tannins, Flavonoids and Reducing sugars.	Coumarins, Saponin, Phytosteroids and Lignans.
CLMEE	20.3 ± 0.78	Phenolic compounds, Tannins, Coumarins, Flavonoids, Reducing sugars and Saponin.	Phytosteroids and Lignans.
CLCLE	12.54 ± 0.55	Phenolic compounds, Tannins, Coumarins, Flavonoids, Reducing sugars, Phytosteroids and Lignans.	Saponin
CLCHE	13.8 ± 0.53	Phenolic compounds, Tannins, Coumarins, Flavonoids and Reducing sugars.	Saponin, Phytosteroids and Lignans.
CLAEE	13.7 ± 0.04	Phenolic compounds, Flavonoids and Reducing sugars.	Tannins, Coumarins, Saponin, Phytosteroids and Lignans.

CLAQE, *C. leptophloeos* aqueous extract; CLMEE, Methanolic extract; CLCLE, Cloroform extract; CLCHE, Cyclohexane extract and CLAEE, Ethyl acetate extract. Phenolic contents are expressed in mg/g of Gallic Acid Equivalent (GAE).

TABLE 3: Antimicrobial Activity of extracts from barks of *C. leptophloeos* against selected Gram-positive bacteria and Fungi.

Commiphora leptophloeos **Extract**	*Bacillus subtilis*		*Enterococcus faecalis*		*Micrococcus luteus*		*Staphylococcus aureus*		*Aspergillus sp.*		*Candida albicans*	
	MIC	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**
CLMEE	3.125	-	25	-	0.097	12.5	1.56	-	3.15	-	6.25	-
CLCLE	25.0	-	25	-	0.78	25	1.125	-	6.25	-	12.5	-
CLCHE	12.5	-	25	-	0.195	-	3.125	-	6.25	-	6.25	-
CLAEE	12.5	-	25	-	12.5	12.5	12.5	12.5	3.125	-	12.5	-
Control	**MIC**		**MIC**		**MIC**		**MIC**		**MIC**		**MIC**	
KAN	4		0.614		6.8		1.6		N.d.		3.2	
KCZ	N.d.		N.d.		N.d.		N.d.		0.32		N.d.	

MIC, Minimal Inhibitory Concentration; MMC, Minimal Microbicidal Concentration; MIC and MMC values are expressed in mg/mL. CLAQE, C. *leptophloeos* aqueous extract; CLMEE, Methanolic extract; CLCLE, Cloroform extract; CLCHE, Cyclohexane extract and CLAEE, Ethyl acetate extract. KAN, Kanamycin and KCZ, Ketoconazole. N.d., Not determined.

TABLE 4: Antimicrobial Activity of extracts from barks of *C. leptophloeos* against selected Gram-negative bacteria and Mycobacterium.

Commiphora leptophloeos **Extract**	*Pseudomonas aeruginosa*		*Escherichia coli*		*Klebsiella pneumoniae*		*Mycobacterium smegmatis*		*Mycobacterium tuberculosis*	
	MIC	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**
CLMEE	50.0	-	12.5	25	12.5	-	N.d.	N.d.	N.d.	N.d.
CLCLE	50.0	-	12.5	-	12.5	-	12.5	-	54.1	-
CLCHE	6.25	-	12.5	-	12.5	12.5	N.d.	N.d.	N.d.	N.d.
CLAEE	-	-	12.5	12.5	12.5	25	N.d.	N.d.	N.d.	N.d.
Control	**MIC**		**MIC**		**MIC**		**MIC**		**MIC**	
AMP	0.008		0.004		0.008		N.d.		N.d.	
RIF	N.d.		N.d.		N.d.		1.16		2.5	

MIC, Minimal Inhibitory Concentration; MMC, Minimal Microbicidal Concentration; MIC and MMC values are expressed in mg/mL. CLAQE, C. *leptophloeos* aqueous extract; CLMEE, Methanolic extract; CLCLE, Cloroform extract; CLCHE, Cyclohexane extract and CLAEE, Ethyl acetate extract. AMP, Ampicillin and RIF, Rifampicin. N.d., Not determined.

TABLE 5: Antimicrobial Activity of *C. leptophloeos* hinokinin against selected *S. aureus* clinical isolates.

UFPEDA	Source[1]	HKN		VA	TOB	CFX
		MIC	MMC		IDZ	
672	Blood	0.395	0.400	11.4	13.9	14.2
677	Postoperative secretion	0.0485	3.125	15.1	16	14.2
682	Ocular discharge	3.125	-	13.3	12	15
728	Oropharynx	1.560	-	14	14.8	16

MIC, Minimal Inhibitory Concentration; MMC, Minimal Microbicidal Concentration; MIC and MMC values are expressed in mg/mL. IDZ Inhibition Disc Zone are expressed in mm according CLSI. HK, Hinokinin; VA, Vancomycin; TOB, Tobramycin and CFX, Cefoxitin. [1]Source of *S. aureus* strains; 682, ocular discharge; 672, blood; 677, postoperative secretion; 728, oropharynx.

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Antioxidant Activity and Phytochemical Profile of *Spondias tuberosa* Arruda Leaves Extracts

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Abstract

***Spondias tuberosa* Arruda (umbuzeiro), a Brazilian semiarid plant, is a species of great economic, social, and ecological importance. In folk medicine, the leaves have been used in the treatment of diabetes, inflammation, stomach and uterine pains, and constipation. In this study, the antioxidant properties of ethyl acetate and methanol leaves extracts were evaluated *in vitro* using different methods: free radicals elimination by DPPH and ABTS assays, and transition metal reduction by phosphomolybdenium assay. In addition, a phytochemical study was also carried out. The metha- nolic leaves extracts showed the strongest antioxidant activity and the higher values for total phenolic and flavonoids. The results showed that *S. tuberosa* leaves have antioxidant activity and this seems to be related to the phenolic content.**

Keywords **Umbuzeiro, Phenolic Compounds, Flavonoids, Antioxidants**

1. Introduction

The oxidative stress induced by free radicals is considered a primary factor in neurodegenerative diseases as

Alzheimer's, Parkinson's and cardiovascular diseases as atherosclerosis [1]. The *in vitro* ability of phenolic compounds in the free radicals elimination and pro-oxidant metals reduction may explain the effectiveness in the treatment of many chronic non-transmissible diseases [2]. Natural antioxidants derived from plants are generally required to neutralize the damage caused by reactive oxygen species (ROS) to the cells [3].

Antioxidant compounds can be defined as substances that show at low concentrations the capability to inhibit or retard substrate oxidation when compared to oxidizable substrates [4]. Antioxidants such as flavonoids, tan- nins, coumarins, curcumanoids, xanthones, phenols, lignans and terpenoids have been found in various plant products as fruit, leaves, seeds and oils [5].

Spondias tuberosa Arruda (Anacardiaceae), known as "umbuzeiro", is a tropical plant that plays a major role in the Northeast of Brazil as an important nutritional resource [6]. It is endemic to the Caatinga zone and pro- duces flowers and fruits during the dry season [7], when most species of plants remain in a completely deci- duous state [8] [9]. In addition, the fruits represent an investment for the local population as food for humans and animals [10] [11]. This species has great ecological, social, economic, and cultural importance [12]. The "Umbu", the fruit of *S. tuberosa* provides a source of vitamins (B1, B2, B3, A, and C) and minerals (calcium, phosphorus and iron) [13] and possesses antibacterial activity [14]. In popular medicine, leaves are also used for treating several pathologies as diabetes, inflammations, uterine pain, stomach pain and constipation [15].

The aim of this study was the evaluation of the phytochemical profile and the determination of total phenolics, flavonoids, and the antioxidant activity of the ethyl acetate and methanol leaves extract of *S. tuberosa* by differ- ent methods (ABTS, DPPH and phosphomolybdenum assays).

2. Material and Methods

2.1. Plant Material

The plant was collected in Catimbau National Park, Pernambuco, Northeast of Brazil, at April 2014. The botan- ical identification was made by the herbarium staff of the Instituto de Agronômico de Pernambuco (IPA) and a voucher specimen was deposited in the herbarium (No. 91090).

2.2. Extract Preparation

The leaves were dried in an oven at 45˚C. The material was triturated in mill (Tecnal/Willye mill/ET-650) to obtain a powder. The extracts were obtained in a mechanical Accelerated Solvent Extractor (ASE 350 Dionex). The extracts were concentrated under a nitrogen stream in a heating block at 60˚C. 20 g of the powder were transferred to the cells of the ASE and extracted with ethyl acetate and methanol and then dried at 50˚C using a rotary evaporator.

2.3. Phytochemical Analysis

The phytochemical screening of the plant extracts was performed by thin layer chromatography (TLC) accord- ing to Harborne [16] and Roberts *et al.* [17]. Aliquots of ten microliters of the extracts of the extracts were ap- plied on silica gel chromatography plates, using elution systems and suitable developers to investigate the pres- ence of saponins, flavonoids, cinnamic derivatives, phenylpropanoids, triterpenoids, steroids, mono- and sesqui- terpenes, alkaloids, proanthocyanidins and leucoanthocyanidins, coumarins, and quinones.

2.4. Determination of Total Phenolic Content

Total phenolic content was determined by the Folin-Ciocalteu method to Li *et al.* [18] Two hundred microliters of diluted sample were added to 1 mL of 1:10 diluted Folin-Ciocalteu reagent. After 4 min, 800 mL of saturated sodium carbonate solution (75 g/L) was added. After 2 h of incubation at room temperature, protected from light, the absorbance at 765 nm was measured in triplicate. Gallic acid (0 - 500 mg/L) was used for calibration of standard curve. The results were expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of plant extract.

2.5. Determination of Flavonoids

The determination of flavonoids follows the methodology proposed by Woisky and Salatino [19] to 0.5 mL of diluted samples, was added 0.5 mL of 2% $AlCl_3$ (w/v) solution prepared in methanol. After 30 minutes of incu- bation at room temperature, protected from light, the absorbance at 420 nm was measured in triplicate. The re- sults were expressed as milligram quercetin equivalent (mg QE)/g dry weight of plant extract.

2.6. Antioxidant Activity Using 2,2-Azino-Bis-(3 Ethylbenzothiazoline)-6-Sulfonic Acid (ABTS)

According to Silva *et al.* [20], the ABTS assay is based on the generation of chromophore cationic radical ob- tained from the oxidation of ABTS by potassium persulfate. The oxidation reaction was prepared with 7 mM ABTS stock solution plus 140 mM potassium persulfate (final concentration) and the mixture was left in the dark at room temperature (23˚C - 25˚C) for 12 - 16 h (time required for radical formation) before its use. The ABTS + solution was diluted in ethanol to an absorbance of 0.7 (±0.02) units at 734 nm. The effect of extract amount on the antioxidant activity was carried out using aliquots of 30 µL, and mixing with 3 mL diluted ABTS+ solution. The absorbances at 734 nm were measured at different time intervals (6, 15, 30, 45, 60 and 120 min). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a reference standard. The values of oxidative inhibition percentage were calculated and plotted as a function of the reference antioxidant concen- tration (Trolox) and expressed as Trolox equivalent antioxidant capacity (TEAC, µM). All determinations were carried out in triplicate.

2.7. DPPH Radical Scavenging Assay

The DPPH free radical scavenging activity of the extracts was performed according to Brand-Williams *et al.* [21] with some modifications. A methanolic DPPH stock solution (200 µM) was

further diluted in methanol to obtain a UV-VIS absorbance between 0.6 - 0.7 at 517 nm, obtaining the DPPH working solution. Different concentra- tions of the extracts (40 µL) were mixed with DPPH solution (250 µL) and after 30 min incubation in darkness the absorbances were read at the same wavelength mentioned above. The measurements were triplicated and their scavenging activities were calculated based on the percentage of DPPH scavenged.

2.8. Total Antioxidant Capacity by Phosphomolybdenum Assay

According to Pietro *et al.* [22], the total antioxidant capacity (% TAC) was evaluated by phosphomolybdenum assay. An aliquot of 0.1 mL of sample solution (100 µg/mL) was combined with 1 mL of reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and in- cubated in a boiling water bath at 90˚C for 90 min. Afterward, the absorbance was measured at 695 nm against a blank (1 mL of reagent and 0.1 mL of solvent). Total antioxidant activity was expressed in relation to ascorbica- cid.

2.9. Statistical Analysis

Pearson correlation analysis was performed using a Statistical (Statistical Statsoft, Tulsa) software; *P*-values <

0.05 were considered significant.

3. Results and Discussion

The phytochemical screening was performed to identify the classes of chemical compounds present in the ex- tracts. Other studies have already demonstrated the antioxidant activity of sterols, terpenoids, oils, flavonoids, alkaloids and other phenolic compounds [23] [24]. The preliminary phytochemical analysis for the *S. tuberosa* leaves (**Table 1**) revealed the presence of high levels of flavonoids, triterpenes. The phytochemical profile results showed that the plant extract has molecules with high technological potential for the development of new drugs with application in the treatment and pre- vention of various diseases.In the determination of total phenolics and flavonoids, the results showed that the methanol solvent was better than ethyl acetate to extract phenolic compounds (**Table 2**) which may be explained by its good polarity and so- lubility for phenolic compounds extracted from plants [25] [26]. The lower polarity solvent, the ethyl acetate, showed much lower capacity for extracting phenolic compounds,

Table 1. Phytochemical profile of the methanolic and ethyl acetate extracts of the *S. tuberosa* leaves.

Secondary metabolites	Extract			
	Standards	Elution system	Ethyl acetate	Methanol
Flavonoids	Quercetin and rutin	A	+++	+++
Cinnamic derivatives	Chlorogenic acid	A	+	+
Triterpenes and steroids	*β*-sitosterol	B	+++	+++
Mono and sesquiterpenes	Thymol	C	−	−
Alkaloids	Pilocarpine	A	−	−
Coumarins	Coumarin	D	−	−
Condensed proanthocyanidins and leucoanthocyanidins	Catechin	A	−	−

A—AcOEt-HCOOH-AcOH-H_2O (100:11:11:27 v/v); B—Toluene:AcOEt (90:10 v/v); C—Toluene:AcOEt (97:03 v/v); D—$CHCl_3$-MeOH (98:2 v/v).

+ = Presence and − = Absence; + = low, ++ = intermediate, +++ = high.

Table 2. Total phenolic and flavonoids compounds quantification from *S. tuberosa* leaves extracts. Media ± SD (n = 3).

Sample	Total phenolic content (mgGAE/g)	Flavonoid content (mgQE/g)
Ethyl acetate extracts	75.69 ± 0.73	10.24 ± 0.66
Methanolic extracts	100.07 ± 0.02	15.74 ± 0.04

GAE: gallic acid equivalent. QE: quercetin equivalent.

compared with the more polar solvent. Numerous studies have shown a strong relationship between total phe- nolic content and antioxidant activity in fruits, vegetables and medicinal plants [27]. Phenolic compounds have been reported to have multiple biological effects including antioxidant activity. In addition, they can act in the free radical elimination or prevent its formation [28].

It has been reported that most of the antioxidant activity may be associated with phytochemicals such as fla- vonoids, isoflavones, anthocyanins, flavones, catechins and other phenolic compounds [29].

There are several methods to determine the antioxidant capacity of phytochemical compounds. The methods used in this study to determine the antioxidant capacity of the ethyl acetate and methanolic extract were ABTS, DPPH and phosphomolybdenum assay. Antioxidants act in many of biological responses as inflammation and immunity, they function as signaling mechanisms for redox regulation. Even at minimal levels of oxidative stress, they are strongly detected and then the protective antioxidant mechanism is put into action, which is es- sential for maintaining the structural integrity of proteins. Recently, particular attention has been made on the antioxidant properties of plants derived from dietary food constituents [29].

The ABTS assay results were shown in **Figure 1**. All extracts (1 mg/ml) of *S. tuberosa* had antioxidant activ- ity as a function of the time according to the Pearson correlation coefficient ($r = 0.958$, $p < 0.05$), which means that the beneficial antioxidant effect of the extracts improves significantly over time.

The methanol leaves extract had the highest antioxidant capacity with oxidation inhibition rate of 70.25% ± 0.49% after 120 min equivalent to TEAC of 1489.99 ± 12.02 µM of Trolox, while the ethyl acetate extract showed 58.724% ± 0.93% inhibition with TEAC of 1211.11 ± 22.68 µM of Trolox after 120 min.

The assay of the DPPH radical elimination expressed as a percentage of the radical reduction was presented in **Table 3**. The radical elimination activity was detected only in the methanolic leaves extract (68.12% ± 2.67%). The percentage of radical reduction for the ethyl acetate extract cannot be calculated due to the formation of precipitates in the sample in contact with the DPPH radical. According to the Pearson correlation coefficient, there is a positive correlation between the methanol leaves extract concentration and the free radicals elimination ($r = 0.967$, $p < 0.05$).

It was observed positive results in the ABTS and DPPH test sample, indicating that the extracts had compara- ble activities in both assays. However, only the methanolic leaves extract showed antioxidant activity in both assays (ABTS and DPPH).

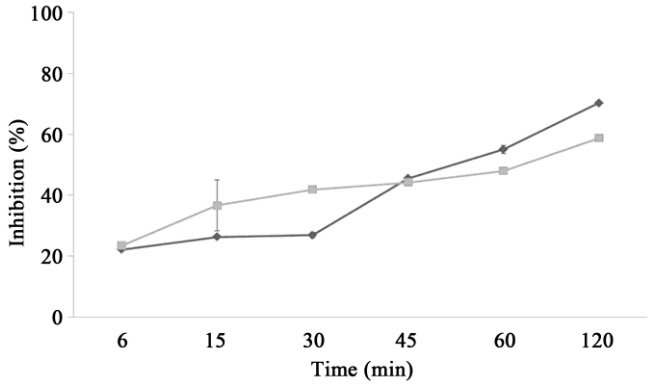

Figure 1. Effect of the incubation time on antioxidant activity of *S. tuberosa* leaves extracts in ABTS assay. Ethyl acetate

Table 3. Antioxidant activity of methanolic extract of *S. tuberosa* leaves in different concentrations. Gallic acid was used as standard. Mean ± SD (n = 3).

Extract Concentration (µg/mL)	DPPH RSA%
1000	68.12 ± 2.67
500	49.66 ± 1.66
250	35.29 ± 1.17
125	18.41 ± 1.40
62.5	18.03 ± 0.79
31.25	9.47 ± 0.36

RSA %: Percentage of DPPH radical reduction activity after 30 min.

Regarding the applicability of each assay reported by our results, DPPH is a free radical that is obtained di- rectly without preparation (ready to dissolve), while the ABTS is a cation (ABTS • +), which should be gener- ated by enzymatic activity (peroxidase and myoglobin) or chemical (manganese dioxide and potassium persul- fate) reactions [30] [31].

The total antioxidant activity (CAT) assay performed by phosphomolybdenum, which molybdenum ion re- duction capability of *S. tuberosa* extracts indicating that both extracts (methanolic and ethyl acetate) leaves were antioxidants. However, differences were observed in the antioxidant activity between these two types of extracts. The methanolic extract showed better activity (31.02% ± 0.01 TAC) than the

ethyl acetate extract (22.58% ± 0.03% TAC) using ascorbic acid as standard. Likewise the other methods, the methanol was the most effective solvent for extracting the antioxidant with potential secondary metabolites from *S. tuberosa* leaves in comparison with ethyl acetate.

According to the results, we note that there was a significant difference between the results of phosphomo- lybdenum and ABTS/DPPH assays, which can be explained by the fact that the hydrogen transfer electrons from antioxidant varies with its chemical structure [32]. In addition, non-phenolic compounds such as tocopherols and ascorbic acid may also act as reducer, thus cannot be observed a positive relationship between phenolic content and phosphomolybdenum reduction activity [33]. Other compounds, such as carotenoids, which were not meas- ured in this study, can be present in the extract and could contribute to antioxidant activity in the samples.

4. Conclusion

These results showed that leaves extracts of *S. tuberosa* possess antioxidant activity in all methods analyzed. The use of methanol was an efficient method of extraction of secondary metabolites with antioxidant activity compared to the use of ethyl acetate. The antioxidant properties of the secondary metabolites of the leaves ex-

tract of this plant may represent a potential source of components that could improve the health, being applied as functional foods or incorporated biomolecules into pharmaceutical or nutraceutical preparations.

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