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ANÁLISE DA COMPOSIÇÃO QUÍMICA E POTENCIAL BACTERICIDA DE TRÊS ÓLEOS ESSENCIAIS DA FAMÍLIA MYRTACEAE: POTENCIAL MOLUSCICIDA E LEISHMANICIDA DO ÓLEO ESSENCIAL DE *Algrizea minor*

Recife
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Universidade Federal de Pernambuco, como requesito parcial à obtenção do título de Doutor em Ciências Biológicas.

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Dedico à conclusão desta etapa importante em minha vida, ao meu **Deus** razão da minha existência**.**

"Porque dEle e por Ele, e para Ele, são todas as coisas; glória, pois, a Ele eternamente." Rm 11:36.

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RESUMO

As terapias naturais são originárias de medicinas tradicionais, que foram acrescidas de novas tecnologias e pesquisas, comumente chamadas de terapias complementares ou alternativas. Os óleos essenciais são compostos naturais, voláteis e complexos, caracterizados por um forte odor, sendo sintetizados por plantas aromáticas no metabolismo secundário. A importância de identificar os metabólitos secundários vegetais deve-se ao fato dos mesmos apresentarem um grande valor do ponto de vista social e econômico, além disso, alguns dos medicamentos aprovados são obtidos diretamente de espécies vegetais. Na presente pesquisa objetivou-se avaliar a composição química e atividade bactericida do óleo essencial das folhas de três espécies da família Myrtaceae: *Algrizea minor* (OEAm), *Algrizea macrochlamys* (EOAmac) e *Eugenia punicifolia* (EOEpu). Da espécie *A. minor* avaliaram-se também a citotoxicidade, o potencial artemicida, moluscicida e leishmanicida. Os óleos essenciais foram obtidos por hidrodestilação e analisados por cromatografia gasosa e espectrometria de massa (CG-EM). A atividade antibacteriana foi determinada por concentração mínima inibitória (CMI) e concentração mínima bactericida (CMB). Os rendimentos dos óleos foram: 0.36 EOEpu), 2.45 (EOAm) e 3.4% (EOAma) (p/p). Verificou-se na composição química dos óleos das espécies avaliadas, compostos comuns como α e β-pineno, (*E*)-caryophyllene, γ-eudesmol e guaiol. Os melhores valores de CMI foram 0.25mg/mL (*A. minor* contra *S. aureus* e *A. macrochlamys* contra *S. sarohyticus*) e MBC em 16mg/mL (*A. minor* e *E. punicifolia* contra *S. aureus*, e *A. minor* e *A. macrochlamys* contra *S. saprophyticus*). O óleo essencial de *A. minor* (0.25mg/mL) erradicou 50% do biofilme formado por *S. aureus*. Os efeitos tóxicos do EOAm frente a embriões e adultos de *Biomphalaria glabrata* mostraram LC_{50} de 6.1 e 34.9µg/mL, respectivamente. O valor da LC_{50} para *A. salina* foi de 42.02µg/mL. A IC_{50} para as formas promastigotas de *Leishmania amazonensis* do OEAm e do composto β pineno foi de 25.0 e 126.8 µg/mL, respectivamente. Para as formas amastigotas intracelulares a LC_{50} do EOAm foi de 53.62 µg/mL. A análise de microscopia eletrônica de scannning de promastigotes mostrou que o tratamento com EOAm induziu alterações morfológicas drásticas e lise aos parasitas em comparação com as células não tratadas. Os resultados apontam para o potencial dos óleos essenciais testados.

Palavras-chaves: Óleo essencial. Myrtaceae. Composição química. *Biomphalaria glabrata*. *Leishmania amazonensis*.

ABSTRACT

Natural therapies originate from traditional medicines, which have been supplemented by new technologies and research, commonly called complementary or alternative therapies. Essential oils are natural, volatile and complex compounds, characterized by a strong odor, synthesized by aromatic plants during secondary metabolites. The importance of identifying the plants secondary metabolites is due to the fact that they have a great value from a social and economic point of view, in addition, some of the approved drugs are obtained directly from plant species. The chemical composition and bactericidal activity of the essential oil from leaves of three Myrtaceae species was evaluated: *Algrizea minor* (EOAm), *Algrizea macrochlamys* (EOAmac) and *Eugenia punicifolia* (EOEpu). From EOAm we also evaluated cytotoxicity, potential molluscicidal, artemicide and leishmanicide. The essential oils were obtained by hydrodistillation and analyzed by gas chromatography and mass spectrometry (GC-MS). Antibacterial activities were determined by minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The yields of the oils were: 0.36 (EOEpu), 2.45 (EOAm) and 3.4% (EOAma) (p/p). The Composition of oils shared common compounds as α and β-pinene, (*E*)-caryophyllene, γ-eudesmol, and guaiol. The best MIC values were 0.25mg/mL (*A. minor* against *S. aureus* and *A. macrochlamys* against *S. sarohyticus*) and MBC at 16mg/mL (*A. minor* and *E. punicifolia* against *S. aureus*, and *A. minor* and *A. macrochlamys* against *S. saprophyticus*). The essential oil of *A. minor* (0.25mg/mL) eradicated 50% of the biofilm formed by *S. aureus*. The cytotoxicity of *A. minor* EOAm showed CC_{50} = 99.9 and 190.58 μg/mL to peritoneal macrophages and VERO cells, respectively.The toxic effects of EOAm on embryos and adults of *Biomphalaria glabata* showed LC_{50} of 6.1 and 34.9μg/mL, respectively. The LC_{50} value for *A. salina* was 42.02μg/mL. The IC_{50} to promastigote forms of *Leishmania amazonensis* from EOAm and the β-pinene compound was 25.0 and 126.8μg/mL, respectively. For the intracellular amastigote forms the LC_{50} of the EOAm was 53.62μg/mL. The scnanning electron microscopy analysis of promastigotes showed that EOAm treatment induced drastic morphological changes and lysis to the parasites comparing to untreated cells. The results point to the potential of the essential oils tested.

Keywords: Essential oil. Myrtaceae. Chemical composition. *Biomphalaria glabrata*. *Leishmania amazonensis*.

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LISTA DE ABREVIATURAS E SIGLAS

EOAm	Óleo essencial de *Algrizea minor*
EOAma	Óleo essencial de *Algrizea macrochlamys*
EOEpu	Óleo essencial de *Eugenia punicifolia*
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
CC_{50}	Concentração citotóxica que inibe 50% das células de mamíferos
IC_{50}	Inhibitory concentration of 50 cells
LC_{50}	Lethal concentration of 50%
IS	Índice de sobrevivência
TSB	Tryptone Soya Broth
MTT	3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina
BHI	Brain Heart Infusion
MH	Mueller-Hinton
NO	Óxido Nítrico
OsO_4	Tetróxido de Ósmio
CaCo	Cacodilato
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
DMSO	Dimetilsulfóxido
GC-MS	Gas chromatography and mass spectrometry

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1 INTRODUÇÃO

A família das Myrtaceae pertence ao grupo das angiospermas, com diversas espécies endêmicas do Brasil. As espécies apresentam os seguintes hábitos de crescimento, tais como: arbusto, árvore, erva, trepadeira ou subarbustro. Crescem em diferentes substratos, podendo ser rupícola, saprófita ou terrícola. A família está distribuída geograficamente em todas as regiões do Brasil sendo distribuídos em diferentes domínios fitogeográficos como Amazônia, Caatinga, Cerrado, Mata Atlântica, Pampa e Pantanal. No Brasil, são aceitos 23 gêneros e 1025 espécies (SOBRAL et al., 2016). Essa família é caracterizada por possuir glândulas transparentes contendo óleos etéreos (APG III, 2009).

Várias espécies dessa família apresentam valor econômico, como o eucalipto (*Eucalyptus* spp.), utilizado na produção de madeira e aromatizantes, e a goiabeira (*Psidium guajava*), frutífera cujos frutos são apreciados *in natura* e industrializados (JUNIOR et al., 2014).

Na família Myrtaceae, o gênero Algrizea apresenta duas espécies conhecidas, *Algrizea macrochlamys* (DC. Proença & Nic Lugh) e *A. minor* (Sobral, Faria & Proença). Estas espécies são arbustos com substrato terrícola, encontradas na região Nordeste, nos estados da Bahia e de Pernambuco. A espécie *A. minor* é endêmica do Brasil, encontrada na Caatinga e no Cerrado (SOBRAL; FARIA; PROENÇA, 2010).

Várias espécies dessa família têm sido relatadas como fontes naturais de compostos bioativos. Eugenia é um gênero importante da família das Myrtaceae devido ao seu potencial econômico e farmacológico. No Brasil, já foram descritas cerca de 383 espécies, das quais 296 são endêmicas. *Eugenia punicifolia* (Kunth DC), uma das espécies endêmicas da família das Myrtaceae, é distribuída em todo o país e todos os biomas, que vão desde a Amazônia tropical e florestas atlânticas até a Caatinga e Cerrado (FLORA DO BRASIL, 2017).

De acordo com Machado e Junior (2011), as terapias naturais têm como origem as chamadas medicinas tradicionais, tendo essas sido acrescidas de novas tecnologias e pesquisas, sendo comumente chamadas de terapias complementares ou alternativas. A utilização de substâncias naturais com ação fungicida, inseticida e herbicida, atualmente, tem sido foco de pesquisas (SARMENTO-BRUM et al., 2014). Dentre as fontes de compostos naturais com pontecial biotecnológico estão os óleos essenciais.

Os óleos essenciais são compostos naturais, voláteis e complexos, caracterizados por um forte odor que é sintetizado por plantas aromáticas durante o metabolismo secundário, e normalmente, são extraídos de diferentes órgãos das plantas de países quentes, como as do

mediterrâneio e dos trópicos, onde representam parte importante da farmacopeia tradicional. Podem ser extraídos por meio da técnica de arraste a vapor e também pela prensagem do pericarpo de frutos cítricos, que no Brasil dominam o mercado de exportação. São compostos principalmente de monoterpenos, sesquiterpenos e de fenilpropanoides, metabólitos que conferem suas características organolépticas (MACHADO; JUNIOR, 2011; BIZZO; HOVELL; REZENDE, 2009).

Além de serem aromatizantes, os óleos essenciais são naturais e biodegradáveis. Geralmente, apresentam baixa toxicidade para mamíferos, pode ser utilizados na proteção de culturas agrícolas, contra doenças e pragas, com a vantagem de não se acumularem no ambiente e terem um largo espectro de ação, o que diminui o risco de desenvolvimento de estirpes patogênicas resistentes (FIGUEIREDO; BARROSO; PEDRO, 2007).

Óleos essenciais possuem várias propriedades biológicas, entre elas têm sido testadas as ações antioxidante, fungicida, bactericida, leishmanicida, entre outras. De acordo com Ootoni et al. (2013), os produtos botânicos como os óleos essenciais e os extratos de plantas medicinas e plantas silvestres têm demonstrado que, além de ter uma variedade de espécies, os compostos são eficazes no controle de micro-organismos patogênicos.

As propriedades dos óleos essenciais são determinadas pela estrutura básica do componente principal e seus grupos funcionais. Embora a quantidade total entre os quimiotipos nos óleos essenciais, extraídos da mesma espécie, não demonstre diferenças, a concentração do componente principal pode diferenciar significativamente. Os óleos essenciais são, principalmente, terpenos hidrocarbonados (isoprenos) e terpenóides (isoprenóides). Os derivados oxigenados dos hidrocarbonetos terpenos, tais como álcoois, aldeídos, cetonas, ácidos, fenóis, éteres e ésteres têm atividade antimicrobiana variável (MARQUES-CALVO et al., 2017).

Diversos estudos científicos mostram testes biológicos utilizando óleos essenciais oriundos de várias espécies da família das Myrtaceae: antimicrobiano (MARTINELLI et al., 2017), antifúngico (YOKOMIZO; NAKAOKA-SAKITA, 2014), antioxidante (SILVA et. al., 2017), inseticida (TIETBOHL et al., 2014), anti-Leishmania (KAUFFMAN et al., 2017), anti-trypanossoma (AZEREDO et al., 2014), entre outros.

Diante do exposto, os óleos essenciais mostram-se ser bastante promissores, como por exemplo, no controle do crescimento de micro-organismos e parasitas. Vários estudos atestam o potencial bactericida e leishmanicida de vários óleos essenciais. Não há relatos na literatura sobre a composição química e atividades biológicas do óleo essencial extraído de *A. minor, A. macrochlamys* e *E. punicifolia*. Diante do exposto, torna-se importante conhecer a

composição química desses óleos, bem como a atividade antibacteriana e o potencial moluscicida e leishmanicida do óleo essencial de *A. minor*.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Avaliar a composição química e o potencial bactericida de óleos essenciais da famíllia das Myrtaceae e a atividade moluscicida e leishmanicida do óleo essencial de *Algrizea minor*.

1.1.2 Objetivos Específicos

- ✓ Extrair óleos essenciais pelo método de hidrodestilação em aparelho Clevenger;
- ✓ Quantificar e caracterizar os compostos químicos de três óleos essenciais da família das Myrtaceae, utilizando cromatografia gasosa acoplada à espectrometria de massa (CG/MS);
- ✓ Avaliar a atividade antibacteriana dos óleos essenciais;
- ✓ Avaliar a atividade antibiofilme, artemicida e moluscicida do óleo essencial de *A. minor*;
- ✓ Avaliar a toxicidade por ensaios de citotoxicidade celular *in vitro* do óleo essencial de *A. minor* e o composto majoritário;
- ✓ Avaliar a atividade anti-Leishmania do óleo essencial de *A. minor* e o composto majoritário.

2 REFERENCIAL TEÓRICO

Caatinga – Vale do Catimbau

Em relação à área ocupada do bioma caatinga no Brasil é de cerca de 844.453Km^2, correspondendo a 11% do território nacional, abrangendo parte dos estados do Maranhão, Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia (região Nordeste do Brasil) e parte do Norte de Minas Gerais (região Sudeste do Brasil). Quanto à vegetação, abrangem, com diferentes extensões, todos os estados da região Nordeste do Brasil e o Norte de Minas Gerais, único estado localizado na região Sudeste (MINISTERIO DO MEIO AMBIENTE, 2016; LOIOLA; ROQUE; OLIVEIRA, 2012).

No que diz respeito à diversidade de espécies, o bioma caatinga abriga 178 espécies de mamíferos, 591 de aves, 177 de répteis, 79 espécies de anfíbios, 241 de peixes e 221 de abelhas. Abriga uma população de cerca de 27 milhões de pessoas, a maioria carente e dependente dos recursos do bioma para sobreviver. É o único bioma exclusivamente brasileiro, cuja riqueza biológica não é encontrada em nenhum outro lugar do planeta. O nome caatinga é devido à paisagem esbranquiçada característica do período seco, no qual a maioria das plantas perde as folhas e os troncos tornam-se esbranquiçados e secos (MINISTERIO DO MEIO AMBIENTE, 2016; AVANCINI; TEGA, 2013; DRUMOND; SCHISTEK; SEIFFARTH, 2012).

Em relação às características climáticas as regiões do semiárido apresentam temperatura elevada variando entre 25ºC a 30°C, em média, com baixa incidência de chuvas e irregularidade da precipitação. Com chuva anual, em média, entre 300 mm e 500 mm, mas nas áreas de maior altitude tende a chover mais – a média pode chegar a 1.200 mm/ano, fazendo com que os rios sejam temporários e que na vegetação predominem as plantas xerófitas (cactáceas e outras espécies espinhosas). A riqueza da diversidade de espécies torna a caatinga à região semiárida mais rica em fauna e flora do mundo, essa riqueza, contudo, só começou a ser valorizada recentemente. Essa biodiversidade permite a realização de diversas atividades econômicas voltadas para fins agrosilvopastoris e industriais, especialmente nos ramos farmacêutico, de cosméticos, químico e de alimentos, entretanto, existem preocupações com o desmatamento, que chega a 46% da área do bioma, como forma de minimizar o problema o governo busca concretizar uma agenda de criação de mais unidades de conservação federais e estaduais no bioma, além de promover alternativas para o uso sustentável da sua biodiversidade (AVANCINI; TEGA, 2013; MINISTERIO DO MEIO AMBIENTE, 2016).

Para a criação de unidades de conservação alguns fatores são levados em consideração, tais como - físicos: recursos hídricos e formas de relevo, por exemplo; biológicos: fauna e recursos significativos da flora, entre outros; e antrópicos: áreas urbanas, uso e ocupação do solo, sócio-cultural, arqueológico e etc., sendo todos esses essenciais (LIMA; ARTIGAS, 2013).

De acordo com Júnior et al. (2013), vários estudos de inventários fitossociológicos e florísticos foram realizados nos últimos anos, em áreas de caatinga, tendo fornecido importantes informações sobre o número de indivíduos por hectare, a área basal ocupada e riqueza florística. No entanto, existe uma escassez de estudos sobre regeneração natural na vegetação da caatinga em Pernambuco. Todavia, tais conhecimentos são essenciais para a elaboração e aplicação correta dos planos de manejo e tratamentos silviculturais, permitindo uma exploração racional dessa vegetação.

O Vale do Catimbau, localizado entre o Agreste e o Sertão de Pernambuco, parte do município de Buíque (Figura 1), estende-se por áreas semi-áridas de Tupanatinga, Inajá e Ibimirim. Devido a essa grande expansão territorial, apresenta desde vegetação típica de Caatinga até vegetação de Mata Atlântica.

Figura 1: Vale do Catimbau, Buíque/PE.

Fonte: autora - SILVA (2016).

Quanto à vegetação natural predominante da região é do tipo caatinga arbórea aberta, que, devido às variações de relevo e micro-clima, apresenta também espécies de Cerrado e

Mata Atlântica, com ocorrência de bromélias, cactos e palmitáceas (FERREIRA; SILVA, 2009).

Metabolismo das plantas: metabólitos primários e secundários

O metabolismo primário é o conjunto de processos metabólicos que desempenham uma função essencial no vegetal, tais como a fotossíntese, a respiração e o transporte de solutos. Os compostos envolvidos no metabolismo primário possuem uma distribuição universal nas plantas. Em contrapartida, o metabolismo secundário origina compostos que não possuem uma distribuição universal, pois não são necessários para todas as plantas. Como consequência prática, esses compostos podem ser utilizados em estudos taxonômicos (quimiosistemática) (PERES, 2016).

A distinção entre metabolismo primário e secundário leva em consideração uma definição funcional, definindo como primário, produtos participantes em nutrição e essenciais processos metabólicos no interior da planta, e produtos naturais (secundários) e os que influenciam interações ecológicas entre a planta e o ambiente. Desempenham funções ecológicas específicas como atraentes ou repelentes animais. Muitos são os pigmentos que proporcionam cor para flores e frutos, desempenhando um papel essencial na reprodução devido à capacidade de atrair insetos polinizadores, ou para atrair os animais para utilizar a fruta como uma fonte de alimento, contribuindo assim para a dispersão de sementes. Outros compostos têm função protetora contra os predadores, agindo como repelentes, fornecendo às plantas sabores amargos, tornando-as indigestas ou venenosas. Também estão envolvidos nos mecanismos de defesa das plantas contra patógenos diferentes, atuando como pesticidas naturais (CROTEU; KUTCHAN; LEWIS, 2000; GARCIA; CARRIL, 2009).

É importante ressaltar que os metabólitos secundários são acumulados em altas concentrações (1-3% peso fresco), podem apresentar toxicidade elevada, podem ter efeitos biológicos em outros organismos. Apresentam também diferentes sítios de produção e acúmulos, que podem ser acumulados em vesículas, em forma glicosídica ou se acumulam em estruturas secretoras especiais tais como tricomas, ductos e canais laticíferos (FIGUEIREDO et al., 2008).

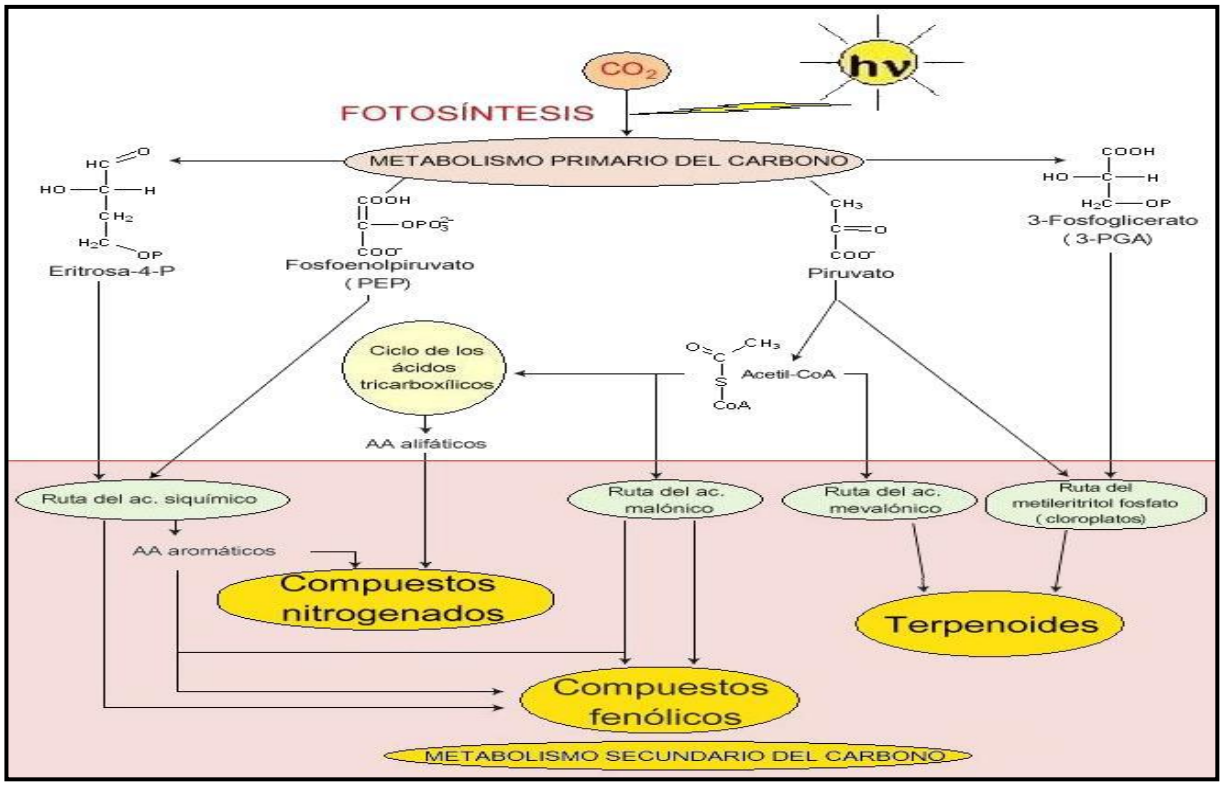
Os estudos dos fatores que podem coordenar ou alterar a taxa de produção de metabólitos secundários geralmente têm se limitado a um grupo restrito de espécies, predominando as que são encontradas em regiões temperadas, muitas delas têm importância comercial e provavelmente sofreram seletividade antrópica na busca de características

pretendidas. Quando comparadas com plantas selvagens ou de outros tipos de habitat seu comportamento nem sempre é o mesmo de plantas que não sofreram pressão antrópica (GOBBO-NETO; LOPES, 2007).

A importância de identificar os metabólitos secundários vegetais deve-se ao fato dos mesmos apresentarem um grande valor do ponto de vista social e econômico e, como exemplo, na década de 80 foram identificados 121 compostos de origem vegetal, provenientes de 95 espécies, os quais têm sido usualmente empregados como terapêuticos nos países ocidentais. Além disso, do total de medicamentos aprovados no período 1983-1994, 6% foram obtidos diretamente de espécies vegetais, sendo denominados produtos naturais, 24% foram compostos derivados e 9% foram desenvolvidos a partir de compostos vegetais cuja estrutura molecular serviu como unidade precursora em processos de síntese (MARASCHIN; VERPOORTE, 2016).

As principais vias de biossíntese de metabolitos secundários oriundos do metabolismo primário do carbono são mostradas na figura 2.

Figura 2: Elementos do metabolismo de carbono em relação às rotas sintéticas de metabólitos. secundários.



Fonte: GARCIA e CARRIL (2009).

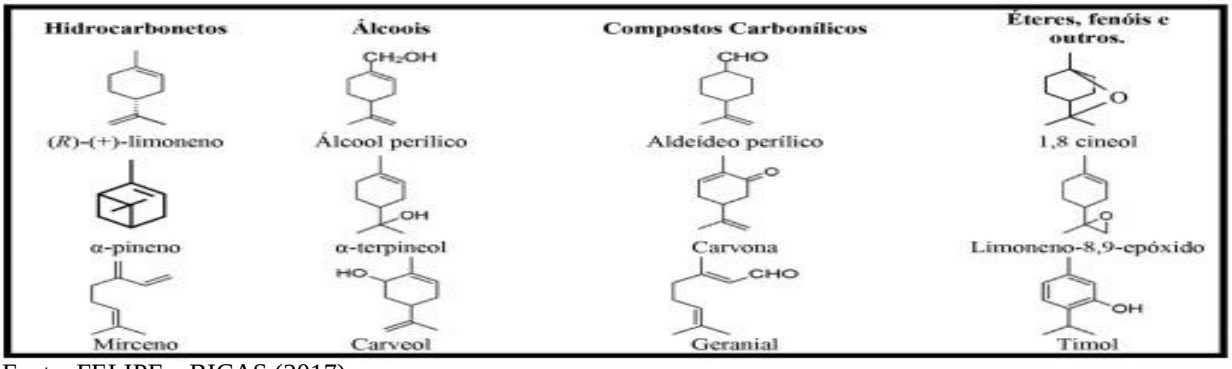
Os metabólicos secundários são divididos em três grandes grupos (terpenos, compostos fenólicos e alcaloides), dos quais, os terpenos são parte importante da constituição dos óleos essenciais.

Terpenos

Terpenoides são a classe estruturalmente mais variada de produtos vegetais naturais. O nome terpenóide, ou terpeno, deriva do fato de que os primeiros membros da classe foram isolados da terebentina (terpentin em alemão). Como mostrado na figura 2 os terpenóides são originados da rota metabólica do ácido mevalónico e do metileritritol-fosfato. De forma geral, os óleos essenciais são constituídos majoritariamente por terpenos ou seus derivados. Se um terpeno contém oxigênio, o mesmo é denominado de terpenoide, podendo apresentar diferentes funções químicas, entre as quais: ácidos, álcoois, aldeídos, cetonas, éteres, fenóis ou epóxidos terpênicos (Figura 3) (VIZZOTTO; KROLOW; WEBER, 2010; FELIPE; BICAS, 2017).

Quanto à origem os terpenos são formados pela justaposição sucessiva de isopentenilpirofosfato (IPP-C5) e este dá origem a todos os terpenos (monoterpenos (C_{10}), sesquiterpenos (C_{15}), diterpenos (C_{20}), triterpenos (C_{30}) e os tetraterpenos (C_{40}) (VIZZOTTO; KROLOW; WEBER, 2010).

Figura 3: Diferentes funções químicas atribuídas a monoterpenos e monoterpenoides.



Fonte: FELIPE e BICAS (2017).

Óleos essenciais

- **Breve histórico**

Inicialmente os óleos essenciais eram utilizados como bálsamos, ervas aromáticas e resinas, sendo estas empregadas para embalsamar cadáveres em cerimônias religiosas há milhares de anos. Em cerca de 2700 a.C, eram usados pelos chineses como essências. Atribui-se ao físico árabe Avicena (980 – 1073) o mérito de ser o primeiro a extrair óleo de rosas. Em

1558 o italiano Giovanni Battista della Porta (1535 – 1615), documentou uma forma de separar os óleos essenciais que até então eram apenas soluções alcoólicas. Em 1928 o químico francês René-Maurice Gattefossé criou o termo “aromaterapia”, associado a óleos essenciais, ficando conhecido como “pai da aromoterapia” (TRANCOSO, 2013).

No Brasil, antes mesmo da utilização de óleos essenciais pelas indústrias, vários pesquisadores no século XIX já trabalhavam com este tema, como, por exemplo, o farmacêutico Theodor Peckolt. Oriundo da Silésia alemã, atual Polônia, chegou ao Brasil em 1847, onde publicou uma vasta literatura sobre a flora brasileira, incluindo dados sobre o rendimento e a composição de vários óleos essenciais. A extração de óleo essencial na indústria brasileira teve início em 1925, com a extração de óleo essencial do pau-rosa (*Aniba rosaeodora*), entre outros óleos. Mas só foi no final da década de 30, com a ocorrência da Segunda Guerra Mundial, que a indústria nacional passou a se desenvolver, isto porque a guerra desorganizou todo o comércio europeu, fazendo com que as empresas daquela região buscassem outros fornecedores (AZAMBUJA, 2016).

- **Extração**

Existem diferentes métodos de extração dos óleos essenciais dos diferentes órgãos das plantas, tais como: utilização de solventes, enfleurage, processo mecânico (prensagem a frio), arraste a vapor, fluídos supercríticos e hidrodestilação. Entretanto, de acordo com a ISO 9235:2013, óleo essencial é o produto obtido a partir de uma matéria-prima natural de origem vegetal, por destilação a vapor, por processos mecânicos a partir do epicarpo de citrinos ou por destilação seca, após separação da fase aquosa - eventualmente - por processos físicos. De acordo com Busato et al. (2014), no processo de hidrodestilação o material vegetal arromático fica em contato com a água fervente, permitindo a evaporação do componentes voláteis, o ar quente ao encontrar o ar frio condensa e o líquido é coletado. Enquanto que na destilação a vapor, o material a ser destilado é apoiado sobre uma placa perfurada ou inserido em uma rede e colocada logo acima do fundo do destilador, a água não é mantida na parte inferior do destilador e o vapor é introduzido através de serpentinas.

O rendimento e a composição dos óleos essenciais podem variar devendo ser considerada a ontogenia de uma planta para se determinar o tempo de colheita. Há estudos que mostram diferenças o teor e composição química dos óleos devido à variação climática, a diferença de temperatura, distribuição de chuvas e características geográficas são fatores que favorecem as diferenças no quimiotipo de certas plantas portadoras de óleo essencial (LEE; DING, 2016).

Há relatos na literatura que evidenciam o uso de óleos essenciais para diversas finalidades. Os óleos essenciais, obtidos pela destilação a vapor de plantas aromáticas, têm uma longa história de uso humano como agentes aromatizantes em alimentos e bebidas, e na indústria de perfumes . Mais recentemente, eles tornaram-se populares como agentes de aromaterapia (ISMANN; MIRESMAILLI; MACHIAL, 2011).

Existem inúmeros conglomerados internacionais que negociam óleos essenciais, os mais importantes empregando-os como matéria-prima para a produção de aromas e fragrâncias (BIZZO, HOVELL; REZENDE, 2009). Estes mesmos autores relatam os principais óleos essenciais no mercado mundial (Tabela 1).

Tabela 1: Principais óleos essenciais no mercado mundial.

Óleo essencial	**Espécie**	**Óleo essencial**	**Espécie**
Laranja (Brasil)	*Citrus sinensis* (L.) Osbeck	Spearmint (nativa)	*Mentha spicata* L.
Menta japonesa (Índia)	*Mentha arvensis* L. f. *piperascens* Malinv. ex Holmes	Cedro (China)	*Chamaecyparis funebris* (Endl.) Franco
Eucalipto (tipo cineol)	*Eucalyptus globulus* Labill., *E. polybractea* R.T. Baker e *Eucalyptus* spp.	Lavandim	*Lavandula intermedia* Emeric ex Loisel
Citronela	*Eucalyptus globules* Labill., *E.polybractea* R. T. Baker e *Eucalytus* spp.	Sassafrás (China)	*Cinnamomum micranthum* (Hayata) Hayata
Hortelã-pimenta	*Mentha* x *piperita* L.	Cânfora	*Cinnamomum camphora* (L.) J. Presl.
Limão	*Citrus* x *limon* (L.) N.L. Burm.		
Eucalipto (tipo citronela)	*Eucalyptus citriodora* Hook.	Coentro	*Coriandrum sativum* L.
Cravo-da-Índia	*Syzygium aromaticum* (L.) Merr. e L. M. Perry	Grapefruit	*Citrus paradisi* Macfady
Cedro (EUA)	*Juniperus virginiana* L. e *J. ashei* Buchholz	Patchouli	*Pogostemon cablin* (Blanco) Benth.
Lima destilada	*Citrus aurantifolia*		

(Brasil)	(Christm. & Panz.) Swingle	

Adaptada/LAWRENCE (1993).

Segundo Souza et al. (2010), o Brasil ocupa posição de destaque na produção mundial de óleos essenciais, entretanto, há problemas crônicos no que refere ao padrão de qualidade dos óleos essenciais, a representatividade nacional e baixos investimentos governamentais no setor, o que não permite uma evolução nesse cenário. Um importante passo para minimizar o caráter estacionário entre a produção e aplicabilidade foi à criação da ABRAPOE (*Associação Brasileira de Produtores de Óleos Essenciais*) que busca, entre outras metas, colaborar na aproximação entre os produtores e os centros de pesquisa nacionais para agregar qualidade aos óleos. Os fatores econômicos são os únicos a governar o setor, uma vez que o domínio destas conversões é importante para tornar um empreendimento de obtenção de óleos essenciais mais rentáveis, agregando-se a um produto primário por meio de tecnologia química.

- **Composição química**

A composição química dos óleos essenciais, dentre outros fatores, depende do órgão vegetal

coletado (folhas, flores, caules ou raízes) e o estágio de desenvolvimento do órgão. Os locais onde são produzidos os compostos naturais são conhecidos como tricomas, canais ou bolsas, tais estrututras secretoras ocorrem mais nas flores, folhas e caules, outros órgãos também podem possuir substâncias aromáticas (FIGUEREDO; PEDRO; BARROSO, 2014).

Quanto à composição química dos óleos essenciais são constituídos principlamente por fenilpropanóides e terpenóides, sendo os últimos os mais abundantes e mais comumente encontrados. Tais substâncias é foco de interesse de pesquisa como fonte de princípios ativos ou como precursores de outros compostos, por exemplo, o safrol, eugenol, citral, citronelal, dentre outros (OOTONI et al., 2013).

- **Atividades biológicas**

Os óleos essenciais têm sido estudados para os mais diversos fins como: inseticida, bactericida, tripanocida, leishmanicida, antibiofilme, antioxidante, acaricida, cupinicida, antiplasmodial, entre outras.

Bactericida e Fungicida

Estudos comprovam que os óleos essenciais são considerados eficientes contra uma gama de agentes patogênicos. Podem agir alterando a membrana celular dos agentes patogênicos, aumentando a permeabilidade da membrana, induzindo vazamento de constituintes intracelulares vitais e interrompendo o metabolismo celular dos patógenos alvos (SWAMY; AKHTAR; SINNIAH, 2016).

As atividades dos óleos essencias são desempenhadas pelo efeito sinérgico dos compostos presentes no mesmo, e em alguns casos, alguns compostos isoladamente são responsáveis pelas atividades biológicas.

Foi testado o potencial fungitóxico dos óleos essenciais de *Cymbopogon citratus*, *Lippia sidoides*, e de seus constituintes majoritários, os óleos essencias inibiram totalmente o crescimento micelial, entretanto, os compostos majoritários cravacol (*L. sidoides*) e citral (*C. citratus*) foram mais efetivos, provocando a ausência de crescimento micelial dos fitopatógenos *Rhizoctonia solani* e *Sclerotium rolfsii* (GONCALVES et al., 2015).

Já Miladinovic et al. (2015) ao trabalhar com óleo essencial de *Thymus glabrescens* Willd. (thyme) e seus compostos majoritários contra *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Proteus mirabilis* (ATCC 12453), *Pseudomonas aeruginosa* (ATCC 27853) e *Staphylococcus aureus* (ATCC 29213), associados ao antibiótico tetraciclina, veriicaram que a combinação óleo essencial + tetraciclina e timol + tetraciclina apresentaram maior interação sinérgica, reduzindo a dose mínima efetiva do antibiótico e, consequentemente, minimizando os efeitos adversos do antibiótico.

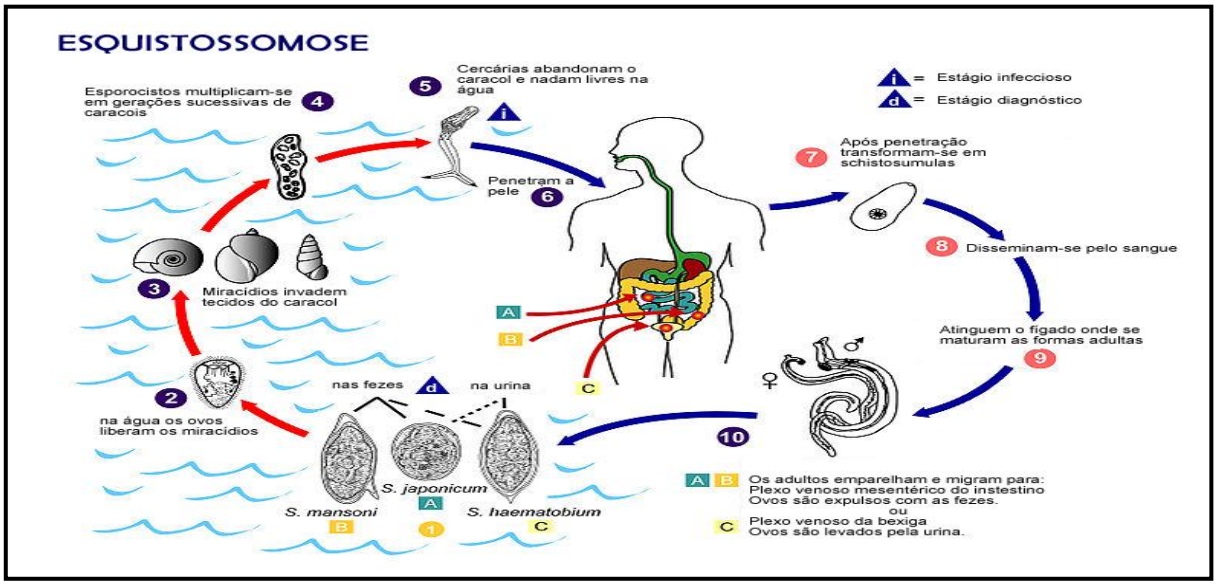
A análise dos efeitos citotóxicos e mutagênicos do óleo essencial de *Piper gaudichaudianum* Kunth e de seu componente majoritário (E) – nerolidol demonstrou que ambos não são mutagênicos, entretanto, induzem efeitos citotóxicos significativos em *Saccharomyces Cerevisiae*, pela produção de ROS e quebra de cadeia simples de DNA, sendo o (E) – nerolidol o principal composto responsável por tais efeitos biológicos (SPEROTTO et al., 2013). De acordo com Machado e Júnior (2011), a capacidade citotóxica de óleos essenciais, baseada em sua capacidade pró-oxidante, pode fazer dos óleos esssenciais excelentes antissépticos e antimicrobianos para uso pessoal, purificação do ar, para higiene pessoal, inseticida para preservação de grãos e estoques de alimentos, além do que alguns óleos essenciais demonstrarem clara capacidade antimutagênica que pode estar ligada, assim, a sua atividade anticarcinogênica.

Óleos essenciais como alternativas para o controle de doenças neglienciadas: esquistossomose e leishmaniose

- **Esquistossomose mansônica**

A esquistossomose é uma doença causada pelos Platelmintos (vermes achatados) da classe dos trematódeos (de forma foliacea), família *Schistosomatidae*, que apresenta sexos separados, com nítido dimorfismo sexual. Das seis espécies de Schistosoma que parasitam o homem, somente o *S. mansoni* existe nas Americas do Sul e Central. É endêmica em vasta extensão do território nacional, ocorrendo nas localidades sem saneamento ou com saneamento básico inadequado, e é adquirida através da pele e mucosas em consequência do contato humano com águas contendo formas infectantes do *S. mansoni*. A transmissão da doença depende da presença do homem infectado, excretando ovos do helminto pelas fezes, e dos caramujos aquáticos, que atuam como hospedeiros intermediários (MINISTERIO DA SAUDE, 2017). As etapas do ciclo de vida do Schistosoma estão ilustradas na Figura 4.

Figura 4: Ciclo de vida do Schistosoma nos hospedeiros intermediário e definitivo.



Fonte: https://commons.wikimedia.org/wiki/File:Esquistossomose-2.png

Quanto às características do *S. mansoni*, são vermes de cor esbranquiçada, que se alojam em vasos do plexo hemorroidário ou na porção intra-hepática da veia porta. No interior desses vasos, encontram-se geralmente o macho e a fêmea, acasalados (Figura 5). A fêmea aloja-se no canal ginecóforo do macho e, por ser mais longa, ultrapassa-o para diante ou para trás e se recurva em uma ou duas flexões. Geralmente esses vermes realizam

migrações dentro do mesmo vaso ou de um para outro, através de anastomoses (MINISTERIO DA SAUDE, 2017).

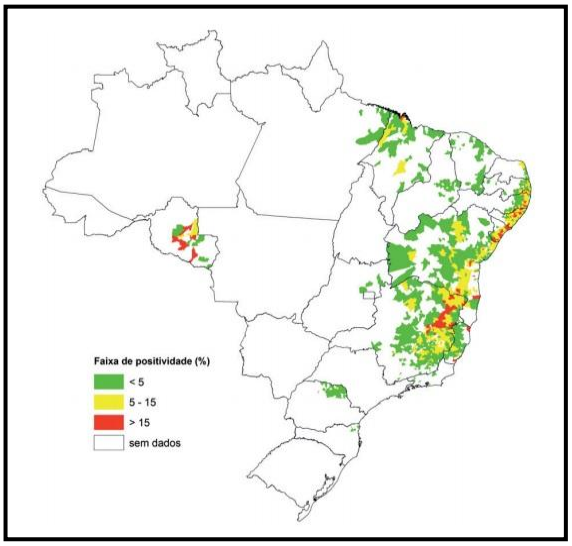
Figura 5: Casal de *S. mansoni*, mostrando a fêmea do verme no canal ginecóforo do macho.

Fonte: Ministério da Saúde, 2017. José Roberto Machado e Silva – UERJ, Renata Heisler Neves e Delir Corrêa Gomes – IOC (2008).

Epidemiologia, manifestações clínicas, diagnóstico e tratamento da esquistossomose mansônica

A esquistossomose é considerada um grave problema de saúde pública no país e no mundo. Há diversas áreas no Brasil que são endêmicas para a moléstia, constituindo importante causa de morbidade e mortalidade da população. Na figura 6 pode-se observar o percentual de positividade da doença nas regiões do Brasil. Esta doença pode acometer vários órgãos e sistemas, é importante ressaltar a severidade das formas crônicas, que são responsáveis por sérias implicações para os indivíduos portadores. A fase aguda é dividida em dois períodos evolutivos: o pré-patente (antes da oviposição) e pós-patente (após a oviposição). Em relação aos aspectos imunológicos acredita-se que a resposta imune do tipo Th1 seja responsável pelas lesões teciduais e manifestações clínicas da fase aguda. Outro aspecto interessante é a citotoxicidade celular dependente de anticorpo (adcc), com ação efetora sobre esquistossômulos, mas aparentemente inócua para os helmintos adultos (SOUZA et al., 2011).

Figura 6: Distribuição da esquistossomose segundo percentual de positividade em inquéritos coproscópicos – Brasil, 2012.



Fonte: MINISTERIO DA SAUDE (2017).

A fase aguda caracteriza-se pela dermatite cercariana, sua intensidade varia desde um quadro assintomático até o surgimento de dermatite urticariforme, com erupção papular, eritema, edema e prurido, persistindo até cinco dias após a infecção. Ainda na fase aguda, após três a sete semanas de exposição caracterizada por febre, anorexia, dor abdominal e cefaléia, o paciente pode apresentar, em menor freqüência, diarréia, náuseas, vômitos, tosse seca. Durante o exame físico, pode-se detectar a hepatoesplenomegalia, isto é, o fígado e o baço aumentados de tamanho. A fase crônica da doença, a partir de seis meses após a infecção, pode evoluir por muitos anos. Quanto às manifestações clínicas, variam a depender da localização do parasito e da intensidade da carga parasitária, podendo apresentar as formas intestinais, hepatointestinal, hepatoesplênica e até neurológica (PORDEUS et al., 2008).

O diagnóstico clínico é mais difícil em áreas não edêmicas, pelo fato das manifestações serem plurais faz-se necessário um diagnóstico diferencial para cada fase evolutiva da infecção. Se houver a suspeita, com base nos dados clínicos e epidemiológicos, é indicada a realização da avaliação laboratorial, que é relativamente rápida e de fácil execução. A avaliação laboratorial é realizada por métodos e exames parasitológicos, métodos imunológicos e exames inespescíficos como, por exemplo, o hemograma na fase aguda da doença. Ainda pode ser feita a avaliação por imagem, como a telerradiografia de tórax, o ecocardiograma, ultrassonografia abdominal e endoscopia digestiva (VITORINO et al., 2012).

Com relação ao tratamento da esquistossomose, os fármacos que são amplamentes utilizados são oxamniquine e praziquantel. Entretanto, além do tratamento com esses

fármacos, algumas particularidades devem ser consideradas na terapêutica dos diferentes estágios evolutivos da esquistossomose mansônica – agudo e crônico. Já há alguns estudos em andamento para o desenvolvimento futuro de vacina. Levantamentos bibliográficos atestam a toxicidade e baixa eficácia no tratamento da doença devido à resistênncia dos parasitas. Uma alternativa mais eficaz são ações de saneamento amibiental, para as modificações das condições de transmissão (SILVA et al., 2012; VITORINO et al., 2012).

Óleos essenciais no controle de caramujos vetores da esquistossomose

A ocorrência de moluscos que são hospedeiros intermediários de doenças, como a esquistossomose, por exemplo, leva à disseminação destas parasitoses nas regiões em que se encontram e, por isso, deve ser controlada. O ciclo do parasita depende da presença dos hospedeiros intermediários. Há dez espécies e uma subespécie do gênero Biomphalaria e, destas, três são hospedeiras intermediárias naturais (*B. glabrata, B. tenagophila* e *B. straminea)* e duas *(B. amazonica* e *B. peregrina)* são hospedeiras intermediárias potenciais, uma vez que só se infectam experimentalmente. Medidas de controle da transmissão: controle e tratamento de dejetos, abastacimento de água potável, hidráulica sanitária e eliminação de coleções hídricas que sejam criadouros de moluscos (COSTA et al., 2015; MINISTERIO DA SAUDE, 2008; SILVA et al., 2012).

Há relatos na literatura dos efeitos dos óleos essenciais sobre os hopedeiros intermediários do parasita causador da esquitossomose. Como por exemplo a pesquisa de Costa et al. (2015) que utilizaram óleo essencial das folhas de *Cymbopogon winterianus* Jowitt sobre *Biomphalaria tenagophila* e verificaram que as doses DL_{100} e DL_{50} de 80ppm e 60ppm, respectivamente, com o intervalo de tempo de avaliação de 30min, duas, seis, 12 e 24 horas (COSTA et al., 2015) foram eficientes no controle de *B. glabrata* como também o uso do óleo essencial das folhas de *Syzygium cumini* (L.) Skeels foi avaliado frente à *B. glabrata* em 24 e 48 horas e a LC_{50} obtida foi de107 e 90mg/l, respectivamente (DIAS et al., 2013).

Os óleos essenciais das partes aéreas de *Thymus capitatus* Hoff. et Link. e *Marrubium vulgare* L. contra ovos e adultos de *Biomphalaria alexandrina* mostrou LC_{50} e LC_{90} de 200 e 400ppm/3h, respectivamente, enquanto que para *M. vulgare* era de 50 e 100 ppm/3 horas, respectivamente. Além disso, *M. vulgare* mostrou atividade ovicida LC_{100} a 200ppm/24h, enquanto que o óleo de *T. capitatus* não apresentava atividade ovicida (SALAMA; TAHER; EL-BAHY, 2012).

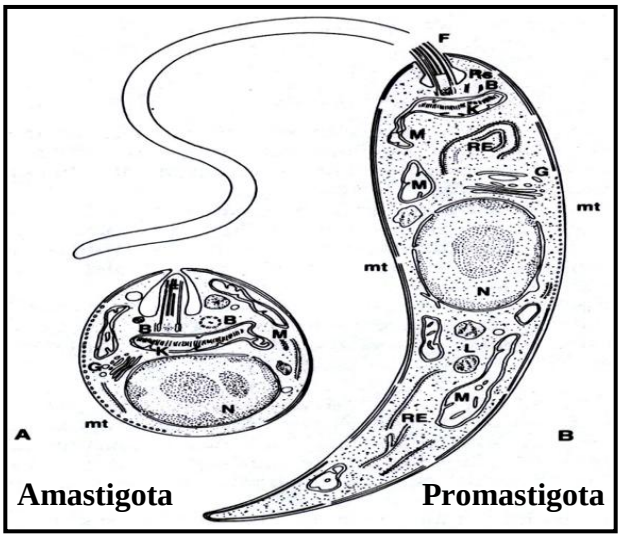
O óleo essencial das flores e folhas de *Porophyllum ruderale*, após 72h de exposição, apresentou atividade moluscicida, frente a ***B. glabrata***, com LC_{10}, LC_{50} e LC_{90} de 738.96, 774.82 e 812.43ppm, respectivamente (FONTE-JR et al., 2012).

O óleo essencial das folhas de *Lippia gracilis* H.B.K. contra *B. glabrata* com tempo de exposição de 24h, os resultados obtidos da LC_{10}, LC_{50} e LC_{90} foram de 36.9, 62.2 e 82.8ppm, respectivamente (TELES et al., 2010).

Leishmanioses

O agente etiológico da leishmaniose é a Leishmania, que é um protozoário pertencente à família Trypanosomatidae, parasito intracelular obrigatório das células do sistema fagocítico mononuclear. Com duas formas principais: uma flagelada ou promastigota, encontrada no tubo digestivo do inseto vetor, e outra aflagelada ou amastigota, observada nos tecidos dos hospedeiros vertebrados (Figura 7) (MINISTERIO DA SAUDE, 2017).

Figura 7: Formas do parasita *Leishmania*.



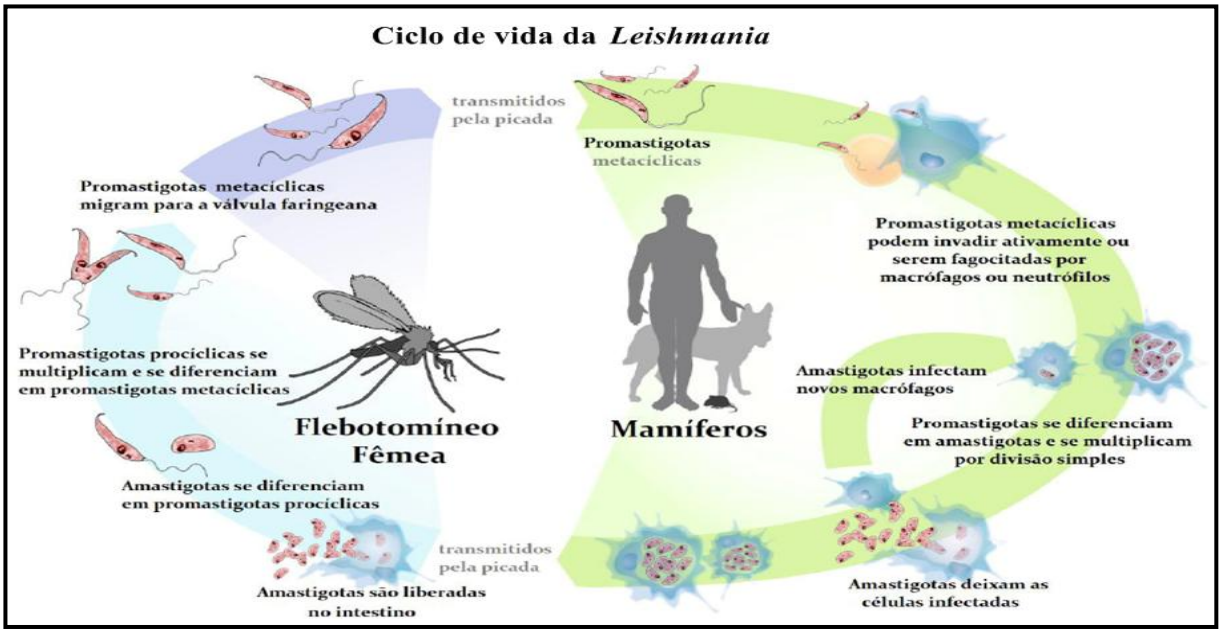
Fonte: http://alunoonline.blogspot.com.br

Leishmania causa uma doença tropical e subtropical, é uma das sete doenças tropicais mais importantes e representa um grave problema de saúde mundial. Encontra-se em todos os continentes, exceto Oceania, sendo edêmica em áreas geográficas circunscritas no Nordeste da África, Sul da Europa, Oriente Médio, Sudeste do México e Central e Sul da América (TORRES-GUERRERO et al., 2017).

Leishmanioses é um grupo de doenças parasitárias causadas por mais de 20 espécies de protozoários de Leishmania. Os parasitas são transmitidos aos seres humanos pela picada do mosquito fêmea de insetos vetores infectados, é sempre um flebotomínio que ao picar o

indivíduo ou o animal parasitado retira junto com o sangue ou com a linfa intersticial as leishmanias, que passarão a evoluir no interior do tubo digestivo, sofrendo muitas modificações. Ao alimentar-se com o sangue de outros animais ou pessoas, o inseto passa a regurgitar o material aspirado. Fica assegurada, desse modo, a inoculação de formas infectantes em um novo hospedeiro vertebrado, completando assim o ciclo evolutivo do parasito e a sua propagação a novos indivíduos suscetíveis (Figura 8) (WHO, 2017; FIOCRUZ, 2017).

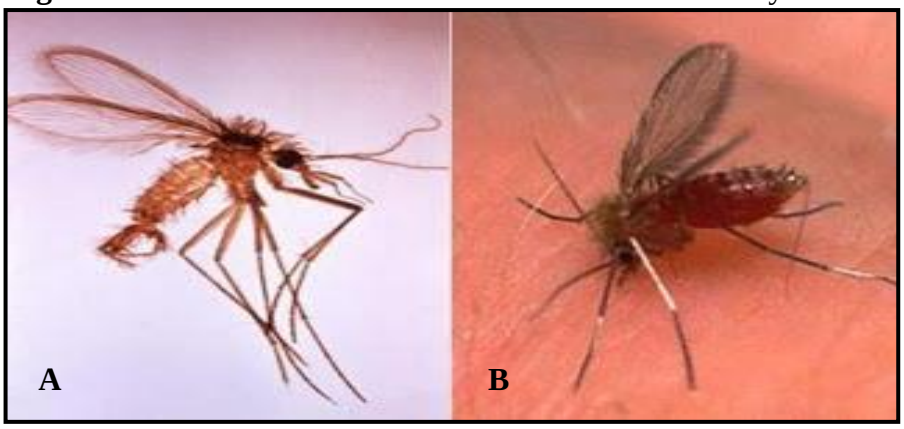
Figura 8: Ciclo de vida da *Leishmania*.



Fonte:http://www.canalciencia.ibict.br/pesquisa/0295_A_caminho_da_cura_da_leishmaniose_visceral_canina.htm

Os flebotomínios são insetos pequenos, muito pilosos, de cor palha ou castanho claros, facilmente reconhecidos pela atitude que assumem quando pousados, permanecendo com as asas entreabertas e ligeiramente levantadas, ao invés de se cruzarem sobre o dorso. São conhecidos como: "cangalha", "orelha de veado", "mosquito palha", "tabuira", etc. Apenas dois gêneros são realmente importantes para a epidemiologia das leishmanioses: Lutzomyia, cujas fêmeas picam o homem (leishmaniose da América) e Phlebotomus, responsáveis pelas leishmanioses da África, da Europa e da Ásia (Figura 9) (WHO, 2017; FIOCRUZ, 2017).

Figura 9: Flebbotomínios. A- Phlebbotomus e B – Lutzomya.



Fonte: http://fpslivroaberto.blogspot.com.br/2009/12/parasitas-leishmania-spp-e-leishmaniose.html

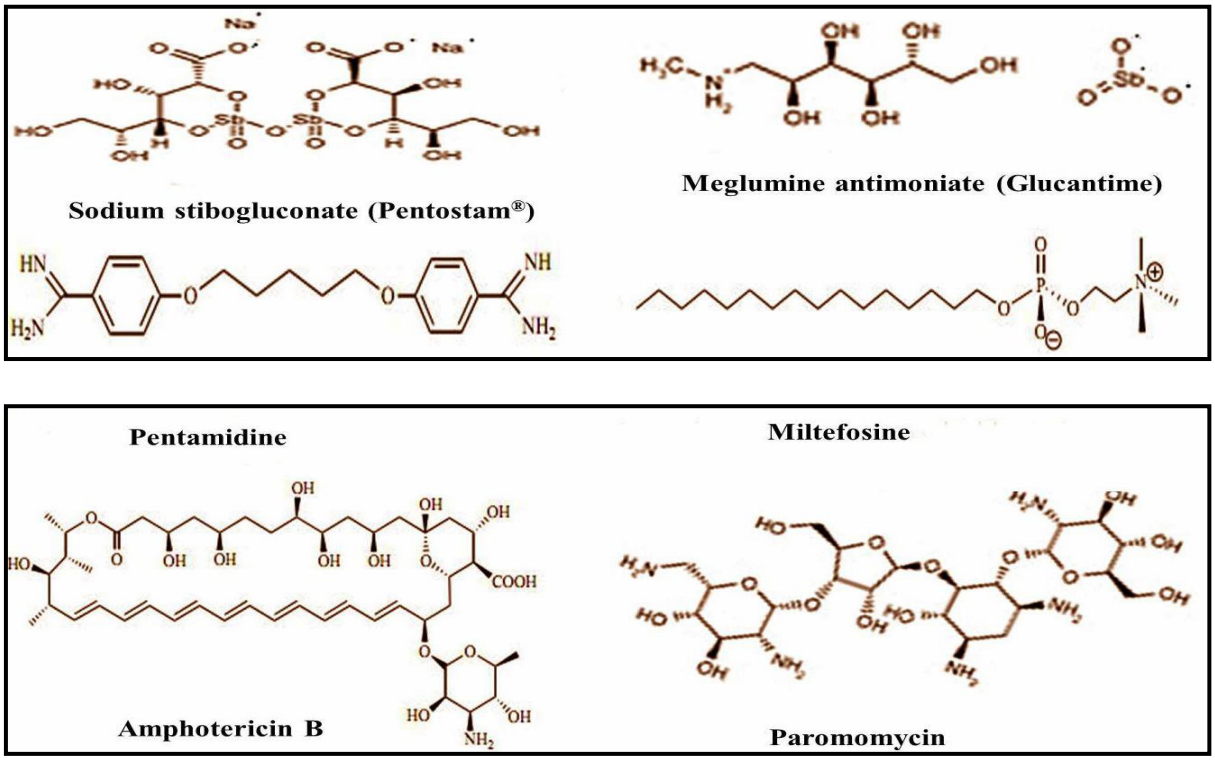
Manifestações clínicas, diagnóstico e tratamento.

As manisfestações clínicas da leishmaniose são caracterizadas por um espectro que varia de simples lesões cutâneas ulcerativas até formas mucocutâneas desfigurantes e formas viscerais que podem ser fatais. Esta doença é dividida em quatro grupos: leishmaniose cutânea, que produz exclusivamente lesões cutâneas, ulcerosas ou não, porém limitadas; leishmaniose cutâneo-mucosa ou leishmaniose mucocutânea, caracterizada por formas que se complicam freqüentemente com o aparecimento de lesões destrutivas nas mucosas do nariz, boca e faringe; leishmaniose visceral ou calazar, formas viscerais em que o parasito tem afinidade (tropismo) com o sistema fogocítico mononuclear (SFM) do baço, do fígado, da medula óssea e dos tecidos linfoides; leishmaniose cutânea difusa, formas disseminadas cutâneas que se apresentam em indivíduos alérgicos ou, tardiamente, em pacientes que foram tratados de calazar (NERIS et al., 2013; FIOCRUZ, 2017).

As espécies de Leishmania variam em sensibilidade às terapias disponíveis, além disso, as manifestações clínicas são variadas, o que dificulta o diagnóstico da doença. Em contraste com muitas outras doenças infecciosas, a identificação do parasita Leishmania costumava ser trabalhosa. Os parasitas de Leishmania agora podem ser identificados de maneira relativamente fácil por novas técnicas de biologia molecular (VRIES; REEDIJK; SCHALLIG, 2015).

Na figura 10 pode-se observar a estrtura das principais drogas utilizadas para o tratamento das leishmanioses.

Figura 10: Estrutura bidimensional de drogas mais comumente usadas contra a leishmaniose.



Fonte: ORYAN (2015).

Dados relatados em levamento bibliográfico realizado por TORRES-GUERERO, 2017, atestam que o único tratamento eficaz é pela administração intravenosa de antimoniais pentavalentes (Sb5+), sob a forma de stibogluconato de sódio (SSG, Pentostam, Reino Unido) ou antimoniato de meglumina (Glucantime, França), exceto no estado de Bihar na Índia. Em Bihar, a resistência do parasita aos antimiários causou um aumento dramático na falha do tratamento de até 65% entre 1980 e 1997. A falha de tratamento para VL com antimonial pentavalente (SSG) foi relatada nos últimos anos no Nepal; como resultado, a anfotericina B lipossomal é atualmente recomendada pelo Programa Nacional do Nepal. A anfotericina B é uma alternativa extremamente eficaz, porém tóxica, é eficaz mesmo em formas resistentes a antimoniais.

Óleos essenciais e atividade anti-Leishmania

Estudos *in vitro* têm demonstrado os óleos essenciais como promissores agentes leishmanicidas. *Artemisia campestris* L. e *Artemisisa herba-alba* Asso – foram eficientes contra à forma promastigota de *L. infatum* com valores de IC_{50} de 68µg/ml e 44µg/ml para *A. herba-alba* e *A. campestris*, respectivamente. A atividade antileishmania dos óleos essencias através da indução de apoptose celular e parada do ciclo celular (ALOUI et al., 2016).

O óleo essencial da espécie *Syzygium aromaticum* (L.) Merrill & Perry possui atividade significativa contra *L. donovani*, com concentração inibitória de 50% (IC_{50}) de 21±0,16µg ml^{-1} e 15,24±0,14 µg ml^{-1}, frente às formas promastigotas e amastigotas intraceulares, respectivamente (ISLAMUDDIN; SAHAL; AFRIN, 2014).

Thymus capitellatus Hoffmanns & Link – Através da citometria de fluxo foi possível mostrar que o óleo essencial induziu a perda de viabilidade de promastigotas de *L. infantum, L.tropica e L. major*, mostrando um efeito marcado sobre *L. infantum* (IC_{50} = 37µg/ml), *L. tropica* (IC_{50} = 35µg/ml) e *L. major* (IC_{50} = 62µg/ml) (MACHADO et al., 2014).

Croton cajucara Benth. o óleo essencial dessa espécie mostrou atividade contra a *L. chagasi*, cuja concentração mínima inibitória (CMI) foi de 250µg/ml e IC_{50} de 66.7µg/ml. As mesmas não são citotóxicas para macrófagos. O óleo essencial reduziu o índice de associação do parasita com os macrófagos, aumentou a produção de NO por macrófagos infectados. O pré-tratamento de macrófagos com 250 e 125 µg/mL de óleo essencial reduziu o número de parasitas aderentes e internalizados em 30,0 e 9,6%, respectivamente (RODRIGUES et al, 2013).

Cymbopogon citratus (DC) Stapf. o óleo essencial dessa espécie a 50µg/ml foi capaz de inibir 65% da forma promastigota de *L. infantum e L. major* e 80% de *L. tropica* (MARCHADO et al., 2012).

Leishmaniose Tegumentar Americana: manifestações clínicas

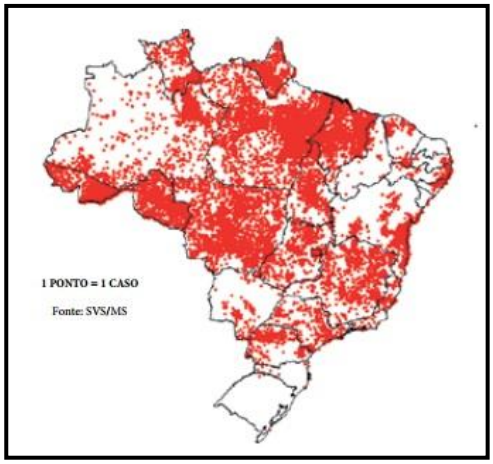
A Leishmaniose Tegumentar Americana (LT) é uma doença infecciosa, não contagiosa que acomete pele e mucosas. São reconhecidas atualmente nas Américas 11 espécies dermotrópicas de Leishmania causadoras de doenças em humanos, entretanto, no Brasil, já foram identificadas sete espécies, sendo seis do subgênero Viannia e uma do subgênero Leishmania. As espécies conhecidas são: *L. amazonensis*, *L. braziliensis*, *L. guyanensis*, *L. lainsoni*, *L. lindenbergi*, *L. naiffi* e *L. shawi*. Os vetores da LT são insetos denominados flebotomíneos, pertencentes à Ordem Díptera, Família Psychodidae, Subfamília Phlebotominae, Gênero Lutzomyia, conhecidos popularmente, dependendo da localização geográfica, como mosquito-palha, tatuquira, birigui, entre outros (MNISTERIO DA SAUDE, 2017; PAHO/WHO, 2015).

De acordo com a Organização Pan-Americana da Saúde (OPAS) e a Organização Mundial de Saúde (OMS, 2017), a leishmaniose cutânea e mucosa ocorre em 20 países nas Américas e é endêmico em 18 deles, mas com transmissão com diferentes intensidades: baixa, média, alta, intensa e muito intensa. De 2001 a 2015, 843.931 novos casos cutâneos e

mucosos foram relatados pela OPAS/OMS com uma média anual de 56.262 casos, distribuídos entre 17 dos 18 países endêmicos. Nos últimos cinco anos, houve uma diminuição dos casos, visto que 2015 teve o menor número de casos registrados, resultando em 10% de redução em relação a 2014.

No Brasil, em 2015, houve a ocorrência de 19395 novos casos 18324 cutâneo (94,5%) e 1071 mucosa (5,5%). O percentual por idade foi de: <5 anos de idade (3%), ≥ 5 <10 anos de idade (3,6%), > 10 anos de idade (92,1%), desconhecido (1,3%) e das crianças abaixo 10 anos de idade (6,6%). Quanto ao sexo: 73% do sexo masculino e 27% do sexo feminino, do total de homens 57% com 20 anos de idade (PAHO/WHO, 2015). Pode-se observar na figura 11 a incidência de leishmaniose no Brasil no ano de 2015.

Figura 11: Casos de leishmaniose tegumentar por município no Brasil em 2015.



Fonte: http://bvsms.saude.gov.br/bvs/publicacoes/manual_vigilancia_leishmaniose_tegumentar.pdf

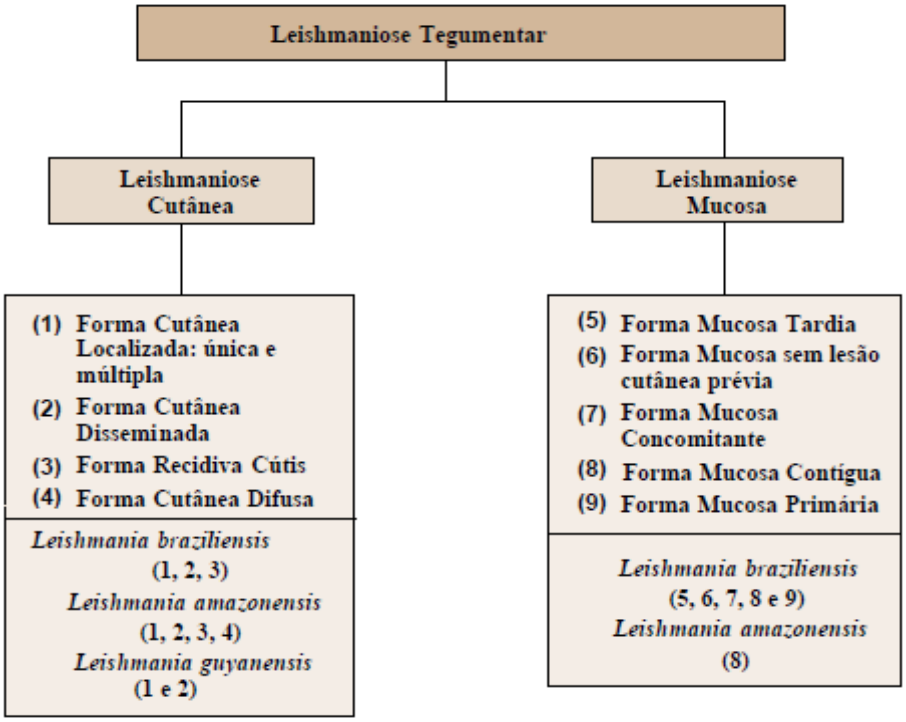
A apresentação clínica exibe polimorfismo e o espectro de gravidade dos sinais e sintomas também é variável, embora exista certa correspondência entre as distintas apresentações clínicas e as diferentes espécies do parasito (Figura 12) (MINISTERIO DA SAUDE, 2017).

Diagnóstico, tratamento e prevenção

O diagnóstico laboratorial pode ser feito pela identificação do parasita por meio do material obtido da lesão: exame direto de esfregaços corados, exame histopatológico, cultura, pesquisa de DNA do parasita e métodos imunológicos. O tratamento é realizado pelo uso de antimoniais de meglubina, os pentavalentes (Sb^{+5}) sendo estas as drogas de primeira escolha. O mecanismo de ação, de acordo com o Manual de Controle de Leishmaniose Tegumentar, ocorre à inibição da glicolise e da oxidação dos ácidos graxos em organelas peculiares, sendo

que esta inibição é seguida da redução na fabricação de adenosina (ATP) e guanosina trifosfato (GTP) no parasita. As drogas de segunda escolha são: anfotericina B e pentamidinas. Ainda não há parâmentros para a precisão de cura da LTA (FERREIRA; MAROCHIO; PARTATA, 2012).

Figura 12: Leishmaniose Tegumentar no Brasil, classificação clínica e respectivos agentes etiológicos.



Fonte: MINISTERIO DA SAUDE (2017).

Segundo levantamentos realizados por ORYAN (2015), os antimoniais pentavalentes têm efeitos inconvenientes e colaterais, incluindo anorexia, vômitos, tonturas, artralgia, mialgia, febre, administração parenteral, alto custo e longo curso de tratamento. Essas drogas têm limitações para mulheres grávidas, idosos e indivíduos com doenças cardíacas, renais e hepáticas devido à toxicidade para estes órgãos e pâncreas.

A pentamidina é a droga de segunda escolha comumente recomendada, embora também apresente efeitos adversos significantes e requeira a administração parenteral. De caráter catiônico, pode apresentar-se na forma de isotionato ou cloridrato (GIL et al., 2008). Na forma difusa, 300mg do medicamento devem ser diluídos em 5ml de água destilada para aplicação intramuscular no glúteo. A dose é de 4mg/kg com uma dose máxima de 240mg por dia em dias alternados. A dose total depende da resposta clínica e os efeitos adversos, incluindo nefrotoxicidade, hepatotoxicidade, hipertensão, hipoglicemia, hiperglicemia

(raramente), alterações eletrocardiográficas, abscesso glúteo, parestesia facial central, cefalea, epigastralgia e vertigem (TORRES-GUERERO et al., 2017).

A forma cutânea tende a ser curada espontaneamente deixando as cicatrizes, que podem evoluir para a leishmaniose cutânea difusa ou leishmaniose mucocutânea (MCL), dependendo das espécies de Leishmania que causaram a infecção. Em vista disso, os pacientes sofrem de consequências estéticas desastrosas (HALDAR; SEM; ROY, 2011).

Uma alternativa de prevenção em áreas que são endêmicas da LC é minimizar o risco de mordida dos flebotomínios no período noturno. O uso de redes de cama, com labirintos três vezes menores que os mosquiteiros usados para prevenção da malária são requeridos. Além disso, as redes de cama devem ser impregnadas com permetrina ou outro repelente de insetos eficaz para reduzir a chance de mordidas (VRIES; REEDIJK; SCHALLIG, 2015).

Potencial de óleos essenciais frente à Leishmaniose cutânea e mucosa

Nos últimos anos tem sido relatada na literatura a utilização de óleos essenciais de diversas espécies e esses tem demonstrado serem promissores frente às espécies que causam a LTA.

Em pesquisa com o óleo essencial de *Myracrodruon urundeuva* (Engl.) Fr. All. – MuEO inibiu o crescimento da forma promastigota de *Leishmania* (Leishmania) *amazonensis* (IFLA/BR/67/PH8) com a IC_{50} de 104.5±11.82µg.mL^{-1}, de amastigotas axênicos (IC_{50} 104.5±11.82µg.mL^{-1}) e diminuiu o percentual de macrófagos infectados e o número de amastigotas por macrófago (IC_{50}44.5±4.37µg.mL^{-1}). Os resultados obtidos por esses autores sugerem que a atividade anti-Leishmania frente à forma amastigota está associada à atividade imunomoduladora de macrófagos devido a um aumento da capacidade fagocítica induzida por MuEO (CARVALHO et al., 2017).

Ao trabalhar com o óleo essencial de *Zataria multiflora* Boiss, Dezaki, 2016, verificou que o EOZm inibiu as formas promastiotas de *L. tropica* (MHOM/IR/2002/Mash2) com IC_{50} de 3.2µl/mL e 8.3 µl/mL para as formas amastigotas (DEZAKI, 2016).

Já estudos de Demarchi et al., 2015, *Tetradenia riparia* (Hochst.) Codd constatou-se que TrEO após 72h de incubação a $LD_{50,}$ para a forma promastigota de *Leishmania (Leishmania) amazonensis*, foi de 0.8 µg/mL. E que houve a redução o índice de macrófagos infectados tratados com 0.03µg/mL.

Ramos et al. (2014), ao trabbalhar com o óleo essencial de *Mangifera indica* L. var. Rosa e Espada observou-se que o óleo essencial de ambas espécies (manga rosa e manga espada) contra as formas promastigotas de *L. amazonensis* (MHOM/77BR/LTB0016) após

72h de incubação, mostrou IC_{50} de 39,1 e 23,0µg/mL, respectivamente. Ambos foram mais seletivos para o parasita do que para os macrófagos.

A avaliação *in vivo*, do óleo essencial de *Chenopodium ambrosioides* L., em camundongos BALB/c infectados com *L. amazonensis* (30mg/kg - via intralesional/14 dias) preveniu o desenvolvimento da lesão comparado com animais não tratados e tratados. Além disso, a eficácia da EO também foi estatisticamente superior em comparação com animais tratados com glucantime (MONZOTE, et al., 2014).

Como descrito anteriormente várias espécies de diversas famílias botânicas têm sido relatadas com potencial leishmanicida. Entre essas famílias, destaca-se a família das Myrtaceae, tanto por apresentar espécies de interesse econômico, como espécies empregadas popularmente com diferentes finalidades, o que faz com esta seja alvo de pesquisas, inclusive sobre a atividade leishmanicida de suas espécies (KAUFFMANN; ETHUR, 2016).

Família das Myrtaceae

A família das Myrtaceae Juss. é representada por árvores, arvoretas ou arbustos com ramos jovens pilosos, tronco em geral com córtex esfoliante. Possui folhas opostas, simples, broquidódromas, pontuações translúcidas presentes e estípulas ausentes. Inflorescência tipo panícula, racemo, dicásio ou flores solitárias, bissexuadas, actinomorfas, diclamídeas, com cálice 4–5-lobado ou caliptriforme. Corola 4–5-mera, hipanto prolongado ou não acima do ovário, androceu polistêmone, estigma capitado e ovário ínfero. Os frutos são do tipo baga, com uma a numerosas sementes, coloração uniforme ou maculada, lisa ou muricada (AMORIM; ALVES, 2011).

De acordo com Gomes et al. (2017), essa família botânica possui distribuição natural por todos os continentes do hemisfério Sul. No Brasil, sua riqueza é representada por 1034 espécies, pertencentes a 23 gêneros, ocorrendo, especialmente, na Floresta Atlântica, ecossistema considerado centro de diversidade da família.

Algumas espécies encontradas no Vale do Catimbau

- **Gênero Algrizea**

***Algrizea minor* e *Algrizea macrochlamys* (DC.)**

O gênero Algrizea é caracterizado pela forma de vida de arbustos, apresentando inflorescências em dicásios, flores com longos pedicelos além do cálice bastante

desenvolvido, atingindo até duas vezes o comprimento da flor. As espécies são encontradas em áreas de Caatinga e Cerrado (STADNIK; PROENÇA, 2017).

Do gênero Algrizea só existem duas espécies descritas até o momento, que são *Algrizea minor* e *Algrizea macrochlamys* (DC.).

A espécie *A. minor*, pertencente à família Myrtaceae, foi descrita e ilustrada por Sobral; Faria-Junior; Proença (2010). De acordo com esses autores, essa espécie ocorre em altitudes mais baixas, cerca de 300-550m, sendo encontrada no norte do Planalto da Bahia. A espécie *A. macrochlamys*, descrita por Proença et al. (2006), é encontrada na Chapada Diamantina em altitudes variando entre 900-1250m, diferindo *A. minor* por possuir folhas, lobos e cálice menores.

Até o momento não há nenhum relato sobre a utilização do uso tradicional dessas espécies e possíveis atividades biológicas.

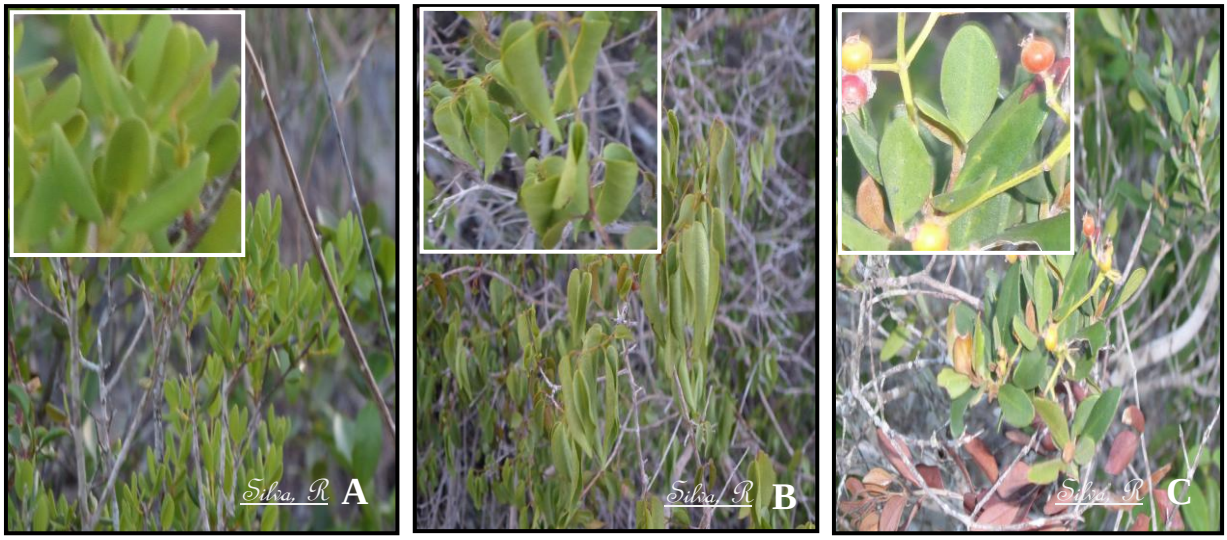
- **Gênero Eugenia**

O gênero Eugenia se caracteriza quanto à forma de vida como arbustos, árvores e subarbusto. O substrato é saprófita ou terrícola. De origem nativa, não sendo endêmico do Brasil. É encontrado em vários estados das regiões do Brasil, com os seguintes domínios fitogeográficos: Amazônia, Caatinga, Cerrado, Mata Atlântica, Pampa e Pantanal. Eugenia é um importante gênero da família Myrtaceae devido ao seu potencial econômico e farmacológico. No Brasil, foram descritas cerca de 388 espécies de Eugenia, das quais 301 são endêmicas (FLORA DO BRASIL, 2017). Entre as espécies, encontra-se a *Eugenia punicifolia* (Kunth) DC uma das espécies endêmicas da família Myrtaceae, distribuída em todo o país e em todos os biomas, que vão desde a Amazônia tropical e florestas atlânticas até à Caatinga e Cerrado.

Não foi encontrado nenhum relato sobre o óleo essencial da espécie *E. punicifolia*. Entretanto, já foi testado o extrato hidroalcólico das folhas, avaliando-se os efeitos: antinociceptivos, antiinflamatórios e gastroprotetores em roedores (BASTING et al., 2014), e o potencial do pó da folha como adjuvante no tratamento de diabetes mellitus tipo 2 (SALES et al., 2014).

Na figura 13 está ilustrado o aspecto geral das folhas das três espécies descritas anteriormente.

Figura 13: A – Aspecto e detalhe das folhas de *A. minor*, B – *A. macrochlamys* e C – *E. punicifolia***.** Encontradas no vale do Catimbau/Buíque-PE



Fonte: autora - SILVA (2015).

Óleos essenciais em espécies da família das Myrtaceae

Na tabela 2 encontra-se listada a composição química dos óleos essenciais de várias espécies da família das Myrtaceae.

Tabela 2: Composição química de óleos essenciais da família das Myrtaceaes.

ESPÉCIES	ÓRGGÃO VEGETAL	PRINCIPAIS COMPOSTOS	REFERÊNCIAS
Eugenia uniflora L.	Folhas e frutos	Frutos (32%) curzerene (19,7%), Selina - 1,3,7 (11) -trien - 8-ona (17,8%), atractylone (16,9 %) e Furanodieno (9,6 %). Folhas Germacrene A, B e C (11,6%, 21,2 e 11,4 respectivamente), Seline-1,3,7--trien-8-one oxide 3 (19,3), Seline 1,3,7 - --trien-8-one (9,7), β cariophyllene (12,6) e curzerene (3,9 %).	Frutos OGUNWANDE et al.,2005 Folhas VICTORIA et al., 2012
Eugenia sulcata Spring ex Mart.	Folhas e caules	Folhas β-cariofileno, (24,6%), a-cubebene (1,1%), β-copaeno (0,5%), cis-Muurola-3,5-dieno (0,6%), cis-Muurola-4 (14), 5-dieno (1,3%), γ -himachalene (2,0%), epizonarene (0,8%), trans-calamenene (4,4%) e trans-cadina-1,4-dieno (3,4%) Caules β-cariofileno, 18,8%.	LIMA et al., 2012
Eucalyptus Gunnii Hook	Folhas	1,8-cineol (67,8%) e α-pineno (14,12%).	BUGARIN et al., 2014
Psidium guajava Var. Pomifera	Folhas	93,77% epiglobulol (19,20%), 1,8-cineol (13,31%), óxido de isoaromadendrene (11,13%), cariofileno álcool (10,21%), e (E)-cariofileno (9,51%).	PINHO et al., 2014

Eugenia aromatica Kuntze	Botões de cravo	α-pineno (13,09%), β-pineno (45,44%), neral (0,20%), geranial (0,43%), gama terpineno (5,08%), cis-ocimeno (2,32%), alo ocimeno (2,78%), 1,8-cineol (16,27%), linalool (0,71%), borneol (0,25%), mirceno (5,13%) e pineno-2-ol (2,34%).	OBOH et. al., 2015
Eucalyptus microtheca e *Eucalyptus viminalis*	Folhas e flores	*E. microtheca* – Folhas (100%): α - felandreno (16,487 %), aromadendreno (12,773%), α - pineno (6,752 %), globulol (5,997%), Ledene (5,665%) , p- Cymen (5,251%) , e β - pineno (5,006%). Flores (100%): α - felandreno (16,487%), aromadendreno (12,773%), α - pineno (6,752%) , globulol (5,997%) , Ledene (5,665%), p- Cymen (5,251%), e β - pineno (5,006 %). *E. viminalis* – Folhas (100%) 1, 8-cineole (57,757%), α-pineno (13,379%), limoneno (5,443%), e globulol (3,054%).	MAGHSOODLOU et al., 2015
Myrtus communis L.	Folhas e flores	93,8% e 92,5% da composição total, foram identificados para as MCI e MCII, respectivamente. α-pineno (50,8 e 33,6%), 1,8-cineol (21,9 e 13,3%), linalol (2,7 e 14,8%), e de linalilo de etilo (0,5 e 9,5%).	BOUZABATA et al., 2015
Eugenia brejoensis Mazine	Folhas	89,3% δ-cadineno (22,6%), β-cariofileno (14,4%), α-muurolol (9,34%), α-cadinol (8,49%) e biciclogermacreno (7,93%).	SILVA et al., 2015

Fonte: ROSIMERE (2016)

Importância econômica e farmacológica da família das Myrtaceaes

Há relatos na literatura que evidenciam o potencial econômico de plantas da família das Myrtaceae. Muitas espécies são utilizadas na alimentação, como as espécies de *Psidium guajava* L. (goiaba), *Eugenia uniflora* L. (pitanga), consumidas *in natura* e na forma de suco, doces, geleias e sorvetes. Na ornamentação, destacam-se as espécies *Eugenia sprengelii* DC. (murta), e *Leptospermum scoparium* J.R.Forst; G.Forst. (Érica). E como medicinais destacam-se *Eucalyptus globulus* L. (eucalipto), empregado no tratamento da gripe, congestão nasal e sinusite; e *Myrciaria dubia* (Kunth) Mc Vaugh (camu-camu) que apresenta alto teor de vitamina C, (MORAIS; CONCEICAO; NASCIMENTO, 2014). Na tabela 3 estão listadas as diversas atividades biológicas de óleos essenciais de diferentes gêneros e espécies da família das Myrtaceae.

Tabela 3: Atividades biológicas de óleos essenciais da família das Myrtaceae.

1. TESTES		
1.1 ANTIOXIDANTE	**MÉTODOS**	**REFERÊNCIAS**
***Eugenia uniflora* L.**	DPPH, ABTS e FRAP.	VICTORIA et al., 2012
***Psidium cattleianum* Sabine**	DPPH, ABTS e FRAP.	CASTRO et al., 2014
Eucalyptus Gunnii	DPPH	BUGARIN et al., 2014
***Myrtus communis* var. Leucocarpa DC and var. Melanocarpa DC**	DPPH e ABTS**.**	PETRETTO et. al., 2016
1.2 ANTIBACTERIANO	**ESPÉCIES**	
***Eugenia uniflora* L.**	*Staphylococcus aureus* e *Listeria monocytogenes*	VICTORIA et al., 2012
Eugenia caryophyllata	*Escherichia coli, Staphylococcus aureus* e *Pseudomonas aeruginosa.*	NUNEZ & D´AQUINO, 2012
***Sygyzium aromaticum* L.**	*Bacillus subtilis, Staphyloccocus aureus, Proteus vulgaris, Pseudomonas aeruginosa* e *Klebsiella pneumoniae*.	ANWER et al., 2014
Eugenia	*Haemophilus ducreyi*	LINDEMAN et al., 2014

Caryophyllus			
***Pimenta pseudocaryophyllus* var. *pseudocaryophyllus* (Gomes) Landrum**	*Escherichia coli* e *Staphylococcus aureus*.		YOKOMIZO & NAKAOKA-SAKITA, 2014
***Myrtus communis* L.**	*Acinetobacter baumannii*		ALEKSIC et al., 2014
***Eucalyptus Gunnii* Hook**	*Staphylococcus aureus* *Staphylococcus epidermidis* *Pseudomonas aeruginosa* *Escherichia coli SY252 e IB112*	*Escherichia coli* *Bacillus subtilis* *Micrococcus flavus* *Klebsiella pneumoniae*	BUGARIN et al., 2014
***Eugenia calycina* Cambess.**	*Streptococcus mutans* *Streptococcus mitis* *Streptococcus sanguinis* *Streptococcus sobrinus*	*Prevotella nigrescens* *Porphyromonas gin-givalis* *Actinomyces naeslundii* *Bacteroides fragilis*	SOUZA et al., 2015
Eucalyptus camaldulensis	*Acinetobacter baumannii*		KNEZEVIC, et al., 2016
Eugenia anômala	*Escherichia coli*		SIMONETTI et al., 2016
Psidium salutare	*Listeria monocytogenes*		
Eugenia caryophyllus	*Pseudomonas aeruginosa* (antibiofilme)		BATISTA et al., 2014
1.3 ANTIFÚNGICO	**ESPÉCIES**		
***Eugenia uniflora* L.**	*Cândida lipolytica* e *Cândida guilliermondii*		VICTORIA et al., 2012
***Pimenta pseudocaryophyllus* var.**	*Aspergillus* Níger e Penicillium *verrucosum*.		YOKOMIZO & NAKAOKA-SAKITA, 2014

***pseudocaryophyllus* (Gomes) Landrum**			
***Psidium cattleianum* Sabine**	*Candida albicans, Candida parapsilosis, Candida guilhermondii, Candida lipolytica* e *Trichosporon asahii*		CASTRO et al., 2014
***Myrtus communis* L.**	*Candida albicans*	*Microsporum gypseum*	
	Candida tropicalis	*Trichophyton mentagrophytes*	
	Candida krusei	*Trichophyton mentagrophytes* var. *interdigitale*	
	Candida guillermondii	*Trichophyton rubrum*	
	Candida parapsilosis	*Trichophyton verrucosum*	
	Cryptococcus neoformans	*Aspergillus niger*	
	Epidermophyton floccosum	*Aspergillus fumigatus*	
	Microsporum canis	*Aspergillus flavus*	BOUZABATA et al., 2015
1.4 INSETICIDA	**ESPÉCIES**		
Myrtus communis	*Anopheles gambiae*		DELL'AGLI et al., 2012
***Eugenia uniflora* L.**	*Atta laevigata* Smith		JUNG et al., 2013
Eugenia sulcata	*Dysdercus peruvianus* e *Oncopeltus fasciatus*.		GONZALEZ et al., 2014
Myrciaria floribunda	*Dysdercus peruvianus* e *Oncopeltus fasciatus*.		TIETBOHL et al., 2014
Syzygium aromaticum	*Aedes aegypti* e *Culex Quinquefasciatus*.		FAYEMIWO et al., 2014
***Psidium guajava* Var. Pomifera**	*Drosophila melanogaster*		PINHO et al., 2014

1.5 Anti-Leishmania	**ESPÉCIES**	
Anti-Trypanossoma		
***Eugenia uniflora* L.**	*Trypanosoma cruzi*	SANTOS et al., 2012
***Eugenia uniflora* L.**	*Leishmania amazonensis*	RODRIGUES et al., 2013
***Eucalyptus globulus* e**	*Trypanosoma cruzi*	AZEREDO et al., 2014
***Eugenia uniflora* L.**		
***Syzygium cumini* L.**	*Leishmania amazonensis*	RODRIGUES et al.,2015
***Myrtus communis* L.**	*Leishmania tropica*	MAHMOUDVAND et al., 2015
OUTROS TESTES		
Eugenia caryophyllata	Cognição e dor em camundongos.	HALDER et al., 2012
Myrtus communis	Antiplasmodial *Plasmodium Falciparum* Antimutagênica *E. coli*	DELL'AGLI et al., 2012
***Eucalyptus Gunnii* Hook**	Antinociceptiva e propriedades anti-inflamatórias em ratos.	BUGARIN et al., 2014
Myrcia ovata	Inibição das enzimas: α - amilase e α-glicosidase. Abordagem terapêutica para o tratamento de diabetes tipo 2.	SANTOS et al., 2014

***Eugenia aromatica* Kuntze**	Anti-inflamatório O potencial anti-inflamatório foi avaliado em um modelo *in vitro* de lipopolissacárido (LPS) estimulada por macrófagos.	OBOH et al., 2015
***Myrtus communis* L.**	Larvicida Larvas de *Aedes Aegypti*	BOUZABATA et al., 2015
***Eugenia brejoensis* Mazine**		SILVA et al., 2015

Fonte: ROSIMERE (2016).

3 MÉTODO

3.1 LOCAL DE ESTUDO E ASPECTOS ÉTICOS

Os testes experimentais foram realizados em laboratórios do departamento de bioquímica da Universidade Federal de Pernambuco e do Instituto Aggeu Magalhães – FIOCRUZ/PE. Os experimentos atenderam as questões éticas e de segurança.

3.2 COLETA E OBTENÇÃO DO MATERIAL VEGETAL

Folhas de *A. macrochlamys, A. minor* e *E. punicifolia* foram coletadas em maio e julho de 2015, e março de 2016, a partir de arbustos no Parque Nacional do Catimbau, Pernambuco, Brasil. Os espécimes de comprovante foram depositados no Herbário do Instituto Agronômico de Pernambuco (IPA, Recife, PE, Brasil), com os números IPA 96257, 84346 e 97008, respectivamente.

3.3 COMPOSIÇÃO QUÍMICA DOS ÓLEOS ESSENCIAIS

Os óleos essenciais foram analisados em um Cromatógrafo a gás (modelo 7890A; Agilent Technologies; Palo Alto, CA, EUA) equipado com uma coluna não polar HP-5ms ™ da Agilent J & W (30 mx 0,25 mm id; 0,25 µm de espessura do filme) e acoplado a um detector de massa seletivo (modelo 5975C; Agilent Technologies; Palo Alto, CA, EUA). Os dados de MS adquiridos para cada componente foram comparados com aqueles armazenados na biblioteca espectral de massa do sistema GC-MS (MassFinder 4, NIST08 e Wiley Registry ™ 9ª Edição) e com espectros publicados em Adams (2007) para confirmar a identidade. Sempre que possível, a identidade dos compostos foi confirmada usando tempos de retenção e espectros de massa de padrões autênticos disponíveis no Laboratório de Ecologia Química (UFPE). As áreas de pico nos cromatogramas foram integradas usando o software Agilent MSD Productivity ChemStation (Agilent Technologies, Palo Alto, EUA) para obter o sinal de corrente de íon total, que foi usado para determinar as porcentagens relativas de cada componente dos óleos.

3.4 TESTES BIOLÓGICOS

- **Antibacteriano**

A atividade antimicrobiana foi determinada pelo teste de sensibilidade contra quatro bactérias patogênicas comumente associadas a infecções hospitalares. As concentrações inibitórias mínimas (CIMs), ou seja, a concentração mais baixa das amostras dos óleos essenciais capaz de inibir o crescimento de microrganismos, foi determinada por um método de microdiluição

em caldo seguindo procedimentos padrão de acordo com Clinical and Laboratory Standards Institute (CLSI, 2017).

Para determinar as concentrações bactericidas mínimas (MBC), as concentrações dos óleos nas quais não se observou crescimento bacteriano foram inoculados em placas de Petri com ágar Mueller-Hinton. As placas foram incubadas a 37 durante 24h. O CBM foi considerado a menor concentração que não permitiu a reativação dos microrganismos no meio sem o agente antimicrobiano.

- **Antibiofilme**

O ensaio foi realizado em placas de 96 poços de acordo com Trentin et al. (2011). O inóculo bacteriano (*S. aureus*) foi ajustado em tubo estéril e lido em OD 600nm, correspondendo a 1 escala MacFarland, 3.10^8 UFC. Foram realizados os seguintes testes: controle de esterilização, controle de crescimento e formação de biofilme, controle de atividade antibacteriana e testes (água, inóculo bacteriano e meio), após montagem a placa foi lida e incubada a 37 ° C. O grupo controle de crescimento e formação de biofilme recebeu água, o grupo de atividade antibacteriana recebeu vancomicina e o grupo teste recebeu o óleo essencial *de A. minor*. O grupo controle de biofilme foi usado para representar 100% da formação de biofilme.

- **Citotóxico**

O ensaio citotóxico do óleo essencial de *A. minor* foi determinada utilizando células VERO através do ensaio colorimétrico com brometo de 3- [4,5-dimetiltiazol-2-il] -2,5-difeniltetrazólio (MTT) (SIGMA, St. Louis, MO, EUA), um protocolo adaptado de Monzote et al. al. (2014). Outro método foi a utilização do reagente CellTiter-Glo (Promega, EUA) para o teste com macrófagos peritoniais.

- **Moluscicida**

O ensaio de embriotoxicidade foi realizado de acordo com Oliveira-Filho et al. (2010) usando massas de ovos com aproximadamente 300 embriões de *Biomphalaria glabrata* para cada tratamento no estágio de blástula (15h após a desova). Os embriões foram selecionados por observação num estereomicroscópio (Leica MZ6; Leica Microsystems, Wetzlar, Alemanha) e colocados em placas de Petri (90x15 mm).

Para a avaliação da atividade moluscicida, moluscos adultos (n = 180) de tamanho uniforme (diâmetro de concha entre 10–16 mm) foram distribuídos em seis grupos: controle

negativo, controle de solvente e quatro grupos expostos a diferentes concentrações do óelo essencial de *A. minor*. A mortalidade dos moluscos foi avaliada pela retração da massa cefalopodal no interior da concha com a liberação ou não de hemolinfa ou a projeção anormal do cefalópode fora da casca, conforme descrito por Mccullough et al. (1980).

- **Artemicida**

O crustáceo Artemia salina foi utilizado como modelo biológico para avaliação da toxicidade do óleo essencial de *A. minor* para organismos não-alvo. As artemias foram colocados em recipiente com água do mar por 24h com aeração até o surto. Os animais foram divididos em três grupos (n = 30 para cada grupo), sendo um grupo controle negativo, um grupo controle com solvente e quatro grupos expostos a diferentes concentrações do óleo durante 24h. Os testes para avaliação da mortalidade e sobrevida foram realizados segundo Santos et al. (2010).

- **Leshmanicida**

Para os testes leshmanicida as formas promastigotas de *Leishmania amazonensis* foram mantidas em meio de Schneider suplementado com 10% de soro fetal bovino, a 26°C e colhidas durante a fase exponencial de crescimento. As formas amastigotas foram obtidas de macrófagos peritoneais BALB/c infectados por *L. amazonensis*.

A concentração do óleo de *A. minor* que inibiu a viabilidade de cultura em 50% (IC_{50}) foi estimada após 48h de incubação por análise de regressão utilizando o software SPSS 8.0. Após os tratamentos, avaliou-se a viabilidade celular utilizando o kit de ensaio de viabilidade celular CellTiter-Glo luminescente (Promega, EUA) seguindo as instruções do fabricante. A luminescência foi medida no aparelho GloMax (Promega, EUA).

Para avaliar o do óleo essencial de *A. minor* contra a forma intracelular do parasita, macrófagos peritoneais de camundongos Balb/c foram colhidos e plaqueados a 1×10^6 células/ml em uma placa de 24 poços, contendo meio RPMI suplementado com 10% de fetos inativados. soro bovino. Os macrófagos foram deixados aderir e então infectados com promastigotas. Os parasitas não-interiorizados foram removidos por lavagem e a cultura infectada foi incubada por 24h em meio RPMI 1640 ou tratada com diferentes concentrações do óleo de *A. minor* correspondentes aos valores de IC_{50} obtidos para promastigotas. A percentagem de macrófagos infectados foi determinada em lâminas de cultura coradas com Giems, contando-se 100 células escolhidas aleatoriamente em triplicata.

3.5 ANÁLISE ESTATÍSTICA

Os dados foram analisados por análise de variância one-way (ANOVA) e teste de Turkey para determinar a significância estatística, p-valor <0,05 foi considerado estatisticamente significativo.

4 RESULTADOS

4.1 ARTIGO 1 - ARTIGO A SER SUBMETIDO AO JOURNAL OF ESSENTIAL OIL RESEARCH.

Chemical composition and antibacterial potential of essential oils from *Algrizea macrochlamys* and *Eugenia punicifolia* (Myrtaceae)

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Abstract

Nosocomial infections represent a serious public health problem worldwide. This work aimed to evaluate the chemical composition and antibacterial activity of the essential oils from leaves of *Algrizea macrochlamys* (EOAma) and *Eugenia punicifolia* (EOEpu) against bacteria commonly associated with hospital infections. EOs were obtained by hydrodistillation and analyzed by GC-MS. The antibacterial activities were determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The yields of the EOs ranged from 0.36 to 3.4% (w/w). The essential oils shared the compounds (*E*)-caryophyllene, γ-eudesmol and guaiol, which are commonly reported as EOs constituents. The best MIC and MBC values observed were 0.25 mg/mL (EOAma against *Staphylococcus sarohyticus*) and 16 mg/mL (EOEpu against *S. aureus* and EOAma against *S. saprophyticus*), respectively. In summary, the essential oils tested showed antimicrobial potential and this effect could be linked with the majoritarian compounds.

Keywords: *Algrizea macrochlamys*; *Eugenia punicifolia*; (*E*)-caryophyllene; γ-eudesmol; guaiol; Antibacterial activity.

INTRODUCTION

The family Myrtaceae comprises about 132 genera and 5671 species of trees and shrubs, occurring mainly in tropical and subtropical regions, particularly in South America, Australia and Tropical Asia (1,2). In Brazil, Myrtaceae is one of the most diverse taxa with 23 genera and 1034 species, distributed in all regions and vegetal formations of the country (3).

Among Myrtaceae, the genus *Eugenia* is one of the most relevant due to its many economic and pharmacological applications. Currently, 388 species of *Eugenia* are recognized for Brazil, of which 301 are endemic. *Eugenia punicifolia* (Kunth) DC, one of this endemic species, occurs across the country, growing in both tropical rain- (Amazon and Atlantic forests) and dry-forest formations (Caatinga and Cerrado) (3). The genus *Algrizea* is consisted of only two shrub species, i.e. *Algrizea macrochlamys* (DC.) and *A. minor*, both endemic to Northeastern Brazil, occurring specifically in the states of Bahia and Pernambuco (4).

Essential oils (EOs) are natural hydrophobic products characterized by a high volatility, strong odor, and high chemical complexity, which are commonly extracted from plants found in tropical countries (5). They are composed mostly by mono-sesquiterpenes and phenylpropanoids, compounds which confer their organoleptic characteristics (6,7). EOs play an important role in plant defense against an astonishing array of natural enemies, such as herbivores and pathogens (5), therefore presenting a wide applicability for biological control. Furthermore, they are natural, biodegradable and commonly have low toxicity to mammals (8). Given the aforementioned peculiarities, the activity of EOs against crop/human diseases has been extensively researched (9-12). Specifically among Myrtaceae, antibacterial (13), antifungal (14), antioxidant (15), leishmanicidal (16), anti-trypanosoma (17), and insecticidal activities (18) have already been reported.

Nosocomial infections are caused commonly by bacteria resistant to multiple antibiotics (or multi-antibiotic resistant bacteria) and represent a serious public health problem worldwide. These infections are very difficult to treat and require very expensive and generally toxic drugs (19). The World Health Organization (WHO) defines antimicrobial resistance as the ability of a microorganism (bacteria, virus and some parasites) to tolerate the effect of antimicrobial agents (antibiotics, antivirals and antimalarials). As a result, standard treatments become ineffective, infections persist and can spread to other patients. The bacteria that develop antimicrobial resistance are popularly known as "superbacteria" (20). This is the case of multi-resistant *Pseudomonas aeroginosa*, considered a problem in Brazil (21). There are also reports of multi-antibiotic resistant bacteria of *Escherichia coli* (22) and *Staphylococcus aureus* (23).

Given this alarming scenario, it is necessary to search continuously for new compounds with antimicrobial activity, which can be used as antiseptics/disinfectants in hospital environment. Therefore, the present work aimed to evaluate the chemical composition and the antimicrobial activity of essential oils of two Myrtaceae in the attempt of finding alternatives to control the dissemination of microorganisms that rapidly evolve resistance against antibiotics and are responsible for infectious diseases. This is the first report about the chemical composition and biological activity of essential oil from *A. macrochlamys* and *E. punicifolia* against microorganisms that represent an important public health problem.

MATERIAL AND METHODS

Collection of Plant material

Leaves of *A. macrochlamys* and *E. punicifolia* were collected in May and July 2015, and March 2016 from shrubs in the *Parque Nacional do Catimbau*, Pernambuco, Brazil. Voucher specimens were deposited at the Herbarium of the *Instituto Agronômico de Pernambuco* (IPA, Recife, PE, Brazil), with numbers IPA 96257 and 97008, respectively.

Extraction of essential oil

The fresh leaves of the two species were separately triturated and submitted to hydrodistillation technique in a Clevenger-type apparatus for 6 hours. After that, essential oil layer was separated, dried over anhydrous sodium sulfate and transferred to amber-glass vials, resulting in EOAma (essential oil of *A. macrochlamys*) and EOEpu (essential oil of *E. punicifolia*). The oil yields were calculated as a percentage based on the plant weight (% w/w) and stored at 4ºC until chemical analysis and biological assays. For each species, we extracted three oil samples.

Chemical characterization of essential oils

The essential oils of the two species were analyzed on a Gas Chromatograph (model 7890A; Agilent Technologies; Palo Alto, CA, USA) equipped with an Agilent J &W non-polar HP-5ms™ column (30 m x 0.25 mm id.; 0.25 μm film thickness) and coupled to a selective mass detector (model 5975C; Agilent Technologies; Palo Alto, CA, USA). Prior to injection, essential olis were diluted in solvent (1μL of the oil in 999μl of hexane). After this, 1μl of each diluted oil, was injected in split mode (1:300) with the injector temperature set to 250°C. GC oven temperature was set at 4°C for 2 minuutes, then increased at a rate of 4°C $minutes^{-1}$ to 230°C. The final temperature was held steady for 5 minutes. Helium (He) carrier gas flow was maintained at a constant pressure of 7.0 psi. MS Source and quadrupole temperatures were set at 230°C and 150°C, respectively. Mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1.0 $scan^{-s}$ from m/z 35–350.

Individual components of the essential oils were identified by comparison of Retention Indices (RI), obtained by co-injection of each oil sample with C_9–C_{30} linear hydrocarbons and calculated according to the Van den Dool and Kratz equation (24), with those reported in the literature. The MS data acquired for each component were compared with those stored in the mass spectral library of the GC-MS system (MassFinder 4, NIST08 and Wiley Registry™ 9th

Edition) and with published spectra (25) in order to confirm identity. Whenever possible, identity of compounds was confirmed using retention times and mass spectra of authentic standards available in the *Laboratório de Ecologia Química* (UFPE). The peak areas on the chromatograms were integrated using the software Agilent MSD Productivity ChemStation (Agilent Technologies, Palo Alto, USA) to obtain the total ion current signal, which was used to determine the relative percentages of each oil component.

Antibacterial activity

Strains studied and growth conditions

In this study, we used two gram-negative strains, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), both provided by the *Instituto Aggeu Magalhães*, and two gram-positive, *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus saprophyticus* (UFPE 823), provided by the *Instituto Aggeu Magalhães* and *Departamento de Antibióticos* of *Universidade Federal de Pernambuco*, respectively.

Stocks of the bacteria strains were maintained at –80°C in cryotubes with BHI broth + glycerol (1:1). Before the assays, to obtain the colonies, the strains were cultivated in Petri dishes containing Mueller-Hinton agar and incubated at 37°C for 24 hours. Subsequently, between three and five colonies were selected and resuspended in Eppendorf tubes (with 1mL of Mueller Hinton broth). The optic density was then determined at 600 nm (OD600), and the inoculum was adjusted to the 0.5 McFarland scale (equivalent to 1-5 x 10^8 CFU/mL), following the standard indicated by Clinical and Laboratory Standards Institute (CLSI) (26).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimal inhibitory concentrations (MICs) of the essential oils, i.e. the lowest concentration of the samples capable of inhibiting the growth of microorganisms, were determined by a serial microdilution method in broth following standard procedures (26). The 96 multi-well plates were prepared with 50 μL of Mueller-Hinton (MH) broth (per well) containing twelve decreasing concentrations of oils, diluted in dimethyl sulfoxide (DMSO) not exceeding 10 %, ranging from 32 to 0.0156 mg/mL. Afterwards, it was added 50 μL of bacterial suspension (10^6 CFU/mL) in each well. All plates included a positive growth control in the absence of EOs. The plates were incubated at 37°C for 24 hours. The MIC's values

were then determined as the lowest concentration capable of visually inhibiting bacterial growth.

To determine the minimal bactericidal concentrations (MBC), the lowest concentration in which there was no bacterial growth, 10 μL of the wells with oil concentrations at which growth of bacteria was not observed were inoculated on Petri dishes with Mueller-Hinton agar. The dishes were incubated at 37°C for 24 hours. The MBC was considered the lowest concentration that did not allow the reactivation of the microorganisms in the medium without the antimicrobial agent. The MIC's and MBC's were expressed in mg/mL. The data were expressed as a mean of replicates ± Standard Desviation (S.D.), which were calculated using Microsoft Office Excel version 14.0 for Windows. All assays were performed in triplicate and repeated twice in different days.

RESULTS

Extraction and chemical composition of essential oils

The yield of essential oils obtained by hydrodistillation of fresh leaves from *A. macrochlamys* and *E. punicifolia* were 3.4±0.70% and 0.36±0.00% (w/w), respectively.

The essential oil of *A. macrochlamys* was composed of 74 constituents, of which 56 were identified. The sum of the identified components represented more than 88% of the essential oil blend. Sesqui- and monoterpenes accounted for 84.50% and 4.03 of the oil, respectively. (*E*)-caryophyllene (15.10%), γ-eudesmol (13.43%), β-eudesmol (7.76%), α-eudesmol (7.36%), guaiol (5.62%) and germacrene D (5.18%) were the major compounds (Tables 1 and 2).

Seventy nine compounds were reported in *E. punicifolia* oil, of which 55 were identified. The sum of the identified components represented more than 85% of oil. Sesqui- and monoterpenes accounted for 59.72% and 25.70% of the oil blend, respectively (Table 1). Major compounds were α-pinene (18.07%), guaiol (11.20%), bulnesol (10.56%), (*E*)-caryophyllene (6.87%) and γ-eudesmol (5.37%) (Tables 1 and 2).

Antibacterial activity

Essential oils of *A. macrochlamys* and *E. punicifolia* were active against two bacteria (Table 3), and the best MIC values observed were 0.25 mg/mL (EOAma against *S. saprophyticus*) and MBC in 16 mg/mL (EOAma and EOEpu against *S. aureus*, and EOEpu against *S. saprophyticus*).

DISCUSSION

The yield of the essential oils from leaves of *A. macrochlamys* and *E. punicifolia* (3.4 and 0.36, respectively) are comparable with those reported for oils of other Myrtaceae that were also obtained by hydrodistillation. In a study investigating seven species of Myrtaceae, Silva et al (27), reported yields ranging from 0.2 in *Callistemon polandii* and 4% in *Melaleuca thymifolia*. *A. macrochlamys* showed better yield compared to species of other families, *Cymbopogon citratus* and *C. nardus* of the Poaceae family, with yields of 1.04 and 0.14%, respectively (28, 29), and the species *Croton Rhamnifolioides*, *C . adamantinus* and *C. campestris* from the Euphorbiaceae family, with yields of 0.80, 0.60 and 0.04%, respectively (10, 11, 30).

Some of the major compounds from EOs of *A. macrochlamys* and *E. punicifolia* were already reported in oils obtained from leaves of other Myrtaceae species. For example, α-pinene is reported in *E. Gunnii*, *E. viminalis*, *E. aromatica* and *E. microtheca* (14.12, 13.38, 13.09 and 6.75%, respectively) (31-33); (*E*)-caryophylene in *E. riedeliana* (10.9%) and *Psidium guajava* (9.51%) (34, 35); γ-eudesmol in *E. riedeliana* (12.9%) (34); guaiol in *Myrcia fallax* (31%) and *E. axillaris* (35.4%) (36, 37); and germacrene D in *M. alagoensis* (11.1 and 6.4%) (38). These data point similarity among the EOs from leaves of *A. macrochlamys* and *E. punicifolia* and other species of Myrtaceae.

These results reinforce the importance of searching for alternative control of microorganism dissemination in hospital environment, since they cause several infectious diseases and rapidly evolve resistance against most commonly used antibiotics. Previous studies demonstrated that EOs show a considerable antimicrobial activity due to the presence of chemical compounds such as monoterpenes, sesquiterpenes and phenolic compounds (11, 38, 39). An alternative to control the dissemination of microorganisms is reported by Pereira et al. (40) through development of disinfectant solution of essential oils from species of family Myrtaceae (*Thymus vulgaris, Elettaria cardamomum, E. caryophyllus* and *Foeniculum vulgare*) against *E. coli, S. aureus* and *Salmonella enteritidis*. The antimicrobial activity showed that the EO of *T. vulgaris* was more effective to all bacteria species with a good MIC value (0.25%). The oil from *E. caryophyllus* was only effective to E. coli (MIC of 0.25%). The other oils did not show the same effectiveness, S. enteritidis was not sensible to the tested oils. Due to this, the oils proved to be suitable for use as disinfectants.

The MICs of EO from *E. globulus* against *P. aeroginosa* (ATCC27853) and *E. coli* (same strain used in this study) were 4 mg/mL for both bacteria and MBCs were 9 and 4 mg/mL, respectively. These results are more effective than the showed in our study (41). Thus, it is necessary higher concentrations of EOs assayed in this study for inhibition of these bacteria.

It is noteworthy mentioning that the MICs and MBCs values of the essential oils tested here varied considerably between gram-positive and gram-negative bacteria. This discrepancy in antibacterial activity of EOs is probably related to the different bacterial architectures (cell membrane compositions) (42).

According to Sarto and Junior (43), the EOs could act in three different ways in bacteria: (I) interference in the double phospholipid layer of the cell wall, (II) by increased permeability and loss of cellular constituents, and (III) by altering a variety of enzymatic activities such as those involved in the production of cellular energy and synthesis of structural components or destruction of genetic material. Besides that, due to the lipophilic characteristic, the essential oils cross the cell wall and plasma membrane, and can affect their structures (5).

Some of the major compounds from EOs of *A. macrochlamys* and *E. punicifolia* showed antimicrobial effect against some bacteria. For example, the monoterpene α-pinene was tested against *S. aureus* (ATCC 13150, ATCC 6538, ATCC 25923 and ATCC LB 126), and *S. epidermidis* (SSI 1 and ATCC 12228) and showed activity against all strains tested, (44). The sesquiterpene (*E*)-caryophyllene showed antimicrobial effect against *S.aureus* and *Enterococcus faecalis* (MIC and MBC: 1 and 4 mg/mL, respectively), *E. coli* (MIC and MBC: 1 and 2 mg/mL, respectively), and *S. typhimurium* (MIC and MBC: 0.5 and 2 mg/mL, respectively) (45). Taken together, these results suggest that the antibacterial effect of the essential oils tested in this study could be associated, at least partially, to these compounds.

CONCLUSION

The EOs tested showed antimicrobial potential, through inhibition of bacterial growth, in acceptable concentrations. The antibacterial effect could be linked to the major compounds of the oils as α-pinene and (*E*)-caryophyllene. Then, these oils could be potentially used as disinfecting agents of surfaces and materials for hospital use. Furthermore, the analysis of the chemical composition of the essential oils from new species contributes to further studies of the antimicrobial potential against microorganisms and other biotechnological purposes.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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TABLE 1

Table 1. Classification of the chemical group of compounds found in the essential oils of *A. macrochlamys* and *E. punicifolia* (Myrtaceae).

	Essential oil	
Chemical groups	**EOAma (%)**	**EOEpu (%)**
Hydrocarbon monoterpenes	3.06	22.30
Oxygenated monoterpenes	0.99	2.4
Monoterpenes	**4.05**	**24.70**
Sesquiterpenes	0.09	0.98
Hydrocarbon sesquiterpenes	44.75	18.86
Oxygenated sesquiterpes	39.75	39.88
Sesquiterpenes	**84.59**	**59.72**
Others compounds	11.35	14.58

TABLE 2

Table 2. Mean relative proportions and standard deviation (SD) of constituents of the essential oils of *A. macrochlamys* and *E. punicifolia* (Myrtaceae). Constituents are listed according to their elution on a non-polar DB-5 column. Marked in bold are compounds accounting for more than 1% of the essential oil of any species.

			EOAma		EOEpu	
Compounds	**RI**	**RI***	**Mean %**	**SD**	**Mean %**	**SD**
α-Thujene	925	924	0.02	0.03	0.21	0.30
α-Pinene	932	932	0.33	0.27	**18.07**	16.55
Camphene	945	946	-	-	0.10	0.16
Sabinene	972	969	0.01	0.01	-	-
β-Pinene	974	979	0.27	0.20	**2.78**	1.01
Myrcene	992	988	0.02	0.02	-	-
α-Phellandrene	1003	1002	0.01	0.02	-	-
δ-3-Carene	1009	1008	-	-	-	-
α-Terpinene	1016	1014	0.02	0.04	-	-
p-Cymene	1024	1022	0.04	0.04	0.18	0.06
Limonene	1028	1024	0.17	0.10	**1.12**	1.20
1,8-Cineole	1030	1026	**2.02**	0.71	0.15	0.05
γ-Terpinene	1059	1054	0.09	0.13	0.05	0.08
(*Z*)-linalool oxide (furanoid)	1073	1067	0.02	0.03	-	-
(Z)-linalool oxide (furanoid)	1088	1084	0.02	0.03	-	-
Terpinolene	1088	1086	0.02	0.04	0.17	0.21
Linalool	1100	1095	0.26	0.18	0.89	1.18
(*E*)-Pinocarveol	1138	1135	-	-	0.27	0.26
Borneol	1166	1165	-	-	0.28	0.37
Terpinene-4-ol	1178	1074	0.09	0.05	0.25	0.17

α-Terpineol	1191	1186	0.64	0.22	0.63	0.45
Myrtenol	1197	1194	-	-	0.08	0.10
Fenchyl acetate	1220	1218	-	-	0.02	0.03
Bornyl acetate	1287	1287	-	-	0.45	0.54
N.I.	1329		0.01	0.02	-	-
δ-Elemene	1339	1335	0.39	0.19	0.14	0.16
α-Cubenene	1351	1345	0.61	0.13	0.04	0.07
α-Ylangene	1373	1373	0.04	0.02	0.09	0.10
α-Copaene	1378	1374	**1.44**	0.22	0.68	0.64
β-Bourbonene	1387	1387	0.27	0.09	0.02	0.04
β-Cubebene	1392	1387	0.23	0.06	-	-
β-Elemene	1394	1389	0.98	0.17	0.25	0.19
α-Gurjunene	1412	1409	0.57	0.15	0.20	0.12
(*E*)-Caryophyllene	1422	1417	**15.10**	2.23	**6.87**	2.63
β-Copaene	1431	1430	0.17	0.04	0.08	0.07
N.I.	1434		-	-	0.05	0.08
γ-Elemene	1436	1434	0.06	0.02	0.53	0.44
N.I.	1437		-	-	0.27	0.48
α-Guaiene	1441	1437	0.18	0.05	**1.06**	1.06
N.I.	1445		-	-	0.05	0.08
Guaia-6,9-diene	1446	1442	0.08	0.03	0.08	0.07
(*E*)-3,5-Muuroladiene	1453	1451	**1.31**	0.32	0.05	0.09
Humulene	1456	1452	**2.98**	0.62	**1.77**	0.52
N.I.	1459		-	0.01	-	-
9-epi-Caryophyllene	1463	1464	0.60	0.17	0.65	0.23
(*E*)-Cadina-1(6),4-diene	1476	1475	**1.42**	0.42	0.17	0.19
γ-Muurolene	1480	1478	0.23	0.04	0.25	0.12
Germacrene D	1483	1484	**5.18**	0.91	0.90	0.64
β-Selinene	1489	1489	0.18	0.03	0.21	0.12

N.I.	1491		0.07	0.04	0.31	0.03
(*E*)-Muurola-4(14),5-diene	1494	1493	0.93	0.46	0.12	0.12
Viridiflorene	1497	1496	0.89	0.41	0.89	0.35
Bicyclogermacrene	1500	1500	**2.49**	1.41	**1.51**	0.97
α-Muurolene	1503	1500	0.57	0.11	0.22	0.13
N.I.	1510		0.11	0.03	0.16	0.14
N.I.	1512		0.16	0.05	0.27	0.09
γ-Cadinene	1517	1513	0.32	0.11	0.22	0.05
δ-Cadinene	1526	1522	**4.02**	1.17	**1.51**	0.27
(Z)-Calamenene	1527	1528	**2.02**	0.58	0.40	0.69
Zonarene	1529	1528	0.89	0.18	0.04	0.07
(*E*)-Cadina-1,4-diene	1536	1533	**1.08**	0.20	0.04	0.07
N.I.	1538		-	-	0.05	0.09
N.I.	1541		0.15	0.01	0.16	0.14
α-Calacorene	1545	1544	0.12	0.04	0.52	0.22
N.I.	1551		**2.75**	0.78	0.63	0.45
N.I.	1556		0.13	0.06	0.22	0.03
Germacrene B	1561	1559	0.09	0.05	0.98	0.81
N.I.	1566		0.04	0.01	0.12	0.07
N.I.	1570		0.26	0.15	0.43	0.21
Spathulenol	1580	1577	0.65	0.45	**4.26**	0.28
N.I.	1587		**1.65**	0.90	**4.06**	0.22
N.I.	1595		0.39	0.19	0.97	0.51
N.I.	1597		0.22	0.08	0.57	0.32
Guaiol	1601	1600	**5.62**	4.48	**11.20**	9.31
N.I.	1606		0.48	0.21	0.93	0.37
5-epi-7-epi-α-Eudesmol	1610	1607	0.51	0.18	-	-
N.I.	1613		0.34	0.11	0.36	0.40

N.I.	1617		0.27	0.04	0.14	0.25
N.I.	1619		-	-	0.38	0.65
10-epi-gama-Eudesmol	1623	1622	**1.34**	0.32	0.16	0.28
N.I.	1626		-	-	0.35	0.24
N.I.	1632		**1.70**	0.80	**1.01**	0.27
γ-Eudesmol	1636	1630	**13.43**	3.65	**5.37**	0.75
N.I.	1644		0.43	0.05	**1.01**	0.35
N.I.	1647		**2.19**	0.66	**1.41**	0.45
N.I.	1652		-	-	0.39	0.11
β-Eudesmol	1656	1649	**7.76**	0.73	**3.68**	1.94
α-Eudesmol	1660	1652	**7.36**	1.46	**4.00**	1.74
N.I.	1661		-	-	0.28	0.34
Bulnesol	1672	1670	**2.48**	2.18	**10.56**	8.90
Total identified			88.64		85.42	

Note: RI* (25)

TABLE 3

Table 3. Mean of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) from essential oils of two species of Myrtaceae (*A. macrochlamys* and *E. punicifolia*).

Microrganisms	**MIC**		**MBC**	
	EOAma	EOEpu	EOAma	EOEpu
S. aureus	4±1.15	4±0.00	16±0.00	16±0.00
S. saprophyticus	2±1.15	0.25±0.12	>32	16±0.00
E. coli	>32	32±0.00	>32	>32
P. aeroginosa	32±0.00	32±0.00	>32	>32

Note: MIC/MBC - mg/mL - MD±SD

4.2 ARTIGO 2 - ARTIGO A SER SUBMETIDO A REVISTA BMC COMPLEMENTARY AND ALTERNATIVE MEDICINE.

First report on chemical composition, antibiofilm potential and molluscicidal activity of the essential oil of *Algrizea minor* (Myrtaceae)

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Abstract

Background: The Myrtaceae family has many species with economic interest that are popularly used for different applications, which make them an object of research. Several essential oils obtained from leaves and fruits of Myrtaceae species have showed biological activities. This work aimed to evaluate the chemical composition, antibacterial, molluscicidal, artermicidal and cytotoxic activity of the essential oil from leaves of *Algrizea minor* (EOAm).
Methods: The fresh leaves were crushed and submitted to the hydrodistillation technique, in a Clevenger type apparatus. The essential oil was analyzed on a Gas Chromatograph coupled to a selective mass detector. Compounds were identified by comparing their mass spectra with those available in mass spectral libraries. Antibacterial activity was determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Colorimetric method using MTT was used for the cytotoxicity assay. The molluscicidal tests were performed against embryos and adults of *Biomphalaria glabrata*. For the test in non-target organism, *Artemia salina* was used.
Results: The yield of EO obtained by hydrodistillation of fresh leaves from *A. minor* were 2.45% (w/w). The major compounds were β-pinene (10%), followed by γ-eudesmol (7.67%), bicyclogermacrene (7.41%), germacrene D (6.58%), (*E*)-caryophyllene (6.50%), bulnesol (6.24%), guaiol (5.11%), α-eudesmol (4.93%), β-eudesmol (3.94%) and α-pinene (3.55%). The best MIC values observed were 0.25 and 1 mg/mL (EOAm against *Staphylococcus aures* and *S. saprophyticus*, respectively) and MBC 16 mg/mL for both species. For the test of biofilm eradication, the same concentration (250 µg/mL) was used and the results showed that EOAm eradicated 50% of the biofilm. The CC_{50} of EOAm in VERO cells was 190.58 µg/mL. The molluscicidal assay showed that the EOAm was effective against adults and embryos of *B. glabrata* with LC_{50} = 34.9 and 6.1 µg/mL, respectively. The non-target organisms assay *A. salina* showed that the EOAm presented toxicity with LC_{50} = 42.02 µg/mL.
Conclusion: The essential oil of *A. minor* showed potential molluscicide and bactericide activity, further studies are necessary to elucidate possible mechanisms of action.

Keywords: *Algrizea minor*; Antibacterial activity*; Biomphalaria glabrata*; *Artemia salina*; β-pinene; γ-eudesmol.

Background

Biologically-active compounds from plants are used for several purposes in medical therapy, to prevent or cure diseases. The Myrtaceae family has many species with economic interest that are popularly used for different applications, which make them an object of research [1,2].

Algrizea minor (Myrtaceae) Sobral, Faria Júnior e Proença, 2010 [3] is a shrub endemic to NE-Brazil, occurring at altitudes ranging from 300 to 550m a.s.l, and it is distributed in areas of Caatinga and Cerrado, especially in the States of Bahia and Pernambuco [4].

According to International Organization for Standardization - ISO 9235: 2013 [5], essential oil is a product obtained from natural raw materials of vegetable origin, by steam distillation, by mechanical processes from the citrus epicarp or by dry distillation, after separation of the phase, eventually, by physical processes. Essential oils are composed mainly of hydrocarbon terpenes (isoprenes) and terpenoids (isoprenoids). The properties of essential oils are determined generally by the basic structure of the main component and its functional groups. Besides, the same plant species can have several chemo-types based on the most abundant secondary metabolite. The chemical structures of the main components can determine their antimicrobial activity [6].

Several essential oils obtained from leaves and fruits of Myrtaceae species are known to play many biological activities, such as antibacterial [7], antifungal [8], antioxidant [9], leishmanicidal [10], anti-Trypanosoma [11], and insecticidal activities [12].

The biological activities aforementioned can be related with the fact that essential oil can alter cell membrane permeability, inducing leakage of vital intracellular constituents, and disrupting cellular metabolism of target pathogens [13]. Among the challenges related to human pathogenic bacteria is the development of resistance to conventional antibiotics and biofilm formation, which makes it difficult to eradicate these microorganisms.

Essential oils have been tested against neglected disease-causing parasites, such as the schistosomiasis. Schistosomiasis is a parasitic disease, caused by the trematode helminth *Schistosoma mansoni*, whose adult forms inhabit the mesenteric vessels of the definitive host (man) and the intermediate forms develop in snail aquatic gastropods of the genus *Biomphalaria* (Gastropoda, Planorbidae). In Brazil, estimates suggest that about 1.5 million people live in areas at risk of contamination. The magnitude of its prevalence, associated to the severity of the clinical forms and its evolution, confer schistosomiasis a great relevance as a public health problem [14]. The states of the Northeast and Southeast regions are the most

affected and the occurrence is directly linked to the presence of the host snails [15]. The occurrence of snail of *Biomphalaria glabrata* SAY, 1818 lead to the spread of this disease in the regions where they are found and, therefore, should be controlled.

Besides the treatment of infected people, the schistosomiasis control involves the combat of the intermediate host (snails) using molluscicidal chemicals. Although there are some molluscicidal agents in use, only one synthetic substance, niclosamide, is recommended by the United Nations Organization (UNO) [15]. However, the high cost of niclosamide use limits its application [16].

Due to the facts showed above, the search for bioactive compounds with antimicrobial and molluscicidal effect is extremely necessary. The main objective of the research was to elucidate potential bactericidal and molluscicidal activity of the essential oil of *A. minor* (EOAm), this is the first report on the chemical composition and biological activity of this essential oil.

Methods

Collection of plant material

Leaves of *A. minor* were collected in May and July 2015, and March 2016 at the Parque Nacional do Catimbau, Pernambuco, Brazil. Voucher specimen was deposited at the Herbarium of the Instituto Agronômico de Pernambuco (IPA, Recife, PE, Brazil), with number IPA 84346.

Extraction of essential oil

The fresh leaves were crushed and submitted to the hydrodistillation technique, in a Clevenger type apparatus for 6h. After that, the essential oil layer was separated, dried over anhydrous sodium sulfate and transferred to amber glass vials, resulting in EOAm (*A. minor* essential oil). Three samples of EOAm were extracted and the yield of each sample was calculated as a percentage based on plant weight (% w / w) and stored at 4 ° C until chemical and biological analyzes.

Essential oil chemical composition

The essential oil was analyzed on a Gas Chromatograph (model 7890A; Agilent Technologies; Palo Alto, CA, USA) equipped with an Agilent J &W non-polar HP-5ms™ column (30 m x 0.25 mm id.; 0.25 μm film thickness) and coupled to a selective mass detector (model 5975C; Agilent Technologies; Palo Alto, CA, USA). The MS data acquired

for each component were compared with those stored in the mass spectral library of the GC-MS system (MassFinder 4, NIST08 and Wiley Registry™ 9th Edition) and with published spectra [17] in order to confirm identity. Whenever possible, identity of compounds was confirmed using retention times and mass spectra of authentic standards available in the Lab of Chemical Ecology (UFPE). The peak areas on the chromatograms were integrated using the software Agilent MSD Productivity ChemStation (Agilent Technologies, Palo Alto, USA) to obtain the total ion current signal, which was used to determine the relative percentages of each oil component.

Citotoxicity

The cytotoxicity was determined using the colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (SIGMA, St. Louis, MO, USA), a protocol adapted from Monzote et al. [18]. The VERO cells were plated at 1×10^5 cells/well in 96-well plate containing 100 μL of RPMI medium, supplemented with 10% inactivated FBS, and incubated for 4h at 37 °C in 5 % CO_2. After this time, the adhered cells were cultivated for 24 hours in RPMI in the absence or presence of the different concentrations of EOAm (25 to 400 μg/mL). Treated and untreated cells were washed and incubated in fresh RPMI culture medium, containing 5 mg/mL of MTT for an additional 3h at 37 °C. After the incubation, the cells were solubilized in DMSO (100 μL/well) and the formazan precipitate derived from MTT reduction was determined spectrophotometrically at 540 nm. The 50% cytotoxic concentration (CC_{50}) was determined by regression analysis using the software SPSS 8.0 for Windows. Each assay was carried out in quadruplicate in two independent experiments.

Antibacterial activity

Strains studied and growth conditions

The strains used in this study were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), provided by the *Instituto Aggeu Magalhães*, and *Staphylococcus saprophyticus* (UFPE 823), provided by the *Departamento de Antibióticos* of *Universidade Federal de Pernambuco*. Stocks of the strains were maintained at –80 °C in cryotubes with BHI broth + glycerol (1:1). To obtain the colonies, the strains were grown in Petri dishes containing Mueller-Hinton agar medium and incubated at 37 °C for 24 hours. Then, the optical density was determined at 600nm (OD600) and the

inoculum was adjusted to the scale of 0.5 McFarland (equivalent to 1-5 x 10^8 CFU / mL), following the standard indicated by Clinical and Laboratory Standards Institute (CLSI) [19].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Antimicrobial activity was determined by susceptibility test against four pathogenic bacteria commonly associated with hospital infections. The minimal inhibitory concentrations (MICs), i.e. the lowest concentration of the EO samples capable of inhibiting the growth of microorganisms, was determined by a microdilution method in broth following standard procedures [19]. 96-well plates were prepared with 50 µL of Mueller-Hinton (MH) broth (per well) containing decreasing concentrations of oil, diluted in DMSO not exceeding 10%, ranging from 32 to 0.0156 mg/mL. Afterwards, 50 µL of bacterial suspension (10^6 CFU/mL) was added in each well. All plates included a positive growth control in the absence of essential oil. The plates were incubated at 37 °C for 24 hours, after that, the MIC's values were determined as the lowest concentration capable of visually inhibiting bacterial growth.

To determine the minimal bactericidal concentrations (MBC), 10 µL of the wells with oil concentrations at which bacterial growth was not observed were inoculated on Petri dishes with Mueller-Hinton agar. The dishes were incubated at 37 °C for 24 hours. The MBC was considered the lowest concentration that did not allow the reactivation of the microorganisms in the medium without the antimicrobial agent. The MIC's and MBC's were expressed in mg/mL. The data were expressed as a mean of replicates ± Standard Deviation (S.D.). All assays were performed in triplicate and repeated twice.

Biofilm eradication activity

The assay was performed in 96-well plates according to Trentin et al. [20]. The bacterial inoculum (*S. aureus*) was adjusted in sterile tube and read in OD 600 nm, corresponding to 1 MacFarland scale, 3.10^8 CFU. The following tests were carried out: sterilization control (80, 80 and 40 µL of water, NaCl 0,85 % and medium), growth and biofilm formation control, antibacterial activity control and tests (water, bacteria inoculum and medium), after assembly the plate was read (time = 0h) and incubated at 37 °C. After 24 hours, the contents were removed and each well received again medium and NaCl 0,85 %. The growth and biofilm formation control group received water, antibacterial activity group received Vancomycin (20 µg/mL) and Test group received 0.25 mg/mL of EOAm. After another 24 hours the plate was

washed three times with NaCl 0,85 % and 0,4 % Crystal violet was used to stain the remaining biofilm layer. The biofilm formatin control group was used to represent 100 % of biofilm formation. Each assay was carried out in triplicate in three independent experiments.

Molluscicidal assay

The snails of *B. glabrata* were obtained at São Lourenço da Mata, state of Pernambuco, NE-Brazil. Snails not infected by trematodes were reared at the Departamento de Biofísica, Universidade Federal de Pernambuco, Brazil. The adult animals were maintained in plastic aquaria (50 x 23 x 17 cm) with filtered, dechlorinated water (pH: 7.0) at 25 °C and fed with lettuce leaves (*Lactuca sativa*).

Embryotoxicity assay

Embryotoxicity assay was performed according Oliveira-Filho et al. [21] using egg masses with approximately 300 embryos of *B. glabrata* for each treatment at the blastula stage (15h after spawning). The embryos were select by observing at a stereomicroscope (Leica MZ6; Leica Microsystems, Wetzlar, Germany) and placed in Petri dishes (90 x 15mm). After that, an oil solution (in 0.3 % tween 80) was prepared to achieve the final concentrations of 0.78, 1.56, 3.12, 6.25 and 12.5 µg/mL. Then, the embryos were exposed to each concentration of the EOAm, filtrated and dechlorinated water (as negative control) and 0.3 % of tween 80 (CSC, as co-solvent control) for 24 hours. Subsequently, the egg masses were washed with filtrated water and maintained in Petri dishes containing filtered and dechlorinated water for 7 days. The number of viable and non-viable (dead and malformed) embryos was recorded. The assays were performed in triplicate.

Adulticidal assay

For the evaluation of molluscicidal activity, molluscs (n = 180) of uniform size (shell diameter between 10 – 16 mm) were distributed into six groups, as following: negative control (purified water, C), solvent control and four groups exposed to different concentrations of the EOAm: 12.5, 25, 50 and 75 µg/mL for 24 hours. Ten adult snails per group were placed in a container filled with 60 mL of the solution with the EOAm. The motility and viability of the molluscs were evaluated after 24 hours. After 24 hours in solution, viable molluscs were individually packed in a plastic container filled with 500 mL of

dechlorinated water, where they were kept for eight days. For each experiment, a solvent control consisting of ten mollusks immersed in 0.3 % aqueous solution of tween 80 was made. The mortality of the snails was evaluated by the retraction of the cephalopodal mass into the shell with the release or not of hemolymph or the abnormal projection of the cephalopod out of the shell and heartbeats absence, as described by Mccullough et al. [22].

Artemicidal assay

The crustacean *Artemia salina* was used as a biological model for assessing the toxicity of EOAm essential oil to non-target organisms. Thus, the eggs (25 mg) of *A. salina* were placed in a recipient with seawater (pH 8.0; 25 – 30 °C) for 24 hours with aeration until their outbreak. After this, *A. salina* was collected for analysis of viability. The animals were divided into four groups (n = 40 for each group), as following: one negative control group (containing only sea water, C), one solvent control group and four groups exposed to different concentrations of EOAm (12.5, 25, 50 and 75 µg/mL) for 24 hours. The tests for evaluation of mortality and survival were performed according to Santos et al. [23]. Each assay was performed in quadruplicate in two independent experiments.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Turkey test to determine the statistical significance, p-value < 0.05 was considered to be statistically significant.

Results and Discussion

The yield of EOAm obtained by hydrodistillation of fresh leaves from *A. minor* was 2.45 % (w/w). Chemical analyzes of the essential oil of *A. minor* showed 76 compounds, of which 53 (representing 69,7 % of the total bouquet) were identified. Sesquiterpenes corresponded to the dominant compound class with about 69.63 % of all oil components, followed by monoterpenes (16.75 %) (Table 1). The major constituents of the oil were β-Pinene (10 %), followed by γ-eudesmol (7.67 %), bicyclogermacrene (7.41 %), germacrene D (6.58 %), (*E*)-caryophyllene (6.50 %), bulnesol (6.24 %), guaiol (5.11 %), α-eudesmol (4.93 %), β-eudesmol (3.94 %) and α-pinene (3.55 %) (Table 1). Some of these compounds were found in other essential oils obtained from leaves of species of Myrtaceae family, such as β-pinene in

Eugenia aromatica and *Eucalyptus microtheca* (45.44 % and 5.01 %, respectively) [24,25], (*E*)-caryophylene in *Psidium guajava* (9.51 %) [26] and bicyclogermacrene in *Eugenia brejoensis* (7.93 %) [27].

The cytotoxicity assay in VERO cells of the EOAm showed a CC_{50} value of 190.58 µg/mL (Figure 1). Due to strict control over the use of laboratory animals, there is a need to develop and standardize *in vitro* tests. *In vitro* methods have advantages over *in vivo* as: limit the number of experimental variables, obtain significant data more easily, and generally have a shorter test period [28]. Other essential oils of Myrtaceae species tested on mammalian cells, e.g. *Eugenia uniflora* – J774 cells (8% to 100 µg/mL e 0% to 10 µg/mL) [29] and *E. calycina* Cambess. HeLa (137.4 ± 9.6 µg/mL), showed low cytotoxicity [30].

The results showed that the EOAm was active against at least two bacteria (Table 2), and the best MIC value observed were 0.25 and 1 mg/mL against *S. aureus* and *S. saprophyticus*, respectively, and MBC 16 mg/mL for two microorganisms. Victoria et al. [31] determined the MIC value of essential oil from *Eugenia uniflora* against *S. aureus* (ATCC 27664) at a concentration of 0.8 mg/mL, which is less effective in inhibiting growth of *S. aureus* than the oil of *A. minor* (MIC of 0.25 mg/mL). The antimicrobial activity may be related to compounds found in EOAm, including α-pinene and β-pinene. The compound β-pinene was tested against *Staphylococcus aureus* (ATCC 25923) whit MIC of 20 µL/mL [32].

Table 2: Mean of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) from essential oil.

Microorganisms	**MIC**	**MBC**
S. aureus	0.25±0.07	16±0.00
S. saprophyticus	1±0.70	16±0.00
E. coli	>32±0.00	>32±0.00
P. aeruginosa	16±0.00	32±0.00

MIC/MBC - mg/mL - MD±SD

The MICs of the essential oil of *Eucalyptus globulus* against *P. aeruginosa* (ATCC27853) and *E. coli* (same strain used in this study) were 4 mg/mL for both bacteria and MBCs were 9 and 4 mg/mL, respectively [33]. These results are more effective than that found in our study. It is possible to realize the difference of MICs and MBCs values between gram-positive and

negative bacteria, the antibacterial activity of essential oils differs because of different bacterial architectures (i.e. cell membrane compositions) [13].

Based on the best MIC result, the biofilm eradication test was performed. EOAm was more effective against *S. aureus* at the concentration of 0.25 mg/mL. For the biofilm eradication test, the same concentration (0.25 mg/mL) was used and the results showed that EOAm eradicated 50 % of the biofilm (Figure 2). Our results showed that EOAm presented eradication percentage similar to that of the broadly used antibiotic vancomycin. Another oil from the same family was also effective, the essential oil of *Eucalyptus smithii* (Myrtaceae) was tested against *S. aureus* (ATCC 25923) for inhibition of the formation and disruption of the consolidated biofilm at concentrations of 5, 10 and 20 µl/mL with percentage of inhibition of 43.3, 68, 2 and 93.1 %, respectively, and degradation percentage of 61.9, 72.8 and 81.8 %, respectively, at the same concentrations [34].

The molluscicidal assay showed that the EOAm was effective against adults and embryos of *B. glabrata* with LC_{50} = 34.9 and 6.1 µg/mL, respectively (Figure 3A,B). The non-target organisms assay *Artemia salina* showed that the EOAm presented toxicity with LC_{50} = 42.02 µg/mL (Figure 4, Table 3).

Table 3: Summary of biological activities of essential oil *A. minor* of the Myrtaceae family.

Compound	Embryotoxic	Molluscicidal	Artemicidal
EOAm	6.1±0.2	34.9±6.33	42.02±1.65

LC_{50} µg/mL

Other essential oils from Myrtaceae have been assayed in intermediate host, for example, essential oils of *Thymus capitatus* and *Marrubium vulgare* were tested on *B. alexandrina* with LC_{50} and LC_{90} 200 and 400 ppm/3h, and LC_{50} and LC_{90} 50 and 100 ppm/3h, respectively. In addition, *M. vulgare* showed LC_{100} ovicidal activity at 200 ppm/24h, however, *T. capatus* oil had no ovicidal activity [35]. For the essential oil of *Syzygium cumini* (L.) Skeels (Myrtaceae) tested against adults of *B. glabrata* the LC_{50} was 90 mg/l, our results were better for both adults and for embryos of the same species of snail [36], EOAm showed better result. Although recommended by the World Health Organization niclosamide is not suitable for aquaculture due to its toxicity to fish and other aquatic animals. Therefore, it is necessary to search for compounds that are less toxic [37]. Our results were better than for niclosamide,

evaluated under laboratory conditions, in a study that evaluated its toxicity against *B. glabrata*, which presented LC_{50} and LC_{90} values of 0.077 and 0.175 mg/l, respectively [38].

The essential oil from *Cymbopogon winterianus* Jowitt leaves showed molluscicidal effect on *Biomphalaria tenagophila* with LD_{100} = 80 ppm and LD_{50} 60 ppm [39]. The previous examples show the potential of essential oils as molluscicides. The EOAm LC_{50} values on adult *B. glabrata* embryos and snails have been shown to be even more promising for mollusk control.

The LC_{50} of *S. cumini* essential oil against *Artemia* was 175 mg/mL, a better result than the EOAm [36]. However, according to the World Health Organization (WHO), substances with LC_{50} values below 1000 ppm are considered to be toxic in *A. salina* [40]. Therefore, our study indicates that caution should be taken regarding the dosage and frequent use of EOAm leaf essential oil against snails of *B. glabrata*, being the same one better indicated for embryos of *B. glabrata*, since it performed better in lower concentration.

Conclusion

Our study points to a multi-target activity of the EOAm against microorganisms and organisms vectors of diseases that threat human health. The results revealed the antibacterial potential of EOAm against human pathogenic bacteria, mainly against *S. aureus*, evidencing the eradication of part of the biofilm. EOAm showed good molluscicidal activity against *B. glabrata* embryos and low cytotoxicity for mammalian cells. Further studies are needed to elucidate the possible mechanisms of action.

Declarations

Competing interests

No potential conflict of interest was reported by the authors.

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Availability of data and material

The raw data used to obtain the results, if necessary, will be distilled by prior request to the corresponding author.

Authors' contributions

RS and IATAR performed the antibactericidal and molluscicidal experiments. JLFS and AMMAM supported the in moluscicide experiments and provided of material. AJM and ALA contributed to biofilm eradication test and data analysis. PMP and DMAFN performed chemical analysis and identified the chemical composition of the essential oil. MVS and AGS collection and identification of plant material. RS collected the data and wrote the manuscript. MTSC designed and supervised the study. All authors contributed to different drafts of the manuscript and approved its final version.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Table 1: Mean relative proportions and standard deviation (SD) of constituents of the essential oil of *A. minor* (Myrtaceae).

Compound	RI	Adams	Sample 1 Relative amount %	Sample 2 Relative amount %	Sample 3 Relative amount %	Mean%	SD
α-Thujene	925	924	0,09	0,26	0,15	0,17	0,09
α-Pinene	932	932	1,85	5,49	3,32	3,55	1,83
Sabinene	972	969	0,00	0,25	0,09	0,11	0,13
β-Pinene	974	979	5,19	15,19	9,65	10,01	5,01
Myrcene	992	988	0,08	0,28	0,17	0,18	0,10
α-Phellandrene	1003	1002	0,00	0,09	0,00	0,03	0,00
δ-3-Carene	1009	1008	0,00	0,07	0,06	0,04	0,04
α-Terpinene	1016	1014	0,07	0,17	0,08	0,11	0,06
p-Cymene	1024	1022	0,10	0,27	0,40	0,26	0,15
Limonene	1028	1024	0,60	1,34	0,88	0,94	0,37
(*Z*)-β-Ocimene	1039	1037	0,16	0,00	0,15	0,10	0,09
γ-Terpinene	1059	1054	0,16	0,37	0,16	0,23	0,12
Terpinolene	1088	1086	0,13	0,21	0,12	0,15	0,05
Linalool	1100	1095	0,00	0,09	0,06	0,05	0,05
Terpinene-4-ol	1178	1074	0,15	0,49	0,35	0,33	0,17
α-Terpineol	1191	1186	0,48	0,52	0,42	0,47	0,05
Unknown	1196	1196	0,00	0,00	0,07	0,02	0,00
δ-Elemene	1339	1335	0,19	0,00	0,35	0,18	0,17
α-Cubenene	1351	1345	0,12	0,05	1,19	0,45	0,64
α-Ylangene	1373	1373	0,00	0,22	0,23	0,15	0,13
α-Copaene	1378	1374	0,22	0,37	1,23	0,61	0,54
β-Bourbonene	1387	1387	0,00	0,12	0,04	0,05	0,06
β-Cubebene	1392	1387	0,00	0,00	0,12	0,04	0,00
β-Elemene	1394	1389	0,34	0,35	0,46	0,38	0,07
α-Gurjunene	1412	1409	0,24	0,47	0,48	0,40	0,14
(*E*)-Caryophyllene	1422	1417	3,57	8,39	7,56	6,51	2,58
β-Copaene	1431	1430	0,00	0,14	0,09	0,08	0,07
γ-Elemene	1436	1434	0,26	0,27	0,28	0,27	0,01
Guaia-6,9-diene	1446	1442	0,52	0,80	0,48	0,60	0,18
(*E*)-3,5-Muuroladiene	1453	1451	0,65	0,11	3,34	1,37	1,73
Humulene	1456	1452	1,55	2,97	2,61	2,37	0,74
9-epi-Caryophyllene	1463	1464	0,61	0,99	0,60	0,73	0,22
(*E*)-Cadina-1(6),4-diene	1476	1475	0,34	0,16	0,82	0,44	0,34
γ-Muurolene	1480	1478	0,26	0,48	0,33	0,36	0,11
Germacrene D	1483	1484	2,16	10,55	7,03	6,58	4,21
β-Selinene	1489	1489	0,17	0,21	0,29	0,22	0,06
Unknown ST2	1491		0,00	0,37	0,28	0,22	0,19
(*E*)-Muurola-	1494	1493	0,35	0,34	1,10	0,59	0,43

4(14),5-diene							
Viridiflorene	1497	1496	1,75	1,50	1,63	1,63	0,13
Bicyclogermacrene	1500	1500	4,96	11,17	6,12	7,42	3,30
α-Muurolene	1503	1500	0,39	0,47	0,43	0,43	0,04
Unknown ST3	1510		0,29	0,54	0,56	0,46	0,15
Unknown ST4	1512		0,25	0,36	0,40	0,34	0,08
γ-Cadinene	1517	1513	0,18	0,39	0,23	0,27	0,11
δ-Cadinene	1526,282	1522	0,43	2,21	0,00	0,88	1,17
(*Z*)-Calamenene	1527	1528	1,66	0,00	6,76	2,81	3,53
Zonarene	1529,05	1528	0,19	0,00	0,62	0,27	0,32
(*E*)-Cadina-1,4-diene	1536	1533	0,48	0,08	2,04	0,87	1,04
Unknown ST5	1541		0,00	0,19	0,24	0,14	0,13
α-Calacorene	1545	1544	0,00	0,09	0,20	0,10	0,10
Unknown ST6	1551		2,91	0,43	0,62	1,32	1,38
Unknown ST7	1556		0,00	0,10	0,09	0,07	0,06
Germacrene B	1561	1559	1,43	2,35	2,60	2,13	0,62
Unknown ST8	1566		0,00	0,08	0,10	0,06	0,05
Unknown ST9	1570		0,47	0,40	0,21	0,36	0,14
Spathulenol	1580	1577	2,20	2,73	1,49	2,14	0,62
Unknown ST10	1587		2,70	2,30	1,67	2,22	0,52
Unknown ST11	1595		1,18	1,30	0,78	1,09	0,28
Unknown ST12	1597		0,58	0,00	9,15	3,25	5,12
Guaiol	1601	1600	7,29	8,05	0,00	5,11	4,45
Unknown ST13	1606		0,94	0,97	0,68	0,86	0,16
Unknown ST15	1613		0,00	0,50	0,00	0,17	0,00
10-epi-gama-Eudesmol	1623	1622	0,79	0,22	0,33	0,45	0,30
Unknown ST16	1626		0,38	0,20	0,13	0,24	0,13
Unknown	1632		0,34	0,30	0,68	0,44	0,21
γ-Eudesmol	1636	1630	18,90	1,23	2,94	7,69	9,75
Unknown ST18	1643		0,52	0,31	0,40	0,41	0,10
Unknown ST19	1647		1,53	0,88	1,69	1,37	0,43
Unknown ST20	1651		0,28	0,21	0,29	0,26	0,05
β-Eudesmol	1656	1649	8,90	1,09	1,85	3,95	4,31
α-Eudesmol	1660	1652	10,58	1,75	2,50	4,94	4,90
Bulnesol	1672	1670	6,59	4,97	7,17	6,24	1,14
Unknown ST21	1684		0,00	0,00	0,14	0,05	0,00
Unknown ST22	1690		0,00	0,15	0,11	0,09	0,08
Unknown ST23	1699		0,29	0,00	0,07	0,12	0,15
Total			100,00	100,00	100,00	100,00	

Chemical Groups	**(%)**
Hydrocarbon monoterpenes	15.88
Oxygenated monoterpenes	0.85

Monoterpenes	**16.73**
Hydrocarbon sesquiterpenes	36.33
Oxygenated sesquiterpenes	31.25
Sesquiterenes	**69.71**
Others compounds	13.56

RI* From Adams (2007)

Constituents are listed according to their elution on a non-polar DB-5 column. Marked in bold are compounds accounting for more than 1% of the essential oil of any species.

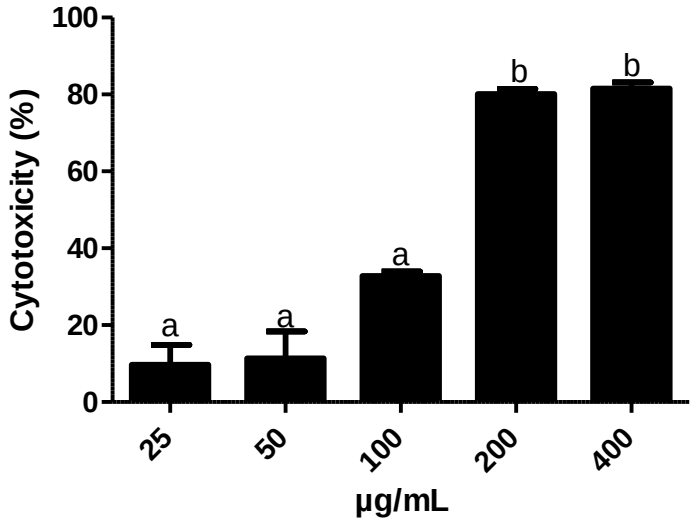

Figure 1: Cytotoxicity of EOAm in VERO cells at time of 24h. Equal letters do not differ statistically between. *Significant values ($p < 0.05$)

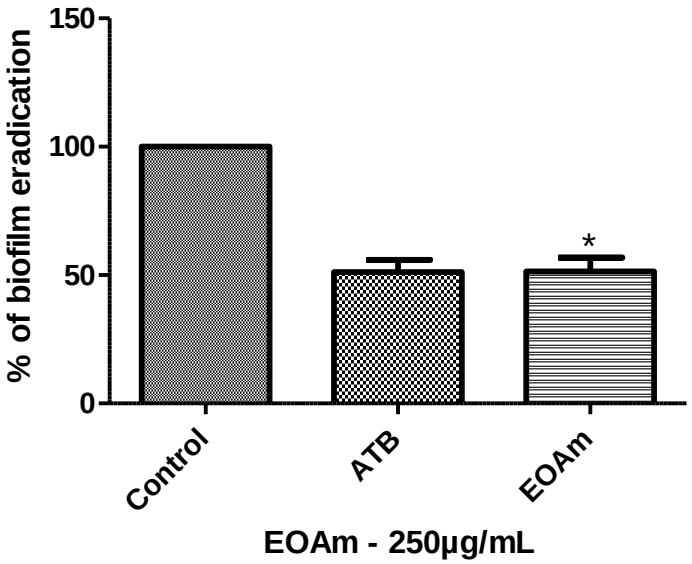

Figura 2: Eradication of the biofilm, formed by *S. aureus*, after 48h of incubation. Untreated bacteria were considered as presenting 100% of biofilm formation. *Significant difference in relation to untreated samples regarding the results about bacterial biofilm formation.

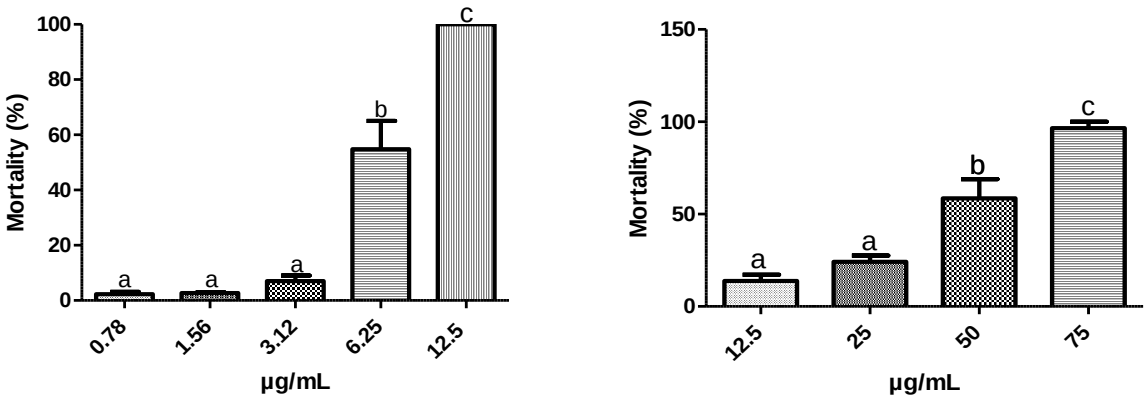

Figure 3: Toxicity caused by EOAm in *B. glabrata* embryos and adults, respectively, the after 24h exposure. Equal letters do not differ statistically between. *Significant values (p < 0.05)

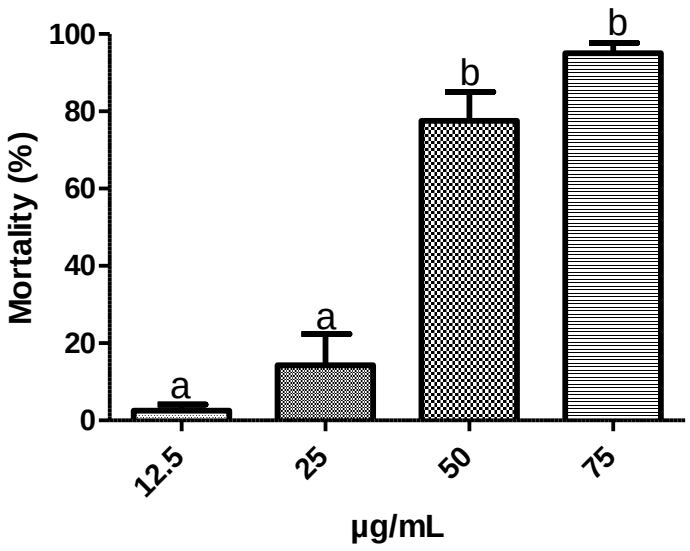

Figure 4: Toxicity caused by EOAm in *A. salina* after 24h exposure. Equal letters do not differ statistically between. Significant values ($p < 0.05$).

4.3 ARTIGO 3 - ARTIGO A SER SUBMETIDO A REVISTA PLANTA MEDICA.

Biological activity of the Essential oil from *Algrizea minor* (Myrtaceae) and its Major Compound β-pinene Against *Leishmania amazonensis*

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Abstract

The essential oils from tropical plants are a source of natural compounds with biotechnological potential. These compounds are biodegradable and usually present low toxicity to mammals. This work aimed to evaluate the leishmanicidal activity and citotoxicity potential of the essential oil from leaves of *Algrizea minor* (EOAm) and its majoritary constituent β-Pinene. The fresh leaves were crushed and submitted to the hydrodistillation and analysed by GC-MS. The cytotoxicity assay was evaluated using CellTiter-Glo viability test (Promega, USA). The main constituents of the EOAm were β-Pinene (10%), followed by γ-Eudesmol (7.69%), Bicyclogermacrene (7.42%), Germacrene D (6.58%), (*E*)-Caryophyllene (6.51%), Bulnesol (6.24%) and Guaiol (5.11%). The CC_{50} of EOAm in peritoneal macrophages was 99.9µg/mL. The EOAm inhibited the promastigote (IC_{50} = 25.0µg/mL) and amastigotes (IC_{50}= 53.62µg/mL) forms of parasite growth in dose-dependent manner, with a selectivity índex in 3.9 and 1.8, respectivelly. The main constituent β-Pinene was less effective to promastigotes than crude essential oils with the IC_{50} of 126.8 µg/mL and more toxic to mammalian cells with CC_{50} value of 77.1µg/mL and SI = 0.6. Scanning electron microscopy showed that treatment with EOAm caused drastic ultrastrucutal changes and cell lysis compared to control cells. In conclusion, the essential oil of *A. minor* showed leishmanicidal potential but further studies are needed to elucidate the mechanisms behind the cytotoxicity towards both mammalian cells and Leishmania.

Keywords: Essential oil, *Algrizea minor*, *Leishmania amazonensis*, chemotherapy, β-Pinene

Introduction

Essential oils (EOs) from tropical aromatic plants are volatile and complex mixture of compounds, characterized by a strong odour, usually utilized in cosmetic industry, as food and beverages flavouring [1]. EOs has been used in the traditional medicine since ancient time due to their promising biological properties and low toxicity to mammals [2,3]. In fact, several reports have demonstrated the potential use of EOs as insecticide [4], bactericide [5], fungicide [6], anti-inflamatory [7], leishmanicide [8].

The species *Algrizea minor*, belonging to the family Myrtaceae, is a shrub, native and endemic of Brazil, occurring at lower altitudes, about 300-550m, being found in the state of Bahia and Pernambuco, in areas of Caatinga and Cerrado ecosystems [9, 10]. The Myrtaceae family is one of the most important representative of Brazilian flora with medicinal interest due to great chemical diversity and biological activities, being a rich source of essential oils with important economic and health benefits [11].

Cutaneous leishmaniasis caused by trypanosomatid protozoa from the genus *Leishmania,* is a severe disease affecting the skin and mucous membranes and leading to severe disfiguration of the patients [12]. According to the Pan American Health Organization (PAHO) and the World Health Organization (WHO) [13], cutaneous and mucosal leishmaniasis occurs in 20 countries in the Americas and it is endemic in 18 of them. In Brazil in 2015 there were 19.395 new cases, being 18324 (94.5%) of localized skin lesions and 1071 (5.5%) of mucosal form of disease.

Current drugs for the treatment of leishmaniais rely on the use of pentavalent antimonials, as drugs of first choice, despite their well-known severe adverse side effects, including anorexia, vomiting, dizziness, arthralgia, myalgia and fever. Furthermore, these drugs require parenteral administration and, long course of treatment which lead to high costs. Pentamidine is the second-choice drug commonly recommended, although it also has significant adverse effects and requires parenteral administration [14, 15, 16].

In this regard special attention has been given to essential oils from plants species used in folk medicine as possible chemotherapeutic agent against cutaneous leishmaniasis [17]. Previous studies have shown that essential oils from Myrtaceae species are promising anti-*Leishmania* agents. The essential oil of *Eugenia uniflora* L, for example, showed significant activity against promastigotes and amastigotes of *Leishmania amazonensis* [18]. In the present work, we investigated at the first time the potential of EO of *A. minor* (EOAm) and its major compound β-pinene against *Leishmania amazonensis*, the etiological agent of cutaneous leishmaniasis in Brazil.

Results and discussion

The chemical characterization of EOAm allowed the identification of β-Pinene (10%), followed by γ-Eudesmol (7.69%), Bicyclogermacrene (7.42%), Germacrene D (6.58%), (*E*)-Caryophyllene (6.51%), Bulnesol (6.24%), Guaiol (5.11%), α-Eudesmol (4.94), (Z)-Calamenene (2.81), β-Eudesmol (3.95%) and α-Pinene (3.55%) as its main constituents. Some of these compounds were also found in EOs from other species of Myrtaceae family used in folk medicine as well as in plants presenting leishmanicidal activity, such as β-pinene identified in *Eugenia aromatica* and *Syzygium cumini* (L.) Skeels (45.44 and 5.57%, respectively) [19, 20], (*E*)-caryophylene found in *Eugenia uniflora* L. (4.33%) [18] and bicyclogermacrene found in *Eugenia brejoensis* (7.93%) [21].

According to Santos et al. [22] the cytotoxic activity of natural products against mammalian cells is an important issue to be considered in a prospection for active compounds with biological activity. Our results showed that the crude EOAm presented cytotoxicity to peritoneal macrophages with CC_{50} of 99.9±13.4μg/mL (Table 1). The essential oil of leaves of *S. cumini* (Myrtaceae) presented a cytotoxic concentration for murine macrophages at 614.1mg/mL, demonstrating to be less cytotoxic than the EOAm [23]. The EOAm inhibited the growth of promastigote forms of *L. amazonensis* in a dose-dependent manner (Figure 1) with IC_{50} of about 25μg/mL after 48h of incubation (Table 1). Although the EOAm presented a relative cytotoxicity to mammalian cell, our results showed that this EO was almost four times more toxic to parasite than host cells, as demonstrated by SI values obtained. (SI = 3.9) (Table 1). It has been demonstrated that essential oil from *Myracrodruon urundeuva* (Engl.) Fr. All. - MuEO inhibited the growth of the promastigote form of *L. amazonensis* (IFLA/BR/67/ PH8) with IC_{50} of 104.5±11.82μg.mL^{-1} [24] and *Tetradenia riparia* (Hochst.) Codd – TrEO, after 72h of incubation at LD_{50}, for the promastigote form of *L. amazonensis*, was 0.8 μg/mL[25]. The activity of essential oils of *Syzygium cumini* (L.) Skeels, which also belongs to the same taxonomic family of *A. minor*, against promastigote forms of *L. amazonensis* have been already reported with IC_{50} values of about 60 mg/mL [20]. Comparing to the results obtained by Dias [20], the EOAm showed to be more effective in inhibiting the growth of infective form of *L. amanazonensis*. The β-pinene also inhibited the growth of *L. amazonensis* promastigotes with an IC_{50} value of 126.8 μg/mL; however, this compound presented high cytotoxicity to macrophages with a CC_{50} value of 77.1μg/mL

(Table 1) and low selectivity index (SI = 0.6). Comparing to the crude EOAm the major compound showed to be less effective. These results suggested that other compounds, found in minor amounts could act decreasing the cytotoxicity of the major compound and increasing the leishmanicidal effect observed.

Because the major compound presented low selectivity to parasite, the further experiments were carried out only with the crude essential oil of *A. minor*. The infection of macrophages with promastigote forms results in the differentiation and proliferation of intracelular amastigote forms which leads to the development of the clinical manifestation of leishmaniasis in mammalian host. In this regard, we evaluated the effect of EOAm on this relevant evolutive form of the parasite. The EOAm significantly inhibited the survival of amastigotes in peritoneal macrophages at the higher concentration tested, as compared to with non-treated infected cells (Fig. 2). The estimated IC_{50} value was about 53.62µg/mL after 48h of incubation and survival index SI=1.8. (Fig. 3, Table 1). The lower efficacy of EOAm against amastigotes comparing with promastigotes could be due to inaccessibility of intracellular amastigote to essential oils. The essential oil of *S. cumini* showed IC_{50} with values of 43.9 and 38.1µg/mL against axenic and intracellular amastigotes, respectively. These authors also tested the inhibitory effect α-pinene the major compound of this oil, which presented IC_{50} of 16.1 and 15.6µg/mL for axenic amastigotes and intracellular amastigotes, respectively. The reference drug glucantime, analyzed in the same study presented a CC_{50} of 412.µg/mL and IC_{50} of 1200, 167.4 and 624.5µg/mL for the promastigote, intramacrophagic amastigotes and axenic amastigotes, respectively. As expected the survival index for intracellular amastigotes was 2.4 and 0.6 for axenic amastigotes in this study was low due to the high toxicity of the drug to mammalian cells [23].

The scnanning electron microscopy analysis of promastigotes showed that EOAm treatment induced drastic morphological changes and lysis to the parasites comparing to untreated cells. Untreated promastigotes (control) presented a typical elongated cell body shape, long anterior flagellum and smooth plasma membrane (Figure 4A-B). Treated cells showed aberrant morphology, ruptured cells, shortening of flagellum and incomplete division process. (Figure 4C-D). Similar results were found by Santos et al. [26] for *L. amazonensis*, treated with elatol (sesquiterpene), isolated from red seaweed *Laurencia dendroide*. At higher concentration tested was possible to observe intense parasite lysis compatible with necrotic cell death (Figures 4E-F).

In conclusion, the essential oils of *A. minor* presented inhibitory effect on the growth of promastigotes and amastigote forms of *L. amazonensis*. Despite the strong cytotoxicity to

mammalian cells, the essential oil of *A. minor* was more toxic to the parasite than to host cells. In this regard, further studies are necessary to investigate the mechanism of action of this oil and its major compounds on both parasite and host cells.

Material and methods

Plant material

Leaves of *A. minor* were collected in September 2016 at the *Parque Nacional do Catimbau*, Pernambuco, Brazil by Alexandre Gomes da Silva. The specimens were identified by Sobral, Faria Júnior e Proença [9]. Voucher specimens were deposited in the Herbarium of the *Instituto Agronômico de Pernambuco* (IPA, Recife, PE, Brazil), under number IPA 84346.

Extraction and chemical characterization of the EO

The fresh leaves of *A. minor* were crushed in little pieces and submitted to hydrodistillation, in a Clevenger-type apparatus for 6h. EO layer was separated, dried over anhydrous sodium sulfate, transferred to amber-glass vials and stored at 4ºC until chemical analysis and biological assays. β-Pinene ($C_{10}H_{16}$, ≥98.5% purity) was purchased from Sigma Chemical (St. Louis, MO, USA).

The EO was analyzed by Gas Chromatography (model 7890A; Agilent Technologies; Palo Alto, CA, USA) equipped with an Agilent J &W non-polar HP-5ms™ column (30 m x 0.25 mm id.; 0.25 μm film thickness) and coupled to a selective mass detector (model 5975C; Agilent Technologies; Palo Alto, CA, USA). The MS data acquired for each component were compared with those stored in the mass spectral library of the GC-MS system (MassFinder 4, NIST08 and Wiley Registry™ 9th Edition) with published spectra [27]. The identity of compounds was confirmed using retention times and mass spectra of authentic standards available in the Lab of Chemical Ecology (UFPE). The peak areas on the chromatograms were integrated using the software Agilent MSD Productivity ChemStation (Agilent Technologies, Palo Alto, USA) to obtain the total ion current signal, which was used to determine the relative percentages of each oil component.

Leishmanicidal activity

Promastigote forms of *Leishmania amazonensis* (MHOM/77BR/LTB0016) were maintained in Schneider's medium supplemented with 10% bovine fetal serum, at 26°C and

harvested during the exponential phase of growth. Amastigote forms were obtained from *L. amazonensis*-infected BALB/c peritoneal macrophages.

L. amazonensis promastigote forms (1×10^6 cells/ml) were incubated at 26 °C in Schneider's Drosophila medium supplemented with 10% FBS in the absence or presence of the different concentrations of EOAm (6.25–100µg/mL) and the major compound β-Pinene (12.5-200 µg/mL). After the treatments the cell viability was evaluated using CellTiter-Glo luminescent cell viability assay kit (Promega, USA) following the manufacturer´s instructions. The luminescence was measured on the GloMax apparatus (Promega, USA). The drug concentration that inhibited culture viability by 50% (IC_{50}) was estimated after 48h of drug incubation by regression analysis using the SPSS 8.0 software. All experiments were performed in quadruple in two independent experiments.

In order to evaluate the effect of EOAm against the relevant intracellular form of parasite, Peritoneal macrophages from Balb/c mice were harvested and plated at 1×10^6 cells/mll in a 24-well plate, containing RPMI medium supplemented with 10% inactivated fetal bovine serum. The macrophages were allowed to adhere for 3h at 37°C in 5% CO_2 and then infected with promastigotes at 1:20 ratio at 37°C for 14h. Non-interiorized parasites were removed by washing and the infected culture were incubated for 24h in RPMI 1640 medium or treated with different concentrations of EOAm corresponding to 0.5x, 1x e 2x the IC_{50} values obtained for promastigotes. The percentage of infected macrophages was determined in culture slide stained with Giems by counting 100 randomly chosen cells in triplicate. The survival index was determined by multiplying the percentage of infected macrophages by the mean number of parasites per infected cell. All experiments were performed in triplicate in three independent experiments.

Cytotoxicity assay

Peritoneal macrophages from Balb/c mice were plated at 1×10^6 cells/well in 96-well plate containing 100µL of RPMI medium, supplemented with 10% inactivated FBS, and incubated for 24 h at 37°C in 5% CO_2. After this time, the adhered macrophages were cultivated for 48 h in RPMI in the absence or presence of the different concentrations of EOAm and the major compound β-Pinene (25 to 400µg/mL). After the treatment the cell viability was evaluated through the CellTiter-Glo reagent (Promega, USA). The 50% cytotoxic concentration (CC_{50}) was determined by regression analysis using the software SPSS 8.0 for Windows. The selectivity index (S.I) was determined as the ratio of CC_{50} for the macrophages to IC_{50} for the protozoa. Each assay was carried out in quadriplicate in two independent experiments.

Ultraestrutural assay

For scanning electron microscopy (SEM) promastigote forms of *L. amazonensis* treated or not with EOAm (1x and 2x IC_{50}) for 48h were obtained by centrifugation at 3200 RPM and washed three times in phosphate buffer (PBS 0.1M pH 7.2). They were then fixed for 2 hours at room temperature and stored at 4°C in a solution containing 2.5% glutaraldehyde in cacodylate buffer (CaCo 0.1M ph7.2, pH 7.2). After two washes in the same buffer, the cells were post-fixed with 1% osmium tetroxide/0.8% potassium ferricyanide/5mM CaCl 2 in 0.1M cacodylate buffer, pH 7.2, for 1h in the dark. After, they were washed again in Cacodylate buffer and dehydrated in increasing concentrations of ethanol, critical-point-dried with CO_2, coated with a 20 nm-thick gold layer, and observed with a JEOL T-200 scanning electron microscope [28].

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Turkey test to determine the statistical significance. The value de p of <0.05 was considered to be statistically significant.

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Conflict of interest

No conflict of interest was reported by the authors.

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Legends to Figures

Figure 1 - Effect of EOAm (A) and its major compound β-pinene (B) on the growth promastigote forms of *Leishmania amazonensis*. *Significant values ($p < 0.05$) compared to the untreated cells

Figure 2 – Peritonial macrophages infected with *L. amazonensis* promastigotas and treated with EOAm - 0.5x, 1x e 2x the IC_{50} values obtained for promastigotes and not treated. (A-C) Cells not treated; A - Uninfected macrophage, B - Infected macrophage and C - Pentamidine treated macrophage. (D-F) Macrophages infected and treated with 0.5x, 1x e 2x, respectively.

Figure 3 - Effect of EOAm on the survival of *L. amazonensis* amastigote within macrophages after 48h of treatment with the EOAm. *Significant values ($p < 0.05$) compared with non-treated infected cells

Figure. **4** - Scanning electron micrographs of *L. amazonensis* promastigotes submitted or not to *A. minor essential* oil treatment. **(A-B)** Untreated cells showing the typical elongated shape and preserved plasma membrane; **C–D** promastigotes treated with IC_{50} of EOAm. **(C)** General aspect of promastigote culture presenting cells with abnormal shape, some of them showing shortening of flagellum. Inset - Detail showing a ruptured cell. (D) High magnification of a completely ruptured cell. (E-F) Promastigote treated with 2 xIC_{50} of EOAm. (E) Low magnification of cell culture showing intense lysis of promastigotes. (F) High magnification showing an intact promastigote and several ruptured cells compatible with loss of membrane integrity and parasite death.

Fig. **1**

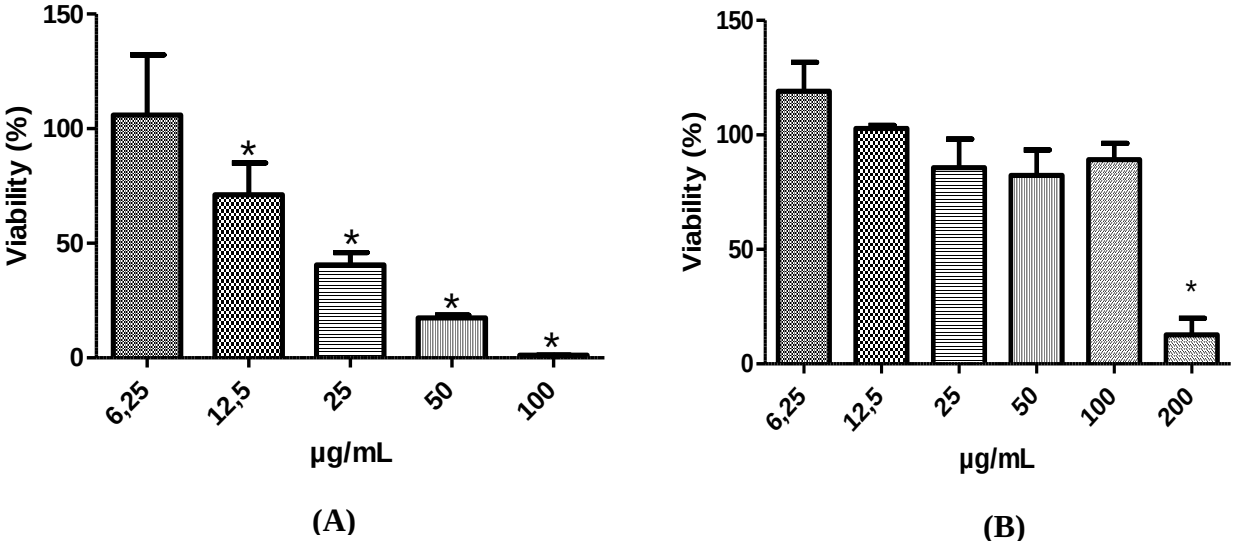
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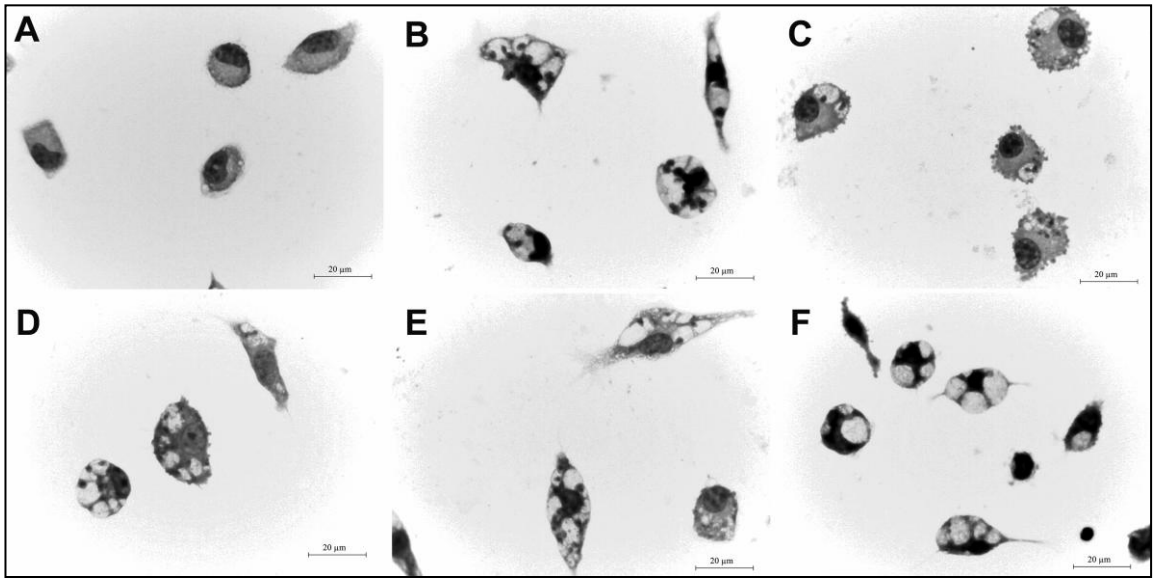
Fig. **2**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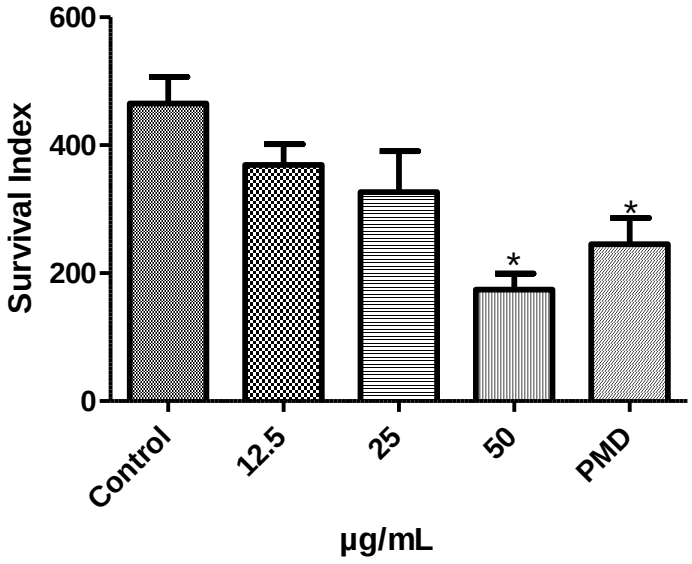

Fig. **4**

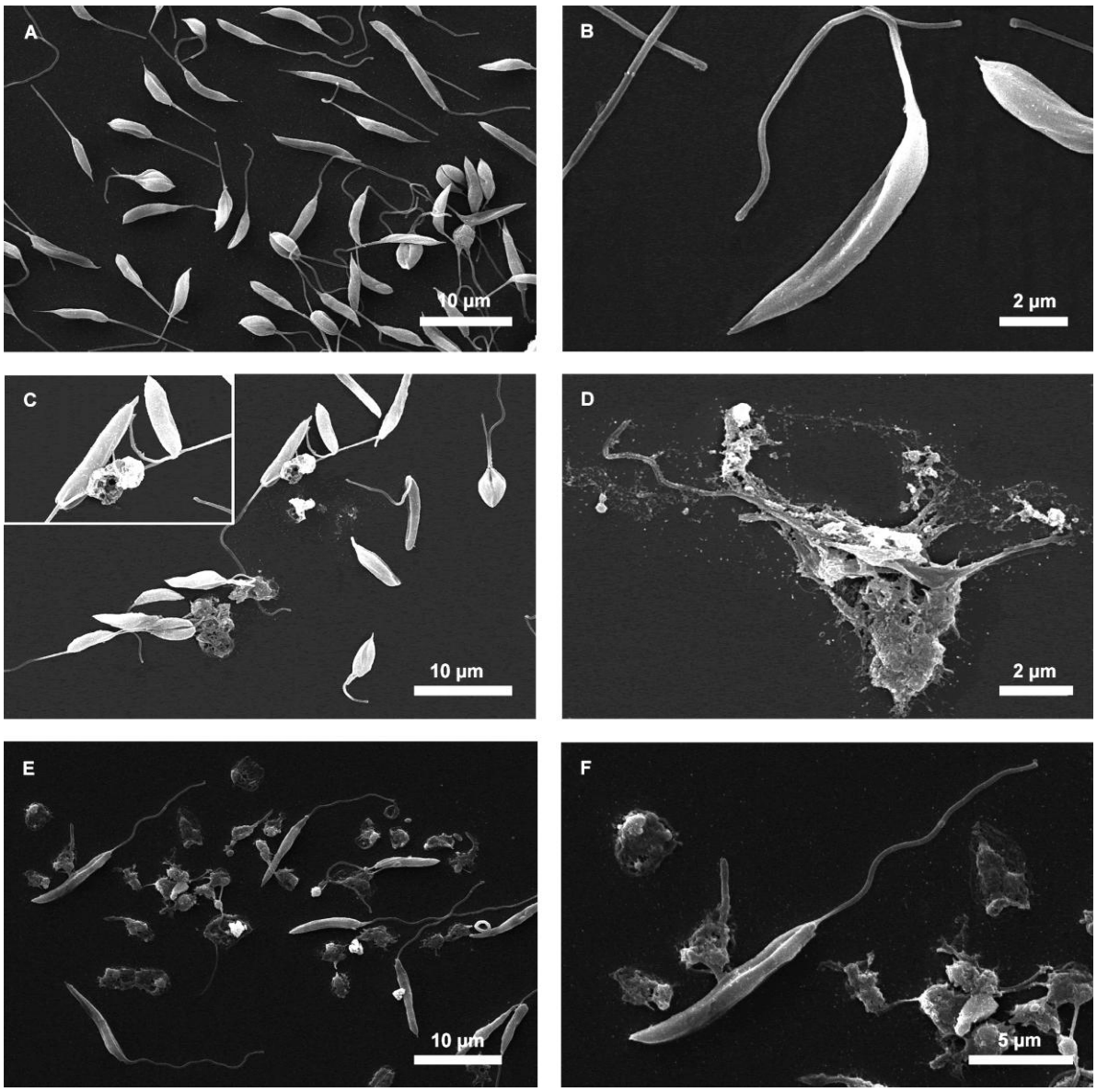

Table 1: Anti-*Leishmania* activity, cytotoxic effects against mammalian cells, and selectivity índex values calculated for *A. minor* essential oil (EOAm) and β-pinene treatment.

Treatment	μg/mL			(SI)	
	IC_{50}/pro	CC_{50}/MØ	IC_{50}/ama	pro	ama
EOAm	25.0 ± 5.9	99.9 ± 13.4	53.62±8.2	3.9	1.8
β-pinene	126.8 ± 37.8	77.1 ± 22.2	-	0.6	-

IC_{50} = Concentration that inhibits by 50% de growth of promastigote and amastigote forms
CC_{50} = Concentration that inhibits by 50 de viability of macrophages (MØ).
pro = promastigote ; ama= amastigote

5 CONCLUSÕES

Os óleos essenciais *A. macrochlamys*, *A. minor* e *E. punicifolia* apresentaram potencial antibacterial frente a bactérias patogênicas humanas. O óleo essencial de *A. minor* foi eficaz frente a embriões de *B. glabrata*, assim como, contra as formas promastigotas e amastigotas de *L. amazonensis*. O composto majoritário β-pineno, do óleo de *A. minor*, não apresentou eficácia em relação às formas promastigotas devido à toxicidade e à baixa seletividade entre o parasita e as células de mamíferos. O óleo de *A. minor* mostrou-se eficaz sendo uma fonte promissora de compostos naturais para controle microrganismos ou hospedeiros de patógenos humanos, bem como para várias aplicações biotecnológicas.

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BMC Complementary and Alternative Medicine

Research article

Criteria

Research articles should report on original primary research, but may report on systematic reviews of published research provided they adhere to the appropriate reporting guidelines which are detailed in our editorial policies. Please note that non-commissioned pooled analyses of selected published research will not be considered.

Preparing your manuscript

The information below details the section headings that you should include in your manuscript and what information should be within each section.

Please note that your manuscript must include a 'Declarations' section including all of the subheadings (please see below for more information).

Title page

The title page should:

- present a title that includes, if appropriate, the study design e.g.:
    - "A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"
    - or for non-clinical or non-research studies a description of what the article reports
- list the full names, institutional addresses and email addresses for all authors
    - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below

- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

- **Background:** the context and purpose of the study
- **Methods:** how the study was performed and statistical tests used
- **Results:** the main findings
- **Conclusions:** brief summary and potential implications
- **Trial registration:** If your article reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be in stated in this section. If it was not registered prospectively (before enrollment of the first participant), you should include the words 'retrospectively registered'. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials

- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

- Ethics approval and consent to participate
- Consent for publication
- Availability of data and material
- Competing interests
- Funding
- Authors' contributions
- Acknowledgements

- Authors' information (optional)

Please see below for details on the information to be included in these sections.

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Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must:

- include a statement on ethics approval and consent (even where the need for approval was waived)
- include the name of the ethics committee that approved the study and the committee's reference number if appropriate

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If your manuscript does not report on or involve the use of any animal or human data or tissue, please state "Not applicable" in this section.

Consent for publication

If your manuscript contains any individual person's data in any form (including individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent for publication.

You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication).

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Availability of data and materials

All manuscripts must include an ‘Availability of data and materials’ statement. Data availability statements should include information on where data supporting the results reported in the article can be found including, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. By data we mean the minimal dataset that would be necessary to interpret, replicate and build upon the findings reported in the article. We recognise it is not always possible to share research data publicly, for instance when individual privacy could be compromised, and in such instances data availability should still be stated in the manuscript along with any conditions for access.

Data availability statements can take one of the following forms (or a combination of more than one if required for multiple datasets):

- The datasets generated and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]
- The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
- All data generated or analysed during this study are included in this published article [and its supplementary information files].
- The datasets generated and/or analysed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.
- Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.
- The data that support the findings of this study are available from [third party name] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [third party name].

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With the corresponding text in the Availability of data and materials statement:

The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS].[Reference number]

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All financial and non-financial competing interests must be declared in this section.

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Please use the authors initials to refer to each author's competing interests in this section.

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Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. Guidance and criteria for authorship can be found in our editorial policies.

Please use initials to refer to each author's contribution in this section, for example: "FC analyzed and interpreted the patient data regarding the hematological disease and the transplant. RH performed the histological examination of the kidney, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript."

Acknowledgements

Please acknowledge anyone who contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials.

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Group authorship (for manuscripts involving a collaboration group): if you would like the names of the individual members of a collaboration Group to be searchable through their individual PubMed records, please ensure that the title of the collaboration Group is included on the title page and in the submission system and also include collaborating author names as the last paragraph of the “Acknowledgements” section. Please add authors in the format First Name, Middle initial(s) (optional), Last Name. You can add institution or country information for each author if you wish, but this should be consistent across all authors.

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Endnotes

Endnotes should be designated within the text using a superscript lowercase letter and all notes (along with their corresponding letter) should be included in the Endnotes section. Please format this section in a paragraph rather than a list.

References

All references, including URLs, must be numbered consecutively, in square brackets, in the order in which they are cited in the text, followed by any in tables or legends. The reference numbers must be finalized and the reference list fully formatted before submission.

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Smith JJ. The world of science. Am J Sci. 1999;36:234-5.

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Book chapter, or an article within a book

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251-306.

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Complete book, authored

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Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

Online database

Healthwise Knowledgebase. US Pharmacopeia, Rockville. 1998. http://www.healthwise.org. Accessed 21 Sept 1998.

Supplementary material/private homepage

Doe J. Title of supplementary material. 2000. http://www.privatehomepage.com. Accessed 22 Feb 2000.

University site

Doe, J: Title of preprint. http://www.uni-heidelberg.de/mydata.html (1999). Accessed 25 Dec 1999.

FTP site

Doe, J: Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt (1999). Accessed 12 Nov 1999.

Organization site

ISSN International Centre: The ISSN register. http://www.issn.org (2006). Accessed 20 Feb 2007.

Dataset with persistent identifier

Zheng L-Y, Guo X-S, He B, Sun L-J, Peng Y, Dong S-S, et al. Genome data from sweet and grain sorghum (Sorghum bicolor). GigaScience Database. 2011. http://dx.doi.org/10.5524/100012.

Figures, tables additional files

See General formatting guidelines for information on how to format figures, tables and additional files.

Planta Medica – Guidelines for Authors

1. Editorial Policy

1.1 Aims and Scope

PLANTA MEDICA – Journal of Medicinal Plant and Natural Product Research is published in 18 issues a year. The following areas of medicinal plant and natural product research are covered:

1. Biological and Pharmacological Activities

2. Natural Product Chemistry & Analytical Studies

3. Pharmacokinetic Investigations

4. Formulation and Delivery Systems of Natural Products

Contributions are not normally considered for publication and will be immediately rejected if:

• The manuscript does not fall into any of the above areas • Activity data are reported without comparison to a recognized positive control

• Extracts have not been characterized by analysis of their major constituents (e.g. HPLC, GC, NMR)

• Predictable bioactivity is reported (e.g. antioxidant properties of phenolic compounds)

1.2 General terms of publication

Only papers of highest scientific quality, concisely written and complying with these Guidelines for Authors can be considered for publication. All contributions are peer-reviewed by independent referees.

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which do not meet acceptable standards will be returned to the authors. Authors investigating the chemistry of a single species should aim to publish their results in a single manuscript rather than in a series of papers. Manuscripts should not report fragmentary parts of a larger study. Pharmacological investigations of extracts require detailed extract characterization (see 4.7.7). Submission of a manuscript signifies acceptance of the journal's Guidelines for Authors. Submissions which are not in line with these principles will be returned directly to the authors by the Editorial Office. A statement clarifying the conflicts of interests of all authors must be included at the end of the manuscript (before the references); this will be published. Conflicts of interest also need to be declared during the submission process. Declaration of conflicts of interest is mandatory; if none, this also needs to be stated. Important: If you intend to use material taken from foreign sources (including figures, tables, etc.), please contact the publisher directly to arrange the next steps (plantamedica@thieme. de).

Planta Medica takes biodiversity and the protection of species very seriously. We support CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) as well as The Rio de Janeiro Convention on Biological Diversity and we expect that during the conduct of the scientific research leading up to the results submitted to Planta Medica these conventions as well as the local rules and regulations have been adhered to.

2. Submission of Manuscripts

Manuscripts can be submitted exclusively online at http://mc. manuscriptcentral.com/plamed. Submissions of hardcopy manuscripts or by e-mail will not be accepted. A sample manuscript (for Original Papers) is available at http:// mc.manuscriptcentral.com/plamed → Instructions and Forms, and at www.thieme.de/plantamedica. In addition to the Guidelines, authors are urged to follow these formats when preparing a manuscript. Commonly used file formats (DOC, DOCX, RTF) should be used for preparation of the manuscripts. PDF files are not accepted. The manuscript has to be accompanied by a cover letter, in which the authors briefly explain the significance of their findings and the interest to the readership of Planta Medica.

The manuscript (main text, including tables) should be submitted as one file. All figures should be submitted separately (detailed layout requirements see 4.11. and 4.12.). Authors are strongly encouraged to provide non-essential but useful data, figures and tables as Supporting Information (see 4.15).

3. Types of Contribution

3.1. Original Papers are research articles describing original experimental results. The material should be arranged in the order: Title Page/Abstract/Keywords/Abbreviations/Introduction/ Results and Discussion/Materials and Methods/Acknowledgements/Conflicts of Interest/References/Figure Legends/Tables. Figures should be uploaded as separate files (see section 4.12., Planta Medica – Guidelines for Authors Planta Med 2018; 84 Graphics). Results and Discussion sections may appear as two separate parts or as a combined "Results and Discussion" section. The normal length of the

main text of an Original Paper, excluding references, tables, figures and figure legends, is < 4,000 words. Original papers should not contain more than 45 references.

3.2. Rapid Communications are intended for the publication of exceptionally significant new and original results, such as unusual structures, bioactivities and innovative analytical techniques that deserve rapid publication, in the format of an Original Paper. If authors want their submission to be considered as a Rapid Communication, they should provide a justification statement for this with their manuscript. However, also regular submissions can be selected by the Editors for rapid communication after the review process.

3.3. Reviews will generally be invited by the Editor-in-Chief. They should be as concise as possible and do not need to include experimental details. The main purpose of reviews is to provide a concise, accurate introduction to the subject matter and inform the reader critically of the latest developments in this area. All reviews should contain an abstract.

3.4. Minireviews and Perspectives will generally be invited by the Editor-in-Chief. Minireviews provide concise and critical updates on a subject of high interest. Perspectives are written by leading experts in an emerging field and provide a concise assessment of the current state-of-the-art and an outlook on future developments. The normal length of the main text of Minireviews and Perspectives, excluding references, tables, figures and figure legends, is about 2000 words.

3.5. Editorials addressing topical issues of general interest to the readership of Planta Medica will be published on an irregular basis. They are written by the Editor-in-Chief, other Editors, or by experts on a specific issue in the form of an Invited Editorial.

4. Preparation of Manuscripts

Please note that papers published in Planta Medica follow the IRDMACR structure: Introduction, Results and Discussion, Materials and Methods, Acknowledgements, Conflict of Interest Statement, References. In addition to the Guidelines, authors should consult the sample manuscript (for Original Papers) at https://www.thieme.de/statics/dokumente/thieme/final/de/dokumente/zw_thieme-en/plantamedica_Sample_Manuscript prior to preparing their contribution. Commonly used file formats (DOC, DOCX, RTF) should be used for preparation of the manuscripts. For submission of all manuscripts, follow the instructions of the on-line submission system. For general instructions on ScholarOne, please follow this link: http://mchelp.manuscriptcentral.com/gethelpnow/training/author/ Before submission, prepare the cover letter, and keep ready all information on the manuscript (title, full name and affiliation of all authors, abstract, name of all files to be submitted). The author submitting the manuscript will be corresponding author.

4.1. The Title Page must contain the title of the manuscript (title should not exceed 20 words), the full names (including the first name) referenced by numerical superscripts and the name of their institute(s), using superscript numbers after name to indicate affiliations. For the corresponding author (to whom all correspondence (including galley proofs) is to be sent),

Name, title, full postal address, telephone and fax number as well as email address has to be displayed.

4.2. The Abstract should contain brief information on purpose, methods, results and conclusion (without subheadings). Abstracts should not exceed 250 words. Please note that during the upload of the manuscript files you will be asked to insert the abstract. This abstract needs to be identical to the abstract in the manuscript file itself.

4.3 The Keywords should include the scientific name and family of the organism(s) investigated. 4–6 keywords should be listed.

4.4. Abbreviations should generally be used sparingly. Abbreviations should be introduced only when repeatedly used. Standard abbreviations such as m. p., b. p., K, s, min, h, µL, mL, µg, mg, g, kg, nm, mm, cm, ppm, mmol, HPLC, TLC, GC, UV, CD, IR, MS, NMR, ELISA, PCR can be used throughout the manuscript (for a more extensive list follow this link: https://www.thieme.de/de/ planta-medica/authors-5605.htm). Non-standard abbreviations must be defined in the text following their first use. Provide a list of all nonstandard abbreviations after the keywords. Define all symbols used in equations and formulas. If symbols are used extensively, provide a list of all symbols together with the list of abbreviations (in alphabetical order).

4.5. The Introduction should state the purpose of the investigation and relate to current knowledge in the specific topic addressed.

4.6. Results should be presented in a concise manner. The Discussion should provide an interpretation of the data and relate them to existing knowledge. The discussion should not be a repetition of the results. Results and Discussion may be combined. No subheadings are allowed within these sections. There should be no separate conclusions paragraph, the conclusion should be incorporated into the discussion.

4.7. In Materials and Methods specific details about test materials and test compounds, instrumentation and experimental protocols should be given. This section should contain sufficient details so that others are able to reproduce the experiment(s). Purity (%) of all reference and standard compounds should be mentioned, as well as the method of how it was determined. Previously reported methods should be referenced only. Suppliers for major equipment, cell lines, chemicals, biochemical reagents and major disposables should be indicated. It should read in the manuscript for example ‘ Quercetin (purity > 98%) was purchased from Sigma’ and not …….‘was purchased from Sigma (St. Louis, USA)’.

4.7.1. Documentation of plants and other organisms or starting materials. Use the correct scientific nomenclature. For plants, the Index Kewensis (electronic Plant Information Centre ePIC, Royal Botanic Gardens, Kew, UK: http://www.kew.org/epic), and/or the International Code of Botanical Nomenclature (www.bgbm.fuberlin.de/iapt/nomenclature/code/tokyo-e/default.htm) should be followed. Give the scientific name (in italics), the author of this name and the family. Indicate the person who identified the material as well as date and place of collection. The manuscript must include references to voucher specimens of the plants

(deposited Planta Medica – Guidelines for Authors Planta Med 2018; 84 in a major regional herbarium) or the material examined including their registration number(s). It should be mentioned which plant parts have been used.

4.7.2. Description of the preparation of extracts and isolation of compounds. The kind and amount of starting material, solvents (including volumes) and extraction methods (including temperature and extraction time) must be indicated. The description of chromatographic systems should contain the quantitative information that allows the reader to repeat the work. Column dimensions, stationary phase, particle size, mobile phase composition, flow rate, sample amount, and elution volumes (or retention times, k' values) of fractions should be given. E. g.: "MPLC on silica gel (40–63 µm; 2 × 50 cm), MeOH/EtOAc 8: 2, 3 mL/min; tR of 1: 60 –70 mL, 2: 120–140 mL, 3: 145–175 mL; detection of eluates by TLC (SiO_2, MeOH/H_2O 9: 1; Dragendorff reagent), Rf 1: 0.35, 2: 0.55, 3: 0.73)." When using gradients the volumes of solvents should be presented; fractions should be defined by their elution volume. Similar information is necessary for HPLC, GLC, DCCC, MLCC and all other methods of purification. Figures of chromatograms will only be accepted if they are essential for understanding the methods or the results described. GC identifications of constituents of essential oils must be supported by retention indices on a polar and an apolar column. Identification by GC-MS is preferred.

4.7.3. Chemical nomenclature used should be based on the systematic rules adopted by Chemical Abstracts and IUPAC. Trivial names should be avoided unless they are definitely advantageous over the corresponding systematic names. Trivial names are not accepted for close analogues and derivatives of known compounds. For reference drug substances the INN names should be used.

4.7.4. Physico-chemical characterisation of compounds. Data provided for new compounds should enable an unambiguous identification of the substance and have to appear in the following order, if available: visual appearance, chromatographic mobility in TLC, GC, or HPLC, mp, UV-vis, specific optical rotation, CD, IR, 1H-NMR, 13C-NMR, low resolution MS, high resolution MS, elemental analysis. Note that for specific optical rotation $[\alpha]D$ temp, the symbol c is defined as mass of substance (in g) in 100 mL of solution. For specific optical rotation no unit should be given; the "degree" symbol "°" should not be used. In case of spectroscopic work on known substances refer, if possible, to published data; the manuscript should then contain the following indication: Copies of the original spectra are obtainable from the corresponding author. Original spectra for new compounds should be provided as Supporting Information (see 4.15), IR, NMR, mass, and UV spectra should normally not be given in the manuscript as figures, unless the listing of characteristic signals is not sufficient.

4.7.5. X-Ray crystallographic data must include a line drawing of the structure, a perspective drawing, and a discussion of bond lengths and angles. A supplement describing full details of the structure and methods and means of its determination in a form suitable for deposition must be submitted to the Cambridge Crystallographic Data Centre, https://www.ccdc.cam.ac.uk/deposit. Deposition of the data has to be prior to submission of

the manuscript, and appropriate reference has to be made in the Materials and Methods section, including the deposition number.

4.7.6. Analytical studies. Key data on method validation must be provided and should typically include information on specificity, linearity, limit of detection, limit of quantification, accuracy, precision, intermediate precision, and some robustness studies. Information on the purity of reference compounds, and on the methods used for the determination of purity must be given. Recoveries of extraction and sample pre-purification steps have to be indicated. Adequate statistical treatment of data is required. For more information regarding validation issues, prospective authors should also refer to ICH guidelines. Analytical studies of a routine nature will not be considered for publication.

4.7.7. Pharmacological investigations. Planta Medica will only consider manuscripts in which conclusions are based on adequate statistics that incorporate the appropriate tests of significance, account for the type of data distribution, and are based on the number of experimental observations required for the application of the respective statistical method. In each case, positive controls (reference compounds) have to be used and the dose-activity dependence should be shown. If IC50 values are given, the dose-response relation should be displayed graphically at least as supplementary data, and the method of calculation should be given. Authors should be conscious of the differences between EC50, IC50, TC50, LC50, ED50, LD50 values. Compounds should follow accepted guidelines when represented as “active”. For example, the cytotoxic effect of a pure substance when tested against a cancer cell line would exhibit an IC50 value of < 10 µM. Authors should pay attention to the following definitions: Compounds that suppress the growth of, or kill, isolated tumor cell lines grown in culture should be referred to as either “cytostatic” or “cytotoxic”, as appropriate. Only compounds that inhibit the growth of tumors in animal-based models should be called “antitumor”. The term “anticancer” should be reserved for compounds that show specific activity in human-based clinical studies. When working with experimental animals, reference must be made to principles of laboratory animal care or similar regulations and to approval by the local ethical committee. The protocol approval number and the exact date of approval (e.g. January 1st 2016) must be provided. Pharmacological investigations of extracts require detailed extract characterization. This includes botanical characterization of plant material, solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). For starting material coming from a company/commercially obtained samples the batch/Lot. Number has to be provided. The drug to extract ratio (DER) must be given. Chromatographic profiling (e.g. HPLC profile with a reference compound recorded at different wavelengths) should be carried out, with at least the major peaks identified, or qualitative and quantitative information on active or typical constituents should be provided. Altogether the phytochemical standardization of an extract and/or fraction(s) requires stateof-the-art methods.

4.7.8. Biological screening. Papers dealing with the biological screening of a meaningful number of extracts of plants or other organisms can be considered for publication in Planta Medica. Identification of the material should properly be documented, and preparation of the extracts should clearly be described (see above, sections 4.7.1 and 4.7.7). Biological activities

should be rePlanta Med 2018; 84 ported by listing IC50 or EC50 values, or a dose-response relationship should be shown by using at least two test concentrations. Positive controls (reference compounds) should be included. Results should be presented in a concise format, and the discussion should be kept to a minimum.

4.8. Acknowledgements should list persons who made minor contributions to the investigation and organisations providing support.

4.9. Conflict of Interest Disclosure. A statement describing any financial conflicts of interest or lack thereof is published with each manuscript. The statement should describe all potential sources of bias, including affiliations, funding sources, and financial or management relationships, that may constitute conflicts of interest (please see the ACS Ethical Guidelines to Publication of Chemical Research). The statement will be published in the final article. If no conflict of interest is declared, the following statement will be published in the article: "The authors declare no conflict of interest."

4.10. References, including those in tables and figure legends, should be numbered in the order in which they are cited in the text, using arabic numbers between square brackets, e.g. [1]; for multiple references, e.g. [1–3] or [1, 2, 5]. The list of references should be arranged consecutively according to the numbers in the text. Use Index Medicus abbreviations for journal titles. Authors bear complete responsibility for the accuracy of the references. Original Research articles should not have more than 45 references. The following examples illustrate the format for references:

a) Journals Trute A, Nahrstedt A. Separation of rosmarinic acid enantiomers by three different chromatographic methods and the determination of rosmarinic acid in Hedera helix. Phytochem Anal 1996; 7: 204–208 Article in press without doi: Lim EK, Ashford DA, Hou B, Jackson RG, Bowles DJ. Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. Biotech Bioeng, in press Note: If reference is made to papers that are in press, authors are requested to add the galley proof or acceptance letter to the online submission. Avoid references to unpublished personal communications. These have to be included in the body of the text as 'unpublished data'. Article in press with doi: Lim EK, Bowles DJ. A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J, advance online publication 8 July 2004; doi: 10.1038/sj.emboj.7600295

b) Books Citation to complete book: Mabberley DJ. The plant book, 2nd edition. Cambridge: Cambridge University Press; 1997: 520 –521

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f) Electronic Sources Agatep R, Kirkpatrick RD, Parchaliuk DL,Woods RA, Gietz RD. Transformation of S. cerevisiae by the lithium acetate/singlestranded carrier DNA/polyethylene glycol protocol. Technical tips online. Available at http://research.bmn.com/tto. Accessed September 22, 2005. If no author is given, the title is used as the first element of the citation.

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