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CÍCERO RAMON BEZERRA DOS SANTOS

AVALIAÇÃO FITOQUÍMICA E ANTIPARASITÁRIA DO ÓLEO ESSENCIAL DE *Eugenia stipitata* McVaugh

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do grau de mestre.

Orientadora: Profª. Drª. Márcia Vanusa da Silva – UFPE

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BANCA EXAMINADORA:

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Profª. Drª. Márcia Vanusa da Silva - UFPE
(Orientadora)

_____

Profº. Drº. Alexandre Gomes da Silva - UFPE
(1º Examinador)

_____

Profº. Drº. Thiago Henrique Napoleão - UFPE
(2º Examinador)

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(William Shakespeare).

RESUMO

Os óleos essenciais (EOs) de plantas utilizadas na medicina tradicional são conhecidos como uma rica fonte de compostos quimicamente diferentes com diversas atividades biológicas. Neste estudo, analisamos a composição química e investigamos o efeito tripanocida, leishmanicida e a citotoxicidade *in vitro* do óleo essencial obtido a partir das folhas de *Eugenia stipitata* McVaugh. O OE foi extraído pelo método de destilação por arraste à vapor e sua composição química analisada por GC/MS e testado em sete concentrações (15.75, 31.25, 62.5, 125, 250, 500 e 1000 µg/mL) contra as formas epimastigotas de *Trypanosoma cruzi* e as formas promastigotas de *Leishmania braziliensis* e de *Leishmania infantum*, bem como contra fibroblastos. De modo geral, 48 componentes foram identificados na análise química do óleo e os constituintes mais abundantes foram: β-Eudesmol (15,28%), γ-Eudesmol (10,85%), Elemol (10,21%), Caryophyllene oxide (6,5%), Clovene (6,18%) e Espatulenol (6,14%). Na avaliação das atividades antiparasitárias, o OE de *Eugenia stipitata* McVaugh em concentrações de 125 µg/mL e 62.5 µg/mL inibiu, respectivamente, 80.69% e 71.91% da forma promastigota de *L. braziliensis.* Inibição semelhante foi observada para as mesmas concentrações do óleo contra *L. infantum*, que inibiu 76.51% da forma promastigona de *L. infantum* na concentração de 125 µg/mL e 73.51% na concentração de 62.5 µg/mL. Contudo, na concentração de 125 µg/mL foi observada uma menor inibição parasitária do óleo essencial de *Eugenia stipitata* McVaugh frente às formas epimastigotas de *T. cruzi*, com 22.85%. O óleo essencial de *Eugenia stipitata* McVaugh apresentou uma baixa citotoxicidade na concentração de 62.5 µg/mL e nenhuma citotoxicidade nas concentrações de 31.25 µg/mL e 15.75 µg/mL. Desse modo, o OE estudado apresentou-se como promissora fonte antiparasitária contra as formas de vida da *L. braziliensis* e *L. infantum*.

Palavras chaves: Óleo essencial. Caatinga. Antiprotozoário. *Eugenia stipitata* McVaugh.

ABSTRACT

The essential oils (EOs) of plants used in traditional medicine are known as a rich source of chemically different compounds with different biological activities. In this study, we analyzed the chemical composition and investigated the trypanocidal, leishmanicide, effect and in vitro cytotoxicity of essential oil obtained from the leaves of *Eugenia stipitata* McVaugh. EO was extracted by the distillation method by steam stripping and its chemical composition analyzed by GC/MS and tested in seven concentrations (15.75, 31.25, 62.5, 125, 250, 500 e 1000 µg/mL) against the forms of *Trypanosoma cruzi* epimastigotes and promastigotes forms of *Leishmania braziliensis* and *Leishmania infantum*, as well as against fibroblasts. Generally, 48 components were identified in the oil chemical analysis and the most abundant constituents were: *β*-eudesmol (15.28%), *γ*-eudesmol (10.85%), Elemol (10.21%), caryophyllene oxide (6.5%), Clovene (6.18%) and Espatulenol (6.14%). In the evaluation of the parasitic activities, EO of *Eugenia stipitata* McVaugh at concentrations of 125 µg/mL and 62.5 µg/mL inhibited, respectively, 80.69% and 71.91% of the promastigote form of *L. braziliensis.* Similar inhibition was observed for the same concentrations of the oil against *L. infantum*, which inhibited 76.51% of promastigote form of *L. infantum* in concentration of 125 µg/mL and 51.1% in the concentration of 62.5 µg/mL. However, in the concentration of 125 µg/mL a smaller parasitic inhibition was observed of essential oil of *Eugenia stipitata* McVaugh bedore the epimastigotes forms of *T. cruzi*, with 22.85%. The essential oil of *Eugenia stipitata* McVaugh presented a low cytotoxicity in the concentration of 62.5 µg/mL and no cytotoxicity in concentrations of 31.5 µg/mL and 15.75 µg/mL. Therefore, the studied EO presented itself as a promising antiparasitic source against the ways of life of *L. braziliensis* and *L. infantum*.

Keywords: Essential Oil. Caatinga. Antiprotozoal. *Eugenia stipitata* McVaugh.

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LISTA DE ABREVIAÇÕES E SIGLAS

cm	Centímetros
CD	Chagas disease
EOS	Essential oils
FBS	Fetal Bovine Serum
GC/MS	Gas chromatography-mass spectrometry
LTA	Leishmaniose Tegumentar Americana
LV	Leishmaniose visceral
µg/mL	Micrograma por mililitro
µM	Micrômetro
mm	Milímetros
nm	Nanômetros
EOESL	Oil from the leaves of *E. stipitata*
OE	Óleo essencial
OMS	Organização Mundial de Saúde
km^2	Quilômetros quadrados
SDTF	Seasonally dry tropical forests
UFPE	Universidade Federal de Pernambuco
URCA	Universidade Regional do Cariri

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1 INTRODUÇÃO

A utilização das plantas como medicamento é uma das mais antigas armas empregadas para o tratamento das enfermidades humanas e muito já se conhece a respeito de seu uso por parte da sabedoria popular. A Organização Mundial de Saúde comenta que, na busca incessante do homem por bem-estar e qualidade de vida, a fitoterapia tornou-se uma alternativa devido à credibilidade terapêutica e ao baixo custo, o que favorece sua utilização por uma grande parcela da população mundial (LUCHESSI et al., 2005).

Em regiões que abrangem a Caatinga, espécies vegetais são amplamente utilizadas pelas comunidades no tratamento de suas enfermidades. Estas comunidades possuem uma vasta farmacopeia natural, grande parte proveniente dos recursos vegetais encontrados nos ambientes naturais habitados por estas populações, ou cultivados em ambientes transformados pelo próprio homem (GOMES et al., 2008). De acordo com Matos (2002), 90% da população economicamente carente do Nordeste utiliza a medicina tradicional, em busca da cura de seus problemas de saúde.

A busca de plantas medicinais e seus derivados como agentes terapêuticos naturais vem sendo priorizada, a fim de amenizar as enfermidades sofridas pela população. A utilização destes fitoterápicos é incentivada por apresentarem um custo mais acessível à população e aos serviços públicos de saúde, em comparação aos fármacos obtidos por síntese química (TOLEDO et al., 2003; SILVA, 2006).

Dentre os agentes terapêuticos vegetais destacam-se os óleos essenciais (OEs), produzidos nas folhas em estruturas chamadas tricomas glandulares, provenientes do metabolismo secundário das plantas que atuam nos mecanismos de defesa contra as condições adversas (MICHELIN, 2008). A obtenção de uma droga vegetal de qualidade tem correlação direta com o conhecimento prévio das classes de componentes químicos encontrados nesses vegetais. Quando confirmada a presença de determinados grupos químicos, o estudo fitoquímico e biológico é direcionado através de ensaios que avaliam substâncias oriundas de plantas, em extratos ou substâncias isoladas. Apesar do interesse na utilização desses produtos vegetais, ainda são poucas as pesquisas que relatam as propriedades biológicas de espécies em vegetações predominantemente brasileiras como a Caatinga (LÔBO et al., 2010).

A família Myrtaceae, no Brasil, é representada por 23 gêneros e cerca de 1000 espécies. Estudos apontam que as plantas pertencentes a essa família são fontes de óleos essenciais responsáveis por importantes benefícios associados à saúde (D'ANGELIS; NEGRELLE, 2014). O gênero *Eugenia* encontra-se representado por 400 espécies nos diversos domínios

fitogeográficos do Brasil, e muitas destas espécies destacam-se pela produção de óleos essenciais que apresentam diversas propriedades farmacológicas (QUEIROZ et al., 2015).

Especificamente, as espécies *E. brasiliensis* e *E. uniflora* já tiveram seus potenciais antiparasitários investigados anteriormente frente ao T. cruzi e o OE das flores de *E. klotzschiana* apresentou satisfatória atividade tripanocida frente às formas tripomastigota desse parasita (AZEVEDO et al., 2014; CARNEIRO et al., 2017; DONATO; MORRETES, 2007). Além disso, atividade leishmanicida de produtos naturais, com atividade citotóxica contra o parasita, obtidos de plantas, tem sido considerado promissor no desenvolvimento de fármacos (RODRIGUES et al., 2015).

Eugenia stipitata McVaugh é uma frutífera, conhecida popularmente como araçá-boi (MCVAUGH, 1956). Na medicina popular, é utilizado no tratamento de desordens intestinais e urinárias, além de também aliviar os sintomas do resfriado, o que indica que os compostos bioativos presentes na planta podem ter potenciais benefícios para a saúde humana (FERNÁNDEZ-TRUJILLO et al., 2011). Entre as propriedades biológicas investigadas, podem-se destacar a atividade antioxidante na eliminação de radicais livres, potencial antimicrobiano do OE produzido a partir de suas folhas e atividades antimutagênica e antigenotóxica de extratos do fruto, sugerindo que estes podem funcionar como agentes preventivos contra o câncer (DUKE, 2017; MEDEIROS et al., 2003; NERI-NUMA et al., 2013).

As infecções por protozoários são consideradas um problema mundial de saúde pública, especialmente em países subdesenvolvidos, onde aproximadamente 14% da população são considerados como risco de infecção (KONDRASHIN et al., 2011; WALDRON et al., 2011). Neste contexto, destacam-se: a doença causada pelo protozoário *Trypanosoma cruzi*, denominada doença de Chagas, cujo tratamento quimioterápico apresenta vários efeitos colaterais, como: anorexia, náuseas, alterações gastrointestinais, dermatopatia alérgica, polineurite, depressão da medula óssea, neuropatia periférica entre outros; e a leishmaniose, causada pelo protozoário *Leishmania*, considerada pela Organização Mundial de Saúde (OMS) como uma das seis principais doenças infecciosas com alta incidência e capacidade de produzir deformidades, cujos tratamentos convencionais são limitados, inseguros e têm uma série de efeitos colaterais, além de contribuírem para a resistência do parasita ao tratamento (ALIZADEH et al., 2008; FERREIRA, 2012; MITROPOULOS et al., 2010; OLIVEIRA et al., 2008).

Devido a estas complicações causadas pelos fármacos disponíveis, torna-se cada vez mais relevante a busca por novos agentes quimioterápicos, com menos efeitos colaterais quando

comparado à terapia alopática e mais acessível à população. Dessa forma, o isolamento, a caracterização e a aplicação biológica de compostos naturais, sobretudo de plantas nativas da Caatinga devido à sua grande biodiversidade, possuem um elevado grau de significância na produção de novas drogas com perfil farmacológico.

Nesse contexto, o presente estudo objetiva avaliar as atividade citotóxica, leishmanicida e tripanocida do óleo essencial de *Eugenia stipitata* McVaugh, além de quantificar os compontentes químicos do mesmo utilizando cromatografia gasosa acoplada à espectrometria de massa.

2 OBJETIVOS

2.1 OBJETIVO GERAL:

Avaliar a composição química e os efeitos tripanocida, leishmanicida e a citotoxicidade *in vitro* do óleo essencial obtido a partir das folhas de *Eugenia stipitata* McVaugh.

2.2 OBJETIVOS ESPECÍFICOS:

- Obter o óleo essencial das folhas de *Eugenia stipitata* McVaugh.
- Quantificar os constituintes químicos do óleo essencial de *Eugenia stipitata* McVaugh através da cromatografia gasosa acoplada à espectrômetro de massas.
- Avaliar a citotoxicidade e as atividades tripanocida, contra a forma epimastigota de *Trypanosoma cruzi* e leishmanicida, contra as formas promastigotas de *Leishmania braziliensis* e *Leishmania infantum* do óleo essencial.

3 REVISÃO BIBLIOGRÁFICA

3.1 O BIOMA CAATINGA

A caatinga é um ecossistema de florestas tropicais secas localizadas na região semiárida do nordeste do Brasil. O termo caatinga (“floresta branca”) se refere à um mosaico de fisionomias vegetais de florestas secas, xerófitas, lenhosas, espinhosas e decíduas, com uma vegetação arbustiva sazonal. Essas variações são atribuídas principalmente em decorrência das alterações climáticas, padrões orográficos, modificações em pequena escala na topografia e nos solos (ANDRADE-LIMA, 1981; SAMPAIO, 1995).

Abrangendo a maioria dos estados do Nordeste como: Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia e no nordeste de Minas Gerais, no Vale de Jequitinhonha, ocupando uma área que se estende por cerca de 850.000 km^2 e está cercada por regiões Amazônicas e da Mata Atlântica a leste e oeste, e de savanas do Cerrado ao sul (Figura 1) (BASSO et al., 2005). Isso corresponde a 10% do território brasileiro e 60% da região nordeste. Sua precipitação média anual varia de 240 a 1500 mm, estando restrita a três meses consecutivos, com temperatura média anual de 27,5 °C (ANDRADE-LIMA, 1981; LEAL et al., 2005; ALVES et al., 2011).

Anteriormente, a Caatinga era considerada um bioma pobre em espécies vegetais e, portanto, indigno de medidas de conservação. Entretanto, é de conhecimento geral que existem 510 gêneros e 5344 espécies de plantas vasculares na Caatinga, dentre as quais 18 gêneros e 318 espécies são endêmicos (GIULIETTI et al., 2002). Dessa forma, o bioma é dominado por um dos poucos tipos de vegetação cuja distribuição é totalmente restrita ao Brasil (SILVA; ALBUQUERQUE, 2005).

Espécies de plantas medicinais pertencentes à Caatinga, como *Myracrodruon urundeuva* Allemão, *Amburana cearensis* (Arr. Cam.) A.C. Smith., e *Anadenanthera colubrina* (Vell.) Brenan var. *cebil* (Griseb) Altschul, são amplamente conhecidas e utilizadas na medicina popular, e para confecção de produtos fitoterápicos (ALMEIDA; ALBUQUERQUE, 2002; ALBUQUERQUE et al., 2007). Apesar da sua diversidade de recursos, a vegetação da Caatinga faz parte de um bioma altamente ameaçado e ainda pouco estudado do ponto de vista etnobotânico, fitossociológico e farmacológico, dada a riqueza cultural e biológica que lá existe (VANDESMET, 2015).

Figura 1: Localização das Caatingas na América do Sul

Fonte: LEAL; SILVA; TABARELLI, 2005.

3.2 CONSIDERAÇÕES GERAIS SOBRE MYRTACEAE

Myrtaceae, uma das maiores famílias de Angiospermas, engloba cerca de 129 gêneros e aproximadamente 4.620 espécies de árvores e arbustos, que por suas características e atributos são incluídas em duas subfamílias: Psiloxyloideae, com duas tribos e Myrtoideae, com quinze tribos (WILSON et al., 2001, 2005). A família apresenta ampla distribuição geográfica, com particular ocorrência na Austrália, Índia, América tropical e várias outras regiões de clima temperado e semiárido (DI STASI; HIRUMA-LIMA, 2002; OLIVEIRA et al., 2005).

Apresenta-se distribuída em todos os domínios fitogeográficos brasileiros, ocorrendo quase 1000 espécies subordinadas a 23 gêneros. As mirtáceas estão entre as 10 famílias com maior riqueza de espécies na flora do país, sendo a Floresta Atlântica um de seus centros de diversidade, onde é a sexta maior família em níveis de diversidade. A mesma engloba os gêneros *Myrtus*, *Psidium*, *Pimenta*, *Eugenia*, *Sygyzium*, *Eucalyptus*, *Leptospermum* e *Malaleuca* (FORZZA et al., 2010; FERNANDES, 2011).

As mirtáceas desempenham um grande papel na economia, uma vez que várias espécies são cultivadas, seja por seus frutos comestíveis, com propósito ornamental, pela extração de essências ou madeiras com alto valor comercial. Seus representantes também possuem várias propriedades medicinais, grande parte destas atribuídas aos óleos essenciais das estruturas secretoras de seus órgãos vegetativos e reprodutivos (BARROSO et al., 1984; MELO, 2009).

Do ponto de vista químico, várias substâncias com atividade farmacológica foram isoladas em estudos a partir de espécies dessa família, tais como o cariofileno, biciclogermacreno, espatulenol, carotenoides, ácido ascórbico e compostos fenólicos (MORENO et al., 2014; MIRANDA et al., 2017; PEREIRA et al., 2017).

O uso medicinal das muitas espécies desta família justifica-se pelas diversas atividades comprovadas, como antimicrobiana, antiviral, hipoglicemiante, antioxidante e anticancerígena. Entre os exemplos mais frequentes, podem-se citar a goiabeira, araçá, jabuticaba, ponhema, sabará, pitanga, uvária, cabeludinha, quimixama, guabiroba e o cambuci (SILVA; CASALI, 2000; APEL et al., 2006; SERAFIM, 2006). Ainda revisando a literatura, encontra-se algumas pesquisas que revelam inúmeras plantas pertencentes a família Myrtaceae com atividade antiparasitária como, por exemplo, o estudo realizado por Silva (2007) que revelou potente atividade tripanocida e leishmanicida da planta *Myrcia hiemalis (Myrtaceae)*, além da pesquisa de Correia et al (2016) que mostrou satisfatória atividade Leishmanicida do extrato de *Myrciaria dubia* (Myrtaceae).

3.3 GÊNERO *EUGENIA*

O gênero *Eugenia* encontra-se bem representado nos diversos domínios fitogeográficos do Brasil, destacando-se pelo vasto potencial medicinal, econômico, alimentício, bem como para exploração comercial de madeiras e óleos essenciais, além de seu uso como plantas ornamentais (ROMAGNOLO; SOUZA, 2006; ALVES; TRESMONDI; LONGUI, 2008; LAGO et al., 2011).

Possuem representantes na forma de arbustos, subarbustos e árvores, nas quais o caule pode atingir de três a doze metros de altura. As flores exibem-se em racemos, dicásios ou isoladas, com antopódio presente e profilos livres, persistentes ou caducos. Os botões florais abertos apresentam quatro sépalas que frequentemente são desiguais, e as pétalas tetrâmeras semelhantes. Os estames são numerosos; ovário bilocular podendo apresentar de quatro a vinte óvulos por lóculo. Os frutos apresentam coloração heterogênea podendo ser amarelos, alaranjados, vermelhos, vináceos e até pretos quando maduros, sendo as bagas globosas a

elipsóides, com cálice persistente. O número de sementes pode variar de uma a três, o embrião é do tipo eugenióide. Entre os cotilédones existe uma linha de separação e o eixo hipocótilo-radícula apresenta-se pouco desenvolvido (QUEIROZ et al., 2015).

As espécies do gênero *Eugenia* são alvos constantes de pesquisas, uma vez que são detentoras de óleos essenciais e diversos metabólitos secundários que apresentam importantes ações biológicas (VICTORIA et al., 2012). Em estudos realizados com a espécie *Eugenia uniflora* L. importantes propriedades terapêuticas foram descritas, tais como: antiparasitária, antibacteriana, antioxidante, antidepressiva, antinociceptiva e hipotérmicas. Já no viés fitoquímico, os principais marcadores descritos são: ácido gálico, ácido elágico e miricitrina, porém, podem ser encontrados nesta planta outros metabólitos, tais como as antacioninas e sesquiterpenos (OGUNWANDE et al., 2005; AMORIM et al., 2009; BEZERRA et al., 2017; OLIVEIRA et al., 2017; SOUZA et al., 2017).

Em consonância, as atividades biológicas de *Eugenia jambolana* L. incluem: antibacteriana, antidiarreica, antiparasitária, antidiabética, antinociceptiva e anti-inflamatória (MUKHERJEE et al., 1988; SAHA et al., 2013; LI et al., 2017; PEREIRA et al., 2017; SOUZA et al., 2017). A avaliação dos constituintes presentes nessa espécie aponta para a presença de compostos fenólicos nos extratos, como taninos, alcaloides, esteroides, flavonoides, terpenóides, ácidos graxos, fenóis, minerais, carboidratos e vitaminas (MARGARET; SHAILAJA; RAO, 2015; VEBER et al., 2015). Além disso, um rastreio químico e quimiopreventivo realizado com extratos e frações de folhas e frutos de *E. jambolana* levaram à purificação de flavonoides (tricetina-4'-O-α-l-rhamnopiranósido e miricitrina) e antacioninas (malvidina-3-O-gentibiosídeo), sugerindo que esta espécie pode agir como um agente preventivo contra danos ao DNA (DAMETTO et al., 2017).

Outras espécies pertencentes a este gênero que vêm sendo estudadas são *Eugenia punicifolia* (Kunth) DC. e *Eugenia dysenterica* DC. apresentando, respectivamente, tais atividades biológicas: neuroprotetora, gastroprotetora, antioxidante, antidiarreica, antileucêmica e antifúngica; e anti-inflamatórias, antinociceptiva, gastroprotetora, anti-diabética e antioxidante. Análises químicas dessas espécies sugerem a presença de terpenos, flavonoides, saponinas e taninos como responsáveis por suas propriedades farmacológicas (LEITE et al., 2014; GALENO et al., 2014; SALES et al., 2014; BASTING et al., 2014; COSTA et al., 2000; PRADO et al., 2014; GALHEIGO et al., 2015; PEIXOTO, 2015; VITEK et al., 2017).

É cabível citar outros estudos, como o de Carneiro et al (2017), cujos resultados demonstraram satisfatória atividade tripanocida do óleo essencial de *Eugenia klotzschiana* e

Santos et al (2013) em que os resultados indicam que *Eugenia uniflora* apresenta atividade leishmanicida.

3.4 CONSIDERAÇÕES GERAIS SOBRE A ESPÉCIE *EUGENIA STIPITATA*

O araçazeiro-boi (*Eugenia stipitata* McVaugh) é uma frutífera pertencente à família *Myrtaceae*. É originária da Amazônia Ocidental, comumente cultivada em pequena escala no Peru, Bolívia, Equador, Colômbia e Brasil (MCVAUGH, 1956).

O arbusto do araçazeiro-boi alcança de três a cinco metros de altura (Figura 2), com abundante ramificação e folhagem, se adaptando muito bem ao clima tropical úmido. Suas folhas são elípticas, verde-escuras e as pequenas inflorescências possuem de três a dez flores hermafroditas, com pétalas brancas e 75 a 100 estames (CHÁVES-FLORES; CLEMENT, 1984; EMBRAPA, 1996).

Figura 2: Arbusto de *Eugenia stipitata*, conhecida popularmente como araçá-boi.

Fonte: http://www.fruitipedia.com/araca_boi.htm

Seu fruto é uma baga globosa, de formato redondo ou achatado, com casca delgada e coloração amarelo-canário quando madura. Sua polpa é suculenta, pouco fibrosa, e bastante ácida, de coloração amarelo-clara, possuindo de 4 a 10 sementes oblongas de 0,5 a 1,0 cm de comprimento (SACRAMENTO; BARRETTO; FARIA, 2008).

Devido sua intensa acidez, a polpa do araçá-boi não é adequada para o consumo *in natura*. Porém, esta possui aroma e sabor agradáveis, podendo ser consumida em forma de

sucos, sorvetes, geleias, néctar, licores e outros (EMBRAPA, 1996; ANDRADE et al., 1997; SOARES, 2009).

Entre as propriedades biológicas investigadas, podem-se destacar a atividade antioxidante na eliminação de radicais livres (DUKE, 2017), potencial antimicrobiano do óleo essencial produzido a partir de suas folhas (MEDEIROS et al., 2003) e atividades antimutagênica e antigenotóxica de extratos do fruto, sugerindo que estes podem funcionar como agentes preventivos contra o câncer (NERI-NUMA et al., 2013).

Na medicina popular local, o araçá-boi é utilizado no tratamento de desordens intestinais e urinárias, além de também aliviar os sintomas do resfriado, o que indica que os compostos bioativos presentes na planta podem ter potenciais benefícios para a saúde humana (FERNÁNDEZ-TRUJILLO et al., 2011).

3.5 *LEISHMANIA*

As leishmanioses são antropozoonoses, causadas por protozoários do gênero *Leishmania*, consideradas um grande problema de saúde pública, representando um conjunto de doenças com grande importância clínica e diversidade epidemiológica (BRASIL, 2007).

A Leishmaniose Tegumentar Americana (LTA) é uma enfermidade causada por protozoários do gênero *Leishmania*, transmitida ao homem pela picada de mosquitos flebotomíneos. Existem atualmente 6 espécies de *Leishmania* responsáveis pela doença humana no Brasil, e mais de 200 espécies de flebotomíneos envolvidos em sua transmissão (BASANO, 2004).

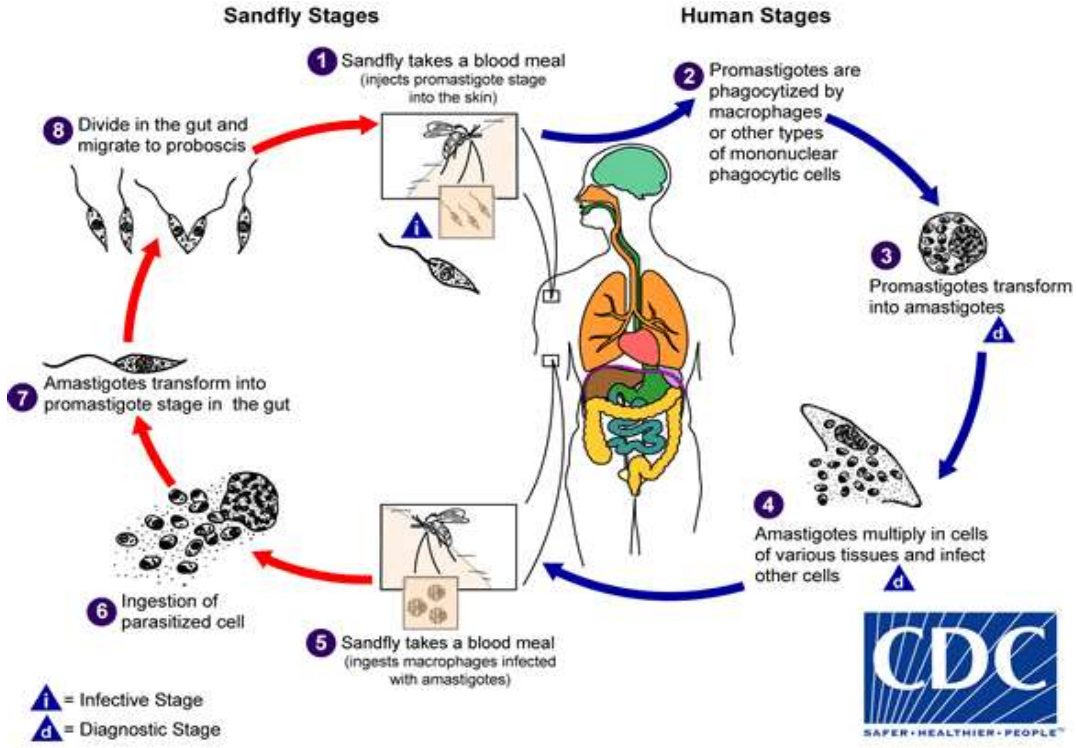
A enfermidade apresenta-se na forma cutânea ou mucosa (PALMEIRO et al., 2007), sendo que a forma mucosa é, na maioria das vezes, secundária às lesões cutâneas, surgindo, geralmente, meses ou anos depois das lesões de pele (BRASIL, 2007). Quando acometem a mucosa bucal, a doença se torna destrutiva ou ulcerovegetativa e granulomatosa, acompanhada pela presença de granulações grosseiras e sulcos profundos. Geralmente, a dificuldade de deglutir e a dor são os principais sintomas relatados, além de sialorréia, odor fétido e o sangramento (GONTIJO; CARVALHO, 2003).

O protozoário *Leishmania infantum chagasi*, no Brasil, é o agente causador da leishmaniose visceral (LV), enfermidade que atinge cerca de 65 países, com incidência estimada de 500 mil novos casos e 59 mil óbitos anuais, e é transmitida por flebotomíneos do gênero *Lutzomyia*, sendo o cão considerado a principal fonte de infecção no meio urbano. Considerada uma doença grave com poucas opções terapêuticas, mesmo quando

adequadamente tratada, a mesma tem letalidade de cerca de 5% (WERNECK, 2010).

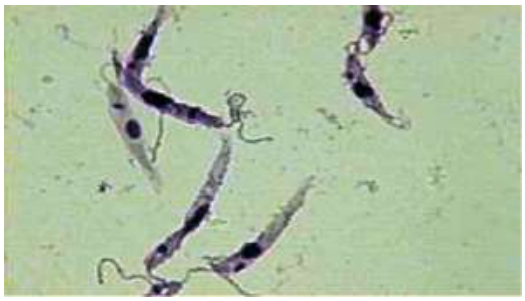
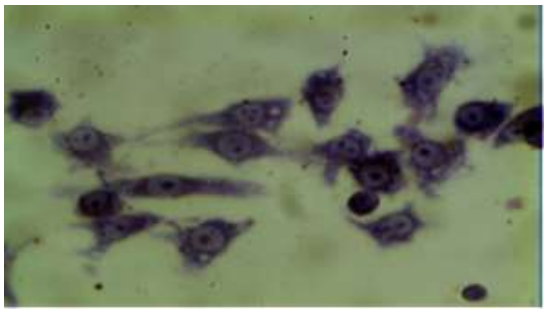
As formas promastigotas metacíclicas de *Leishmania* são responsáveis por causar infecção e são transmitidas aos hospedeiros vertebrados pelos vetores (Figura 3). Além da forma promastigotas metacíclicas, o parasito também apresenta as formas paramastigotas e promastigota não metacíclica, que se multiplicam no trato digestivo do vetor, e as formas amastigotas, que são parasitas obrigatórios das células do sistema fagocítico mononuclear (Figura 4) (ALBERNAZ, 2010).

Figura 3: Ciclo Biológico da *Leishmania*



Fonte: CDC, 2014

Figura 4: A) Forma Promastigota de *Leishmania sp*; B) Forma Amastigota de *Leishmania sp*.

A  B 

Fonte: VANDESMET, 2014.

No tratamento, além da necessidade do desenvolvimento de melhores fármacos para o tratamento da Leishmaniose, ainda não há vacinas contra o parasita *Leishmania* e, também, não

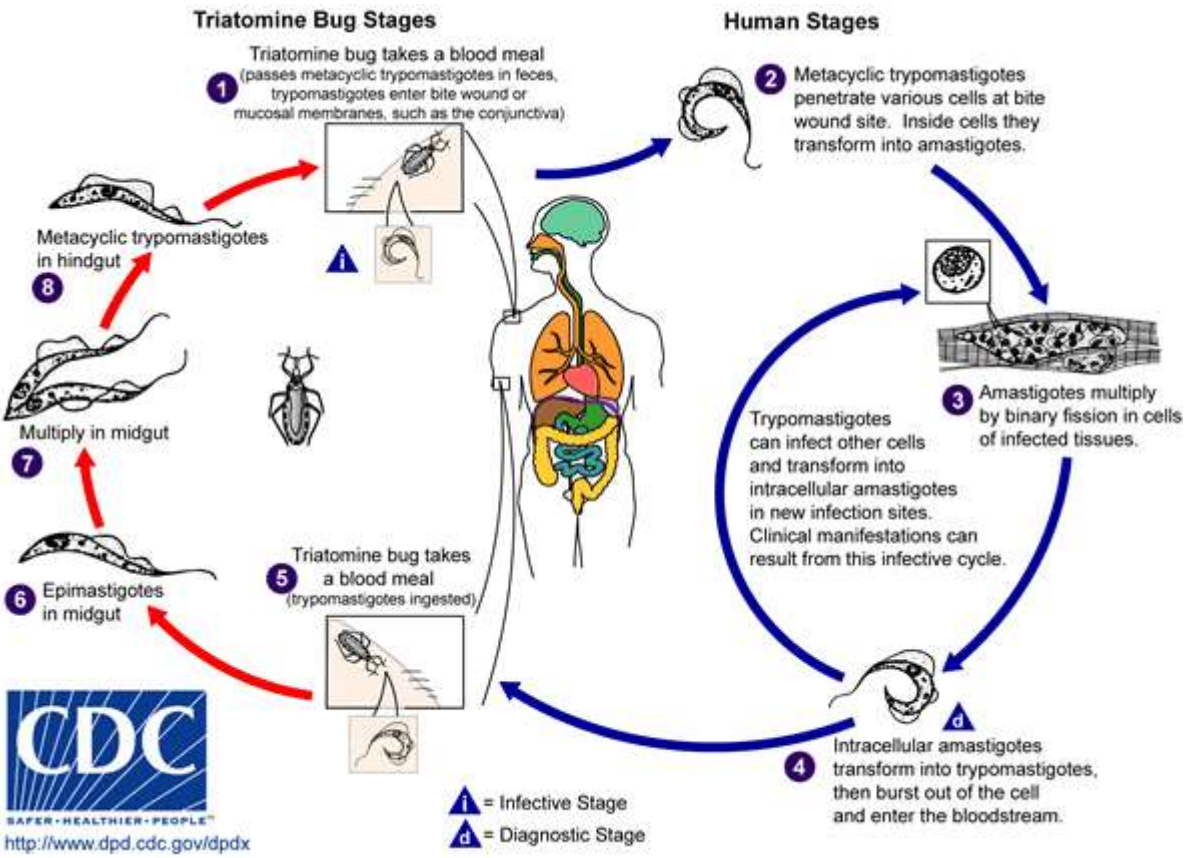
há ações eficazes no controle do vetor. Desta forma, o tratamento utilizado atualmente é baseado na quimioterapia com fármacos caros, tóxicos e ineficientes que não atendem a variedade de agentes etiológicos da doença (REBOLLO; OLIVERO-VERBEL; REYES, 2013).

3.6 *TRYPANOSOMA CRUZI*

Trypanosoma cruzi é o protozoário causador da doença de Chagas, uma infecção sistêmica crônica que atinge cerca de 18 milhões de pessoas na América Latina e 30% do infectados apresentam a forma sintomática da enfermidade. Os problemas cardíacos são os principais responsáveis pelo óbito dos pacientes chagásicos, por arritmias ventriculares ou disfunção ventricular grave (RASSI JÚNIOR et al., 2006). A infecção por *T. cruzi* pode ser transmitida por um vetor invertebrado, os triatomíneos, conhecidos no Brasil como barbeiros, a principal espécie é o *Triatoma infestans* (DE ARAÚJO-JORGE; DE CASTRO, 2000).

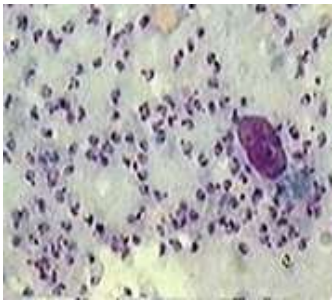
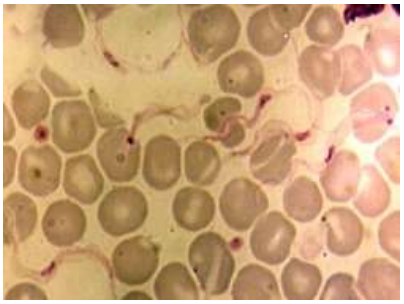
O *Trypanosoma cruzi* possui ciclo de vida complexo que requer passagem obrigatória por um hospedeiro invertebrado, o inseto vetor triatomíneo, e um hospedeiro vertebrado (Figura 5) (BONNEY; ENGMAN, 2010). Durante seu ciclo de vida, o parasito apresenta basicamente três formas evolutivas: 1- epimastigota, forma replicativa não infectante, presente no intestino superior do inseto; 2- tripomastigota, forma não replicativa infectante, presente na porção posterior do intestino do inseto; 3- amastigota, forma replicativa intracelular (Figura 6). As formas são classificadas e diferenciadas a partir da posição do cinetoplasto em relação ao núcleo, flagelo e morfologia (FERRAZ et al., 2007; ABE et al., 2002; ROMERO; MORILLA, 2010).

Figura 5: Ciclo Biológico do *Trypanosoma cruzi*



Fonte: CDC

Figura 6: A: Forma amastigota do *T. cruzi*.; B: Forma tripomastigota do T. cruzi; C: Forma epimastigota do T. cruzi.

A)  B)  C) 

Fonte: VANDESMET, 2014

O ciclo no inseto tem início quando durante o repasto sanguíneo no vertebrado infectado, o vetor ingere os tripomastigotas sanguíneos que, no intestino do vetor, serão transfomados na forma epimastigota (exclusiva do hospedeiro invertebrado) onde se prolifera e, novamente, transforma-se na forma tripomastigota metacíclica que será eliminada com as fezes durante o repasto sanguíneo, podendo penetrar no organismo do hospedeiro vertebrado por meio da picada ou mucosas (DUTRA et al., 2006).

As formas tripomastigotas metacíclicas são capazes de sobreviver e reproduzir-se em uma variedade de células nucleadas (SILVA et al., 2007), onde se diferenciam em formas amastigotas; ocorre proliferação intracelular por sucessivas divisões binárias e diferenciação em formas tripomastigotas que rompem a célula e caem na circulação sanguínea (LIMA et al., 2010). Após a ruptura celular, as formas tripomastigotas liberadas na corrente sanguínea podem infectar células vizinhas, se espalhar para outros órgãos ou serem ingeridas por outro inseto vetor reiniciando o ciclo (MUÑOZ-SARAIVA et al., 2012).

Após a invasão, a doença de Chagas pode ser classificada em fases aguda e crônica, de acordo com o tempo da infecção e características do soro do vertebrado infectado. Por cerca de 30 a 90 dias, observa-se a fase aguda, sendo uma característica marcante desta fase o grande número de parasitas presentes na circulação, enquanto a fase crônica é caracterizada pela parasitemia baixa e pelo elevado número de imunoglobulinas circulantes. As manifestações observadas nesta fase são decorrentes do efeito intracelular do parasitismo (FERRAZ et al., 2007).

A infecção aguda tende a ser uma doença febril autolimitante leve. Aproximadamente 30% dos indivíduos infectados desenvolvem doença de Chagas crônica, que na maioria dos casos afeta o coração (causando cardiomiopatia e disritmias) ou o intestino (causando mega-esôfago ou mega-cólon). A supressão imune de qualquer causa pode resultar na reativação da infecção latente, causando graves problemas cardíacos e sequelas neurológicas (TEIXEIRA et al., 2006).

Nifurtimox e benznidazol são os medicamentos disponíveis para o tratamento de pacientes infectados com *T. cruzi*. Entretanto, devido à sua conhecida toxicidade e efeito limitado para diferentes isolados de parasitas e fases da doença, a produção de novos fármacos é urgentemente necessária (DUSCHAK; COUTO, 2007; MCKERROW et al., 2009; URBINA, 2009; SOEIRO et al., 2009).

3.7 ÓLEOS ESSENCIAIS

Os óleos essenciais (OE) são misturas complexas de substâncias voláteis e lipofílicas usadas desde tempos antigos por suas propriedades farmacêuticas e como aromatizantes de cosméticos e perfumes. São conhecidos cerca de cerca de 3000 OE, dos quais 300 são comercialmente importantes por suas aplicações agronômicas, alimentícias, farmacêuticas, sanitárias, cosméticas e na perfumaria (BAKKALI et al., 2008; FIGUEIREDO et al., 2008).

Essas substâncias podem ser obtidas de diversos órgãos vegetais, tais como flores,

caules, folhas, galhos, frutas, sementes, raízes, madeira ou casca, e são armazenados em células secretórias, canais, cavidades, células epidérmicas ou tricomas glandulares (NIETO, 2017).

Os OE são produtos do metabolismo secundário vegetal e desempenham importantes papéis protetores, atuando como agentes alelopáticos, antivirais, antifúngicos, antibacterianos, inseticidas, sendo capazes de reduzir o apetite de animais herbívoros. Além disso, possuem função crucial para o processo de polinização, atraindo insetos para favorecer a dispersão de pólens e sementes, e repelindo aqueles indesejáveis. As mais bem estabelecidas atividades de óleos essenciais são antimicrobiana, sedativa, anti-inflamatória, bactericida, antiviral, fungicida e como conservante de alimentos (BAKKALI et al., 2008; NIETO, 2017).

Do ponto de vista químico, os OE são constituídos de misturas de 20-60 compostos orgânicos nas mais diversas concentrações, que podem pertencer a classe dos álcoois, éteres ou óxidos, aldeídos, cetonas, ésteres, aminas, amidas, fenóis, heterociclos e principalmente os terpenos. Além destes, também podem conter compostos não-terpênicos como os derivados de fenilpropanóides (DHIFI et al., 2016).

O fato destes óleos serem naturais e biodegradáveis, aliado a baixa toxicidade em mamíferos, podendo atuar sobre várias moléculas-alvo simultaneamente, torna essas substâncias peças-chave na pesquisa de novos medicamentos. Assim, os óleos podem ser explorados como alternativa ou complemento aos compostos sintéticos utilizados pela indústria farmacêutica, diminuindo ou sem induzir os efeitos secundários indesejáveis (FIGUEIREDO et al., 2008; DHIFI et al., 2016).

3.8 ÓLEOS ESSENCIAIS COM ATIVIDADE ANTIPARASITÁRIA

A ação antiparasitária de plantas medicinais, atualmente, pode ser considerada, ao revisar a literatura, como um dos campos de mais interesse aos pesquisadores, principalmente acerca de OE. Dito isto, se faz necessário citar alguns estudos que comprovam a atividade antiparasitária de OE, como Estevam e colaboradores (2018) que fez analise do OE dos frutos verdes de *Protium ovatum (Burseraceae)* que apresentou forte atividade tripanocida contra as formas tripomastigota do *Trypanosoma cruzi* (IC_{50} = 1,2 μg/mL), os principais compostos encontrados no OE dos frutos verdes de *P. ovatum* foram: β-mirceno (62,0 %), α-pineno (11,3 %) e limoneno. Carneiro e colaboradores (2017) onde o OE das flores de *Eugenia klotzschiana (Myrtaceae)* apresentou promissora atividade tripanocida contra formas tripomastigotas de *Trypanosoma cruzi* (20,2 μg/mL) e os principais compostos encontrados no OE das flores foram sesquiterpenoides: β-cariofileno (21,1%), biciclogermacreno (10,2%) e espatulenol (20,9%).

Em especial, o potencial Leishmanicida de OE tem sido descrito na literatura. Por exemplo, Rosa e colaboradores (2003) que relata que o óleo das folhas de *Croton cajucara*, apresentou IC_{50} de 0,008 µg/mL frente a promastigotas da espécie *L. amazonenses* e Dutra e colaboradores (2009) que estudou o OE e frações (6,25 – 100 µg/mL) obtidas das sementes de *P. emarginatus* que apresentou nas frações hexânica (IC_{50} = 50,06 µg/mL) e butanólica (IC_{50} = 46,65 µg/mL) atividade leishmanicida frente às formas promastigotas de *L. amazonensis.*

4 ARTIGO 1 - CHEMICAL COMPOSITION AND EVALUATION OF ANTIPARASITIC ACTIVITY OF THE ESSENTIAL OIL OF *EUGENIA STIPITATA* MCVAUGH (MYRTACEAE)

Chemical composition and evaluation of antiparasitic activity of the essential oil of *Eugenia stipitata* Mcvaugh (myrtaceae)

CÍCERO RAMON BEZERRA DOS SANTOS[a], MÁRCIA VANUSA DA SILVA[a], IRWIN ROSE DE ALENCAR MENEZES[c], ALEXANDRE GOMES DA SILVA[b], MARIANA GOMES VIDAL SAMPAIO[a], LILIAN CORTEZ SOMBRA VANDESMET[a], SAULO ALMEIDA DE MENEZES[d], BENEDITO YAGO MACHADO PORTELA[d], EDUARDO BRANDÃO[f], DOUGLAS WILLYAM RODRIGUES GOMES[d], FERNANDO GOMES FIGUEREDO[e], LEANDRO BEZERRA DE LIMA[g], MARIA CELESTE VEJA GOMEZ[h].

[a] Biochemistry Departament, Biocience Center, Federal University of Pernambuco – UFPE, Recife, Pernambuco, Brazil.
[b] Departament Antibiotics, Biocience Center, Federal University of Pernambuco - (UFPE) – Recife, Pernambuco, Brazil.
[c] Pharmacology and Medicinal Chemistry, Regional University of Cariri, Crato, Ceara, Brazil.
[d] Universitary Center Católica de Quixadá, Quixadá, Ceará, Brazil.
[e] Laboratories of Microbiology and Molecular Biology, Regional University of Cariri, Crato, Ceara, Brazil.
[f] Research Center Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, PE, Brasil
[g] Laboratory of Biotechnology and Ogenic Synthesis, Universidade Federal do Ceará, Fortaleza, Ceara, Brazil.
[h] Centre for the Development of Scientific Investigation - CEDIC, Fundación Moisés Bertoni /Laboratorios Diaz Gill, Asuncion, Paraguay.
***Corresponding Author: Cícero Ramon Bezerra dos Santos. Biochemistry Departament, Biocience Center. Federal University of Pernambuco – UFPE, Recife (PE), Brazil. Address: Av. Prof. Moraes Rego, 1235, Cidade Universitária, CEP: 50.670-901. Phone: +55 (81) 2126.8540**
E-mail: ramonsantosbezerra@hotmail.com

ABSTRACT

The essential oils (EOs) of plants used in traditional medicine are known as a rich source of chemically different compounds with different biological activities. In this study, we analyzed the chemical composition and investigated the trypanocidal, leishmanicide, effect and in vitro cytotoxicity of essential oil obtained from the leaves of *Eugenia stipitata* McVaugh. EO was extracted by the distillation method by steam stripping and its chemical composition analyzed by GC/MS and tested in seven concentrations (15.75, 31.25, 62.5, 125, 250, 500 e 1000 µg/mL) against the forms of *Trypanosoma cruzi* epimastigotes and promastigotes forms of *Leishmania braziliensis* and *Leishmania infantum*, as well as against fibroblasts. Generally, 48 components were identified in the oil chemical analysis and the most abundant constituents were: *β*-eudesmol (15.28%), *γ*-eudesmol (10.85%), Elemol (10.21%), caryophyllene oxide (6.5%), Clovene (6.18%) and Espatulenol (6.14%). In the evaluation of the parasitic activities, EO of *Eugenia stipitata* McVaugh at concentrations of 125 µg/mL and 62.5 µg/mL inhibited, respectively, 80.69% and 71.91% of the promastigote form of *L. braziliensis.* Similar inhibition was observed for the same concentrations of the oil against *L. infantum*, which inhibited 76.51% of promastigote form of *L. infantum* in concentration of 125 µg/mL and 51.1% in the concentration of 62.5 µg/mL. However, in the concentration of 125 µg/mL a smaller parasitic inhibition was observed of essential oil of *Eugenia stipitata* McVaugh bedore the epimastigotes forms of *T. cruzi*, with 22.85%. The essential oil of *Eugenia stipitata* McVaugh presented a low cytotoxicity in the concentration of 62.5 µg/mL and no cytotoxicity in concentrations of 31.5 µg/mL and 15.75 µg/mL. Therefore, the studied EO presented itself as a promising antiparasitic source against the ways of life of *L. braziliensis* and *L. infantum*.

Keywords: Essential Oil. Caatinga. Antiprotozoal. *Eugenia stipitata* McVaugh.

1. INTRODUCTION

Essential oils (EOs) are a complex mixture of compounds arising from plants secondary metabolism, which serve as a protective mechanism, acting as antimicrobial , antiviral and antifungal agents (Bakalli et al., 2008; Sharifi-Rad et al., 2017). Since the middle age, EOS has been widely used as a bactericide, virucides, fungicides and pesticides. Furthermore, their use has been shown to be safe, with few side effects and low toxicity to mammalian cells (Bakkali et al., 2008; Dubey et al., 2010; Santoro et al., 2007).

American trypanosomiasis, also known as Chagas disease (CD), is a neglected tropical disease which is Brazil in Latin America. CD was first described in 1909 and is caused by the parasite *Trypanosoma cruzi* (Moncayo; Silveira, 2017). According to the World Health Organization more than 65 million people live at risk of infection and of 6 to 7 million are infected (WHO, 2018a). The transmission occurs by contact with hematophagous triatomine insects of the genus *Triatoma*, *Panstrongylus* and *Rhodnius* infected by the protozoan parasite, by blood transfusion, via the placenta, by eating contaminated food with the flagellate parasite or, less frequently, ingestion of contaminated raw meat, organ transplants from infected donors and sexual contact (Coura, 2015).

There is no vaccine for CD and the treatment for this disease is based on two chemotherapeutic agents: benzonizadole and nifurtimox. These drugs have a low effectiveness during the chronic phase of the disease and high toxicity (Bermudez et al., 2016). Moreover, the absence of a liquid pharmaceutical form leads to a low adherence of the patient, especially for elderly people and children (Bernardes; Zani; Carvalho, 2013). Therefore, it is necessary to seek more effective and less toxic treatment to this disease (De Paula et al., 2015; Pinheiro et al., 2017). In an attempt to develop new drugs, many compounds of natural and synthetic sources, have been tested against *Trypanosoma cruzi* (Cerecetto; González, 2010; González;

Cerecetto, 2011).

The leishmaniasis is caused by parasites of the genus *Leishmania*, also being considered neglected tropical diseases (Arango, Descoteaux, 2014). Leishmaniasis is classified in cutaneous, subcutaneous and visceral forms, affecting 12 million people in 98 countries (Hamzavi et al., 2018; WHO, 2018b). The treatment occurs through the administration of antimonial mutipurpose drugs, liposomal amphotericin B and pentamidine, paromomycin, miltefosine, among others; all with high cost and a lot of side effects. Therefore, drugs originating from natural products have been studied (Carneiro et al., 2015; Dias; Dassoy, 2013).

Seasonally dry tropical forests (SDTF) present the circum-Amazonian distribution in South America and have been the main focus of many recent biogeographic and conservation studies (DryFlor et al., 2016). These forests are usually associated with fertile soils and climates marked by highly seasonal rainfall with the severe dry season from three to six months, when most of the vegetation is leafless (Mcleod*et al.*,2006; DryFlor et al., 2016). SDTFs are patchily distributed in South America in several "nuclei" of which the Caatinga biogeographical province (Morrone, 2014) from Northeastern Brazil is the largest one (Prado; Gibbs, 1993).

Chapada do Araripe, Ceará, Pernambuco and Piauí state, Brazil, harbors a humid forest enclave within the dry forest mosaic of the Caatinga dryland domain. The humid forest grows in an area with relatively abundant water resources provided by many streams and springs, in spite of the long, regional dry season (DNPM, 1996) constituting a type of oasis in the midst of a semiarid region that supports a wide floral and faunal diversity (FLONA, 2004; Silva et al., 2011). The humid forest acts as a refuge for many specific species to that region, including endemic taxa such*Antilophia bokermanni*(the Araripe Soleil Bleu,"soldadinho do Araripe") which is critically threatened with extinction (Auler et al., 2004; Linhares; Silva, 2015), and other taxa with disjunct distributions between the Atlantic and Amazon forests (*e.g.*,spermatophytes, ferns and angiosperms) (Loiolaet al., 2015; Collinset al., 2015) -

illustrating the biological importance of that area and the necessity of its protection (MMA, 2000).

The genus *Eugenia* is well represented in the various phytogeographic domains in Brazil and are constant targets of research, once they are in possession of essential oils and various secondary metabolites which have important biological actions (Romagnolo; Souza, 2006; Victoria et al., 2012). *Eugenia stipitata* McVaugh, popularly known as araçá-boi, is used in folk medicine in the treatment of intestinal and urinary disorders, in addition to relieving the symptoms of cold, which indicates that the bioactive compounds present in the plant may have potential benefits for human health (Fernández-Trujillo et al., 2011).

This study aimed to investigate the trypanocidal, the leishmanicide effect and *in vitro* cytotoxicity , as well as to identify the chemical components present in the essential oil obtained from the leaves of *E. stipitata*.

2 MATERIALS AND METHODS

2.1 Collection Area

The leaves of the species *E. stipitata* were collected in the area of native vegetation, located in Serra dos Paus - Chapada do Araripe, municipality of Exu, Pernambuco (7°21"S, 39°53"W and altitude 884m), in the month of May in the year 2017.

2.2 Botanical Material

The plant material was identified by PhD. Maria Arlene Pessoa da Silva of the Laboratory of Microbiology and Molecular Biology, Regional University of Cariri - URCA.

Specimens of the species were produced and deposited at the Herbarium Caririense Dárdano de Andrade Lima of the University aforementioned, under the number 13.054.

2.3 Extraction and chemical composition of the essential oil

To obtain the oil from the leaves of *E. stipitata* (EOESL), the plant material was ground into small pieces and subjected to the distillation method by stream stripping (4500g, 3h). The obtained EO was filtered, weighed and the oil yield was calculated in % (w/w). EO was then stored in a dark bottle and chilled (at + 5 °C) until use.

EO was analyzed through the Model of Gas chromatography-mass spectrometry (GC-MS) Shimadzu GCMS-QP2010 using capillary column Rtx®-5MS (30 m x 0.25 mm x 0.25 µm). The MS operation conditions were optimized in the following way: 70 eV, stream stripping gas (He) 13.6 $mL.min^{-1}$ and pressure of 53.5 KPa. The following temperature program was used: 100 °C (3 min) to 310 °C (3.5 °C/min) (Karmeling; Verluis; schauer, 1975).

2.4 Cell lines used

For the in-vitro tests with T. cruzi, CL-B5 clone was used (Buckner et al., 1996). The parasites stably transected with the gene for *β*-galactosidase of *Escherichia coli* (lacZ) were provided by PhD. F. Buckner through Commemorative Gorgas Institute (Panama). Epimastigotes forms were cultured at 28 °C in Liver Infusion Tryptose Broth (Difco, Detroit, MI) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Carlsbad, CA), penicillin (Ern, SA, Barcelona, Spain) and streptomycin (Reig Jofr SA, Barcelona, Spain) as described by Le Senne et al. (2002). The cells were collected for testing in the exponential growth phase. Cultures of *Leishmania* spp. were obtained from the Health Science Research Institute,

Asunción, Paraguay - IICS and identified by isoenzyme analysis. The lines maintenance, forms of cultivation and isolation of promatigotes forms of *Leishmania* spp. followed procedures described by Roldos et al. (2008). The inhibitory action of these promastigotes forms were performed using the *L. braziliensis* (MHOM / CO / 88 / UA301) and *L. infantum* lines (MHOM/ES/92/BCN83), cultured at 22 °C in Schneider's Drosophila supplemented with 20% FBS. In the cytotoxicity assays, the NCTC-929 murine fibroblast line, obtained from the National Collection of Type Cultures and cultivated in Minimal Essential Medium, was used. The culture medium was supplemented with heat inactivated FBS (10%), penicillin G (100 U/mL) and streptomycin (100 mg/mL). Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO^2. The lines viability was evaluated through the use of the resazurin colorimetric method (Rolón et al., 2006).

2.5 Reagents

Resazurin sodium was obtained from Sigma-Aldrich (St Louis, MO) and stored at 4 °C away from light. The Resazurin solution was prepared with 1% phosphate buffer, pH 7 and sterilized by filtration before use. Chlorophenol red-β-D galactopyranoside (CPRG; Roche, Indianapolis, IN) was dissolved in a 0.9% Triton X-100 (pH 7.4) solution. Penicillin G (Ern, S.A., Barcelona, Spain), streptomycin (Reig Jofré S.A., Barcelona,
Spain) and dimethyl sulfoxide (DMSO) were also used.

2.6 Trypanocidal activity

For the trypanocidal assays, 96-well microdilution plates (Sarstedt, Sarstedt, Inc.) were used with cultures that did not reach the stationary phase, as described by Vega et al. (2005).

The *T. cruzi* epimastigote forms were seeded at 1×10^5 per millilitre in 200µL and the plates were incubated with EOESL at the 15.75, 31.25, 62.5, 125, 250, 500 and 1000 µg/ mL concentrations at 28°C for 72h, with 50µL of the CPRG solution to give the final concentration of 200µM. The plates were incubated at 37°C for an additional 6h period and then read at 595nm in a spectrophotometer.

Benzonidazole was the reference drug tested in triplicates at 1, 10, 50 and 100µg/mL concentrations. Each experiment was performed twice separately. The antiepimastigote percentage (%AE) was calculated following the formula: %AE= [(AE - AEB)/(AC - ACB)] x 100, where AE=absorbance of the experimental group; AEB=blank compound; AC=absorbance of the control group; ACB=culture medium blank. The natural product solution to be analyzed was diluted in dimethyl sulfoxide, with the final concentration DMSO never exceeding 0.2% of the final solvent.

2.7 Leishmanicidal activity

The method used in this assay was developed by Mikus; Steverding (2000) and modified, where the promastigote cultures (2.5×10^5 parasites/well) were grown in microdilution plates. The EOESL was dissolved in DMSO at 15.75, 31.25, 62.5, 125, 250, 500, 1000 µg/mL concentrations. After an incubation period of 48h at 26°C, 20µl of resazurin solution was added and the oxidation was quantified in a spectrophotometer with 570–595nm wavelengths. The concentrations were tested in triplicates along with miltefosine which was used as a positive control. The antipromastigote percent (%AP) was calculated following the formula %AP = ((A570 X *117,216* - A_{595} X *80,586*) of test compounds / (A_{570} X *117,216* - A_{595} X *80,586*) of untreated posite growth control))X100. Thus testing the substances efficacy.

2.8. Cytotoxicity test

The clones were seeded (3×10^4) in 96-well flat-bottom microdilution plates with 100µL culture medium. RPMI 1640 per well. Cells were grown overnight at 37°C and 5% CO2 atmosphere. The medium was then replaced with the isolated tested substance and added at different concentrations in 200µL of the medium for 24h. Growth control wells were also included. After the incubation, 20µL of Resazurin solution was added to each well. The plates were incubated again for 3h; The Resazurin reduction was determined by wavelength absorbance measurement at 570 and 595 nm in a microplate reader.

Each concentration of EOESL (15.75, 31.25, 62.5, 125, 250, 500, 1000 µg/mL) was tested three times. The percent cytotoxicity (%C) of the natural products was determined as follows: %C=[(A570×117, 216−A595×80,586) test samples/(A570×117, 216 - A595×80,586) control]×100, where A570 and A595 represented the values 80,586 and 117,216 of optical media density at 570 and 595nm, respectively, recorded for wells with cells containing different natural product doses or the value recorded for wells with cells and without natural product(respectively positive growth controls)(Rolón et al., 2006). Then, the Selectivity Index (SI) was calculated as the 50 inhibitory concentration rate (IC50) of the cells / 50 inhibitory concentration (IC50) of parasites. The value resulting from this calculation reflects how many times the used EO is more selective to parasites than the host cell.

2.9 Statistical analysis

Statistical analysis was performed using ANOVA. The data were analyzed by GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA).

3 RESULTS

The fresh leaves of *E. stipitata* provided oil yield of 0.13%. Table 1 presents the chemical composition of the essential oil (EO) analyzed by GC/MS. In the EO of *Eugenia stipitata* leaves 46 compounds were identified, corresponding to 100% of the total oil, which is composed by 45.83% of sesquiterpene hydrocarbons, 28.26% of oxygenated sesquiterpenes, 15.22% of oxygenated monoterpenes, 4.35% of monoterpenes hydrocarbons and 4.35% other elements. The most abundant constituents of the analyzed EO were *β*-eudesmol (15.28%), *γ*-eudesmol (10.85%), Elemol (10.21%), caryophyllene oxide (6.5%), Clovene (6.18%) and Espatulenol (6.14%).

Table 1. Chemical composition of the essential oil from *Eugenia stipitata* McVaugh

Peak	**RT (min)**	**Compound**	**Area (%)**
1	4.440	*β*-Pinene	0.41
2	5.140	o-Cymene	0.12
3	5.315	Eucalyptol	2.61
4	6.451	Linalool	0.24
5	7.465	Pinocarveol	0.49
6	8.274	Terpinen-4-ol	1.51
7	8.558	*α*-Terpineol	2.62
8	8.717	Myrtenol	0.40
9	8.770	Myrtenal	0.26
10	11.147	(E)-Pinocarvyl acetate	0.24
11	12.076	*δ*-Elemene	0.53

12	12.960	Ylangene	0.19
13	13.077	α-Cubebene	0.95
14	13.330	*β*-Bourbonene	1.99
15	13.431	*β*-Elemene	1.09
16	14.028	*α*-Chamigrene	0.49
17	14.204	Caryophyllene	4.38
18	14.280	*β*-Cedrene	4.33
19	14.460	*β*-Cubebene	4.19
20	14.547	*α*-Amorphene	0.86
21	14.674	Aromadendrene	0.45
22	14.779	*γ*-Cadinene	4.03
23	15.026	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	0.76
24	15.211	Espatulenol	6.14
25	15.818	*β*-Selinene	0.51
26	15.870	Anthracene, 1,2,3,4,5,6,7,8-octahydro-2-methyl, (R)-	0.24
27	16.012	Ledene	0.79
28	16.074	*α*-Muurolene	0.92
29	16.189	*β*-Bisabolene	0.70
30	16.629	Cadina-1,3,5-triene	2.08
31	16.974	*α*-Cadinene	0.52
32	17.126	*α*-Calacorene	0.31
33	10.21	Elemol	10.21
34	17.392	Alloaromadendrene oxide-(1)	1.06

35	17.761	Viridiflorol	1.56
36	18.152	Caryophyllene oxide	6.50
37	18.589	Epiglobulol	0.81
38	18.812	Cubenol	0.48
39	18.969	*γ*-Eudesmol	10.85
40	19,118	8-Cedren-13-ol	1.20
41	19,389	tau-Cadinol	2.53
42	19,688	*β*-Eudesmol	15.28
43	19,730	Clovene	6.18
44	20,063	*β*-Caryophyllene oxide	0.15
45	20,421	Xenitorin A	0.29
46	25,458	1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo[5.3.1.0(4,11)]undec-2-en-8-one	0.18
	Total		100.00

RT: retention time (minutes); Area (%): percentage of compound on the sample

In the evaluation of the antiparasitic activities, OEESL at concentrations of 125 µg/mL and 62.5 µg/mL inhibited, respectively, 80.69% and 71.91% of the promastigote form of *L. braziliensis.* Similar inhibition was observed for the same concentrations of the oil against *L. infantum,* which inhibited 76.51% of promastigote form of *L. infantum* in concentration of 125 µg/mL and 51.1% in the concentration of 62.5 µg/mL. However, in the concentration of 125 µg/mL a smaller parasitic inhibition of EOESL was observed for the epimastigotes forms of T. cruzi, with 22.85%. EOESL presented a low cytotoxicity in the concentration of 62.5 µg/mL and no cytotoxicity in concentrations of 31.5 µg/mL and 15.75 µg/mL. At the dose of 125 µg/mL toxicity of 25.73% was observed, staying on the margin the maximum tolerated

cytotoxicity to mammalian cells (25%). The cytotoxicity against mammalian cells was compared to those of parasite by determining the SI: *L. braziliensis*, SI= 13.59; *L. infantum*, SI= 9.35; *T. cruzi*, SI= 0.50.

It is noteworthy that in relation to the parasitic activities that presented results less than or equal to 20% of inhibition, the results were not considered because they did not have important biological activities.

Table 2: Anti-Leishmani, trypanocidal activity and cytotoxicity of the essential oil of *Eugenia stipitata*.

Natural Product	Conc. (µg/mL)	% AP *L.braziliensis*	% AP *L.infantum*	% AE *T. cruzi*	%C
LC50 (µg/mL)		37.65	54.71	1019	511.5
EOESL	1000	91.04±0.64	84.80±0.45	41.46±0.28	*
	500	88.39±0.39	84.69±0.01	30.85±0.53	*
	250	87.61±0.51	83.82±0.42	27.97±0.81	*
	125	80.69±0.75	76.51±0.32	22.85±0.70	25.73±2.69
	62.5	71.91±0.14	73.91±0.15	-	5.09±0.34
	31.25	41.40±0.42	27.71±8.15	-	0.00±1.12
	15.75	22.17±0.99	0.00±0.14	-	0.00±0.95

EOESL– essential oil from *Eugenia stipitata* McVaugh; SI – selectivity index; Conc. - Concentration; µg/mL - micrograms per milliliters; % AP - Percent Antipromastigote; % AE- Percent Antiepimastigote; % C - Percent of Cytotoxicity in Fibroblasts; * - No determined and ± - Standard Deviation.

Table 3 presents the results for the positive control of benznidazole. At a concentration of 100 μg/mL, the drug inhibited 96.59% of forms of *T. cruzi* epimastigotes and caused the death of 1.43% of the fibroblasts.

Table 3: Positive control of benzonidazole against *Trypanosoma cruzi* (μg/mL)

Drug	Conc. (μg/mL)	%AE (*T. cruzi*)
Benzomidazole	100	96.59±2.13
	50	81.84±2.74
	10	50.61±5.83
	1	10.53±5.87

Conc. - Concentration; μg/mL - micrograms per milliliters; % AE - Percent Antiepimastigote and ± - Standard Deviation.

Table 4 presents the results for the positive control of Miltefosine, which behaved differently before the two species of *Leishmania*. For *L. infantum*, the concentration of 128 μg/mL had the largest percentage inhibition of parasites, however, caused the death of 2.42% of the fibroblasts. In relation to *L. brasiliensis*, the concentration of 64 μg/mL of miltefosine had the highest rate of inhibition, 83.69% of the promastigotes forms without registration of fibroblasts deaths.

Table 4: Positive control of miltefosine against *Leishmania braziliensis* and *Leishmania infantum* (μM).

Drug	Conc. (μg/mL)	% AP *L.braziliensis*	% AP *L.infantum*

Miltefosine	256	97.07±0.41	80.45±0.27
	128	98.75±0.28	81.64±0.19
	64	98.65±0.21	83.69±0.32
	36	95.43±0.79	83.22±0.22
	16	50.49±0.70	73.69±0.03
	8	1.57±0.80	46.45±0.14

μM- Micromols; % AP - Percent Antipromastigote; ± - Standard Deviation;Conc. - Concentration; μg/mL- Micrograms per microliter.

4 DISCUSSION

The analysis of chemical composition performed by GC/MS, of essential oil of *E. stipitata* leaves , collected in São Miguel Island, in Portugal, identified that the main components of the oil were monoterpenes and sesquiterpenes, as α-pinene (14.1%), β-caryophyllene (22.7%), caryophyllene oxide (15.4%) (Medeiros et al., 2003). In another study, the analysis of the essential oil from *E. stipitata* leaves of the city of Macas, in Ecuador, revealed the presence of 30 compounds, of which γ-Muurolene, E-Caryophyllene and δ-Cadinene were the majoritarian (Ronquillo; Galarza, 2016). The mentioned EOs chemical analysis showed distinct chemical elements or the same element in different concentrations of those observed in the present study, as shown in Table 1. According to Douglas et al. (2004), variations in the EO chemical composition of the same species from distinct regions can be attributed to differences in the climatic and geographical parameters, such as temperature, altitude, wind direction, rainfall, soil type, etc.

The chemical analysis of volatile compounds from the fruit of *E. stipitata* collected in the city of Manaus, was characterized by the presence of complex sesquiterpenes and the main

majoritarian elements were germacrene D (37.65%), *β*-pinene (12.2%) and *α*-pinene (10.4%) (Franco; Shibamoto, 2000). According to Garzón et al. (2012) this fruit has important antioxidant activity. This analysis showed different chemical composition of the oil composition of the leaves of this study.

The EO analysis of *Eugenia hiemalis* Cambess leaves collected in Blumenau, Santa Catarina, in four different seasons of the year, revealed that the elements espatulenol, *δ*-cadinene, Bicyclogermacrene and *β*-caryophyllene were among the four main compounds present in at least three of the tations analyzed (Zatelli et al., 2016). Similar results were observed in the EO of *Eugenia brejoensis* Mazine leaves collected in Catimbau, Pernambuco, whose majoritarian constituents were *δ*-cadinene, *β*-caryophyllene and bicyclogermacrene (Silva, 2015). Souza et al. (2017) verified the presence of *δ*-cadinene, trans-caryophyllene and *α*-Muurolol as majority elements of EO of leaves of the species *Eugenia brejoensis*.

The composition of the oil of the *Eugenia natalitia leaves*, collected in the Southern Africa, was dominated by oxygenated sesquiterpenes (54.4%), consisting mainly of selina-1,3,7 (11)-trien8-one (27.5%) and oxidoselina-1,3,7 (11)-trien-8-one (21.9%). The main constituents of a class of compounds of hydrocarbons monoterpenes (33.0%) were *α*-phellandrene (13.0%) and sabinene (9.7%) (Lawal et al., 2016). While the EO of *Eugenia platysema leaves* had in diterpnes the main class of identified compounds (66.05%), followed by the sesquiterpenes (32.95%). No monoterpenes were found in EO, different from other species of the genus Eugenia (Tenfen et al., 2016).

Sesquiterpenes predominate in OE composition of *Eugenia klotzschiana leaves*, collected in Goiás. The following main terpenic components were identified: *α*-copaeno (10.6%), *β*-Bisabolene (17.4%), *α*- (E) -bergamottin (29.9%) and germacrene D (13.3%) (Carneiro et al., 2017). The OE obtained from the leaves of *Eugenia uniflora*, collected in the vicinity of the plant leaves this research shows a high percentage of isoflurane-Germacrene

(65.80%), followed by germacra-3,7,9-trien-6-one (16.19%), *β*-elemenona (4.47%), *γ*-elemeno (3.97%), Germacrene B (2.19%) and (Z)-*β*-elemeno (0.39%) (Pereira et al., 2017). Sesquiterpenos also predominated in the composition of the essential oil of the leaves of Eugenia stipitata of this research.

There are many biological properties attributed to EO. Because of the chemical complexity, it is difficult to correlate these properties to a single substance, since that the same may be determined by the chemical synergism (Chaieb et al., 2007). The synergism of the main chemical EO constituents or the presence of other constituents which may also be active at even lower concentrations is an important factor that can lead to good performance of antiparasitic activity (Melo et al., 2011). The EO activity in this study is probably due to the presence of sesquiterpenes (77.08%), whose biological activities are already well known (Sauter et al., 2011). The *β*-caryophyllene (a sesquiterpenic hydrocarbon) and the nerolidol (a oxygenated sesquiterpene) are examples of terpenic substances with well characterized anti-Leishmania activity, possibly related to the inhibition of the biosynthesis of isoprenoid cells (Arruda et al., 2005; Santos et al., 2008). Two sesquiterpene lactones were isolated from the organic extract of *Ambrosia teunifolia leaves* , both presenting *in vitro* trypanocidal activity against epimastigote forms of *T. cruzi* and promastigotes forms of *Leishmania sp.* (Süsen et al., 2008).

Of the majoritarian components, *β*-eudesmol has important anticancer activity observed in different animal models (Kotawong et al., 2018) and antibacterial activity (Bankova; Trusheva, 2014); Elemol can be considered as one of the main EO antimicrobial components (Mevy et al., 2006). *β*-eudesmol (51.9%) and *γ*-eudesmol (18.9%) were the majoritarian EO elements of *Guatteria friesiana*, plant with excellent antiparasitic activity against *Plasmodium falciparum* and different forms of *T. cruzi* (Meira et al., 2016). The EO chemical composition of *Alpinia zerumbet* presents caryophyllene oxide (18.0%) and *β*-eudesmol (8.9%) and a good

antiparasitic activity (Mendiola et al., 2015). Caryophyllene oxide was also isolated in different species of *Lippia spp*, known by the relevant antiparasitic activity (Escobar et al., 2010).

Phytotherapy has become a promising alternative in the parasitic diseases treatment, especially when it is a question of leishmaniasis, since the existing chemotherapy is considered inadequate due to the high drugs toxicity, to the high costs and the growing resistance of pests to these drugs (González; Cerecetto, 2011; Tasdemir et al., 2006). It is likely that the multicomponent nature of EOs may reduce the occurrence of resistance to them, because multiple targets need to adapt to hinder their action (Yap et al., 2014).

This is the first report of antiparasitic activity of *Eugenia stipitata* and essential oils from the genus Eugenia are little explored regarding this activity.

The ethanolic extract of *Eugenia uniflora* L. leaves collected in Campo Grande, Mato Grosso do Sul, showed no leishmanicide activity on the promastigote forms of *L. amazonensis* (Ribeiro et al., 2014). Studies of Braga et al. (2007) with ethanolic extract of *Eugenia uniflora* L leaves, collected in Juiz de Fora, Minas Gerais, verified the inhibition on the growth of 48.33% of the promastigote forms of *L. amazonensis* and *L. chagasi* in the extract concentrations greater than 250 µg/mL for both parasites. According to Croft et al. (2006), different results may be due to the type of solvent used, moreover, it must be taken into account the genomic difference among the used strains.

The essential oil of the *Eugenia uniflora* L. leaves, collected in São Luís, in Maranhão, showed a significant reduction dependent on the concentration on the parasite viability *L. amazonensis*, with 100% of the promastigote growth inhibition at concentrations of 400, 200 and 100 g/mL (IC50= 1.75g/mL). In the determination of cytotoxicity, the oil was 20 times more toxic for amastigotes than for murine macrophages. The hemolytic activity was 63.22% in the highest tested concentration (400g/mL); however, showed no toxicity to $50g \cdot mL^{-1}$ (Rodrigues et al., 2013). The cytotoxic activity evaluation of natural products before the

mammalian cells is an essential target in search of active compounds with biological activity (Santos et al., 2008).

It has been demonstrated that the EO lipophilic components can affect layers of polysaccharides, fatty acids and phospholipids in plasma membranes of promastigotes forms of *Leishmania spp*., inducing cell lysis and the release of macromolecules (Di Pasqua et al., 2007). In the cytoplasm, these substances can break down specific metabolic pathways of lipids and proteins or stimulate the mitochondrial membrane depolarization, leading to cell necrosis or apoptosis (Armstrong, 2006; Tarikou et al., 2010).

Concerning Chagas disease, the current treatment is based on the use of nifurtimox and benznidazole, very toxic drugs to patients with various side effects (Marin-Neto et al., 2009; Urbina, 2002). Thus, the development of new safer and more effective therapeutic agents are necessary.

Borges et al. (2012) proposes that the EOs trypanocidal activity occurs mainly by their terpene composition, responsible for the hydrophobic character of essential oils, which allows their diffusion through the parasite cellular membrane, affecting the metabolic pathways and intracellular organelles.

The ethanolic extract of *Eugenia uniflora* L. leaves showed 80% inhibition of *T. cruzi* epimastigotes forms at a concentration of 100 µg/mL. Low cytotoxicity of this extract against J774 macrophages was observed, 8% in the concentration of 100 µg/mL, and no toxicity in the concentration of 10 µg/mL. Nifurtimox was used as positive control and, in the highest tested concentration, 10 µg/mL inhibited 89.1% of epimastigotes forms (Santos et al., 2012a).

The trypanocidal activity of ethanolic extract of *Eugenia jambolana* L. leaves, harvested in the city of Crato, Ceará, showed activity against the CL-B5 strain of *T. cruzi*, with 100% of inhibition at a concentration of 100 µg/mL. However, it showed cytotoxicity of 37% in the same concentration (Santos et al., 2012b).

Of the six species of Caatinga plants from different families analyzed by Souza et al. (2017), EO of *Eugenia brejoensis* leaves had the best dose-dependent inhibitory effect on the epimastigotes , trypomastigote and amastigote forms of T. *cruzi* .

According to IS, EO was more selective for the promastigotes forms of *L. braziliensis* (IS=13.59) and *L.infantum* (IS=9.35), and not selective for forms of *T. cruzi* (IS=0.50). The higher the selectivity index the more efficient and less toxic the EO used (Azevedo, 2013).

5. Conclusion

The study revealed that the EO from *Eugenia stipitata* leaves showed no relevant antiparasitic effect on the epimastigotes forms of *T. cruzi*. However, presented antiparasitic effect against the ways of life of *L. braziliensis* and *L. infantum*. Probably, the majoritarian compounds of the analyzed EO are responsible for the observed effect, however additional tests are required to elucidate their action mechanisms.

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5 CONCLUSÃO GERAL

Os resultados obtidos nesse estudo permitem concluir que:

- O OE de *Eugenia stipitata* tem atividade Leishmanicida;
- O OE não apresentou efeito antiparasitário relevante frente as formas epimastigotas de *T. cruzi*;
- O OE da pesquisa apresentou moderada citotoxicidade frente a fibroblastos;
- Foi o primeiro relato de atividade antiparasitária do OE extraído das folhas de *Eugenia stipitata* e a primeira caracterização química do óleo dessa espécie encontrada no Brasil;
- Os constituintes químicos mais abundantes do OE das folhas de *Eugenia stipitata* extraídas do bioma Caatinga foram: β-eudesmol (15.28%), γ-eudesmol (10.85%), Elemol (10.21%), caryophyllene oxide (6.5%), Clovene (6.18%) e Espatulenol (6.14%).

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ANEXO A – Normas da Revista

FOOD AND CHEMICAL TOXICOLOGY

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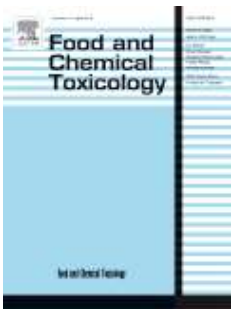

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