



REDE NORDESTE DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

JOICY CORTEZ DE SÁ

**Estudo fitoquímico e avaliação da capacidade leishmanicida e cicatrizante
cutânea das espécies *Arrabidaea chica* Verlot. (pariri) e
Vernonia brasiliiana Less. (assa-peixe)**

São Luís - MA

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia, com sede na Universidade Federal Rural de Pernambuco e ponto focal na Universidade Federal do Maranhão, como requisito para obtenção do Título de Doutor em Biotecnologia.

Área de Concentração: Biotecnologia em Agropecuária

Orientadora: Profa. Dra. Ana Lúcia Abreu Silva

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“Tudo posso naquele que me fortalece”

(Filipenses 4:13)

“Jamais considere seus estudos como uma obrigação, mas como uma oportunidade invejável para aprender a conhecer a influência libertadora da beleza do reino do espírito, para seu próprio prazer pessoal e para proveito da comunidade à qual seu futuro trabalho pertencer”.

(Albert Einstein)

*“Dedico esta pesquisa ao meu amado filho Davi.
Um amor incondicional, puro e sincero.
Seu sorriso alegra meus dias e conforta minhas aflições.”*

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LISTA DE ABREVIATURAS E SÍMBOLOS

% - por cento

°C – graus Celsius

µm – micrômetro

µL – microlitro

µM - micromolar

µg/mL – micrograma por mililitro

a.C. – antes de Cristo

ACR2 - antimoniato redutase

AlCl₃ - cloreto de alumínio

ANOVA – Análise de Variância

ANVISA - Agência Nacional de Vigilância Sanitária

ATP – adenosina trifosfato

BOD - Biochemical oxygen demand

CC₅₀ – citotoxicidade celular de 50%

CDs- células dendríticas

CEUA - Comitê de Ética em Uso Animal

CFMV - Conselho Federal de Medicina Veterinária

CG-EM - cromatografia gasosa acoplada à espectrometria de massa

CGEN - Conselho de Gestão do Patrimônio Genético

CIM - concentração inibitória mínima

Cis - cisteína

cm- centímetros

CO₂ – Dióxido de carbono

CRMVs - Conselhos Regionais de Medicina Veterinária

Cys-Gly - cisteinil-glicina

d.C. – depois de Cristo

DMSO - Dimetilsulfóxido

DNA – ácido desoxirribonucléico

EC - extratos clorofórmicos

EDTA - ácido etilenodiamino tetra-acético

EEBs – extratos etanólicos brutos

EM – extratos metanólicos

EUA - Estados Unidos da América

FeCl₃ - cloreto de ferro

FGF2 - fator de crescimento de fibroblastos 2

FIOCRUZ – Fundação Oswaldo Cruz

G - giros

°GL – Gay Lussac

GP63 – glicoproteína 63

GSH - glutathione

GTP – guanosina trifosfato

h- horas

HCl – ácido clorídrico

HE- Hematoxilina-Eosina

HIV- Vírus da Imunodeficiência Humana

H₂O₂ – peróxido de hidrogênio

IBAMA - Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

IC₅₀ – concentração inibitória de 50%

IFN- γ - interferon- γ

IGF-I Fator de crescimento semelhante à insulina tipo 1

IL- interleucina

IM- intramuscular

iNOS – óxido nítrico sintase induzível

IS- Índice de seletividade

k-DNA - DNA circular do cinetoplasto

LC – leishmaniose cutânea

LCD - leishmaniose cutânea difusa

LC-ESI-MS/MS- cromatografia líquida acoplada à espectrometria de massa em tandem com ionização eletrospray

LMC - leishmaniose mucocutânea

LT- leishmaniose tegumentar

LTA- leishmaniose tegumentar americana

LV - leishmaniose visceral

MAPA - Ministério da Agricultura, Pecuária e Abastecimento

MF- medicamento fitoterápico

mg/kg – miligramas por quilograma

mg/mL – miligramas por mililitro

mM - milimolar

m-RNA – ácido ribonucleico mensageiro

MTT- (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)

NaOH – hidróxido de sódio

NK - células natural killer

Nm- nanômetro

NO – óxido nítrico

pb – pares de base

OE – óleo essencial

PBS - Tampão Fosfato-Salino

PCR – reação em cadeia da polimerase

pH – potencial hidrogeniônico

PTF - produto tradicional fitoterápico

RDC – Resolução da Diretoria Colegiada

RENISUS - Relação Nacional de Plantas Medicinais de Interesse ao SUS

RPMI- Meio Roswell Park Memorial Institute

RMN - Ressonância magnética nuclear

Sb (III) - antimoniais trivalentes

Sb (V) - antimoniais pentavalentes

SISBIO - Sistema de Autorização e Informação em Biodiversidade

SUS - Sistema Único de Saúde

TCD4+ - linfócito T auxiliar

TDR1 - tiol redutase

TGO - transaminase glutâmico oxalacética

TGP - transaminase glutâmico pirúvica

TGF- β fator de crescimento transformante beta

Th1- célula T auxiliar tipo 1

Th2 - célula T auxiliar tipo 2

Th17 - célula T auxiliar tipo 17

TNF- α – fator de necrose tumoral - α

[T(SH)₂] - tripanotona

US\$ - dólares

VEGFA – fator de crescimento vascular endotelial A

WHO - World Health Organization

RESUMO

As leishmanioses englobam um complexo de doenças que afetam o homem e outros mamíferos, cujo espectro varia desde a forma cutânea até a forma visceral grave. O tratamento preconizado são os antimoniais pentavalentes que devido aos seus efeitos colaterais e tóxicos, aumentam o nível de preocupação quanto ao seu uso. A busca por tratamentos alternativos, eficazes e com menor toxicidade em relação as drogas convencionais, tem impulsionado o desenvolvimento de pesquisas utilizando produtos naturais. Desta forma, este trabalho objetivou avaliar os constituintes químicos, além do potencial leishmanicida, citotóxico e cicatrizante cutâneo das espécies vegetais *Arrabidaea chica* e *Vernonia brasiliiana* sob o parasita *Leishmania amazonensis*. Para tal, procedeu-se a elaboração do extrato etanólico bruto (EEB) e frações clorofórmica, metanólica e acetato de etila de *A. chica* e *V. brasiliiana*, com subsequente análise fitoquímica e cromatografia gasosa acoplada a espectrometria de massa. Adicionalmente, também foi realizado a extração do óleo essencial de *V. brasiliiana*. Após a análise fitoquímica, procedeu-se as avaliações: citotóxica *in vitro*, com determinação do CC₅₀, via ensaio colorimétrico MTT; triagem leishmanicida *in vitro* e determinação da capacidade inibitória de 50% das formas promastigotas e amastigotas de *L. amazonensis* após 24, 48 e 72h sob efeito dos EEBs, frações e óleo das plantas em estudo; e cicatricial da lesão cirúrgica em camundongos Swiss Webster submetidos a tratamento tópico com 10 mg/g do EEB de *A. chica* durante 21 dias, com posterior análise histológica. As análises de prospecção fitoquímica demonstraram que as plantas em estudo possuem flavonoides, taninos, antocianinas, estéres, triterpenos e chalconas. O potencial leishmanicida do EEB e frações de *A. chica* apresentaram resultados de inibição do parasito em concentrações de 60 a 155,9 µg/ml conforme o tipo de fração. O EEB, óleo e frações das folhas da *V. brasiliiana* possuem atividade leishmanicida *in vitro* mais acentuada que o pariri, com um IC₅₀ 15 vezes inferior ao da *A. chica*, sendo verificado através da microscopia eletrônica danos expressivos nas membranas celulares, citoplasma e bolsa flagelar do parasita após tratamento com *V. brasiliiana*. O teste de citotoxicidade, após 24 h de incubação com os EEBs, demonstrou que as duas espécies vegetais em estudo não são citotóxicas em concentrações de ação leishmanicida, apresentando um índice de seletividade maior para o parasita do que para a célula do hospedeiro. A preparação tópica com o EEB de *A. chica* apresentou na fase inicial, angiogênese, collagenização e reepitelização mais acentuada que o grupo controle, contudo que não persistiu ao longo dos 21 dias de observação. Esses resultados demonstram que os compostos extraídos das espécies vegetais *A. chica* e *V. brasiliiana* são potenciais agentes leishmanicidas, que impulsionam estudos posteriores no intuito de melhor fundamentar a ação sob o parasita, com isolamento das substâncias bioativas para elaboração de novos fármacos.

Palavras-chaves: *Arrabidaea chica*; *Vernonia brasiliiana*; Fitoquímico; Leishmanicida; Citotoxicidade; Cicatrizante.

ABSTRACT

Leishmaniasis comprises a complex of diseases that affect humans and other mammals, ranging from a mild cutaneous type to a severe visceral form. The standard therapy using pentavalent antimonials is concerning due to its side effects. The search for alternative, effective and less toxic treatments has driven to the development of studies using natural products. Thus, this study aimed to evaluate the potential cytotoxic, leishmanicidal and healing effects of *Arrabidaea chica* and *Vernonia brasiliiana*. For this were prepared the crude ethanolic extracts and chloroform, methanol and ethyl acetate fractions of *A. chica* and *V. brasiliiana*, followed by phytochemical analysis and gas chromatography and mass spectrometry. Additionally, it was also extracted the essential oil of *V. brasiliiana*. After phytochemical analysis, the following evaluations were made: *in vitro* cytotoxic assay, with the determination of cytotoxic concentration via MTT colorimetric assay; *in vitro* antileishmanial screening and determination of the inhibitory capacity of 50% of *L. amazonensis* promastigotes and amastigotes forms after 24, 48 and 72 h under the effect of crude ethanolic extracts, oil and fractions of *A. chica* and *V. brasiliiana*; and wound healing activity in surgical lesions of Swiss Webster mice treated topically with 10 mg/g of the crude ethanolic extract of *A. chica* for 21 days, with further histological analysis. Phytochemical screening showed that the plants under study have flavonoids, tannins, anthocyanins, esters, triterpenes and chalcones. The leishmanicidal potential of the crude extract and fractions of *A. chica* showed satisfactory results in concentrations from 60 to 155.9 µg/ml, depending on the type of fraction. The ethanol extract, oil and fractions of the leaves of *V. brasiliiana* have a markedly *in vitro* leishmanicidal activity than *A. chica*, with an IC₅₀ 15 times lower. In electron microscopy significant damage was observed in cell membranes, cytoplasm and flagellar pocket of the parasite after treatment with *V. brasiliiana*. The cytotoxicity assay showed that both plants are not cytotoxic in leishmanicidal concentrations, with a selective ratio higher to the parasite than to the host cell. Topical preparation with the crude extract of *A. chica* presented in the initial phase, angiogenesis, collagen deposition and epithelialization more pronounced than the control group, but that did not persist throughout the 21 days of observation. These results demonstrate that compounds extracted from *A. chica* and *V. brasiliiana* are potential leishmanicidal agents, which stimulates further studies in order to improve its action on the parasite, identifying bioactive substances for the development of new drugs.

Keywords: *Arrabidaea chica*; *Vernonia brasiliiana*; Cytotoxicity; Healing potencial; Leishmanicidal; Phytochemical screening.

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1 CONSIDERAÇÕES INICIAIS

O trabalho está estruturado da seguinte forma:

Parte I - Compreende a explanação da temática: Introdução; Revisão de Literatura; Justificativa; Hipótese e Objetivos.

Parte II - Composta de dois capítulos referentes aos artigos subtraídos dos objetivos propostos neste estudo:

- Capítulo I - Potencial leishmanicida, citotóxico e cicatricial da *Arrabidaea chica* Verlot.
- Capítulo II - *Vernonia brasiliiana* leaves harbour components with potential leishmanicidal action.

Parte III - Considerações finais, Conclusões, Referências e Anexos.

Os periódicos utilizados para submissão desta pesquisa foram: *Journal of Ethnopharmacology* e *BMC Complementary and Alternative Medicine*, cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) é B1, com fator de impacto 2,939 e 1,88, respectivamente.

Parte I

Explicação da temática

2 INTRODUÇÃO

A flora brasileira detém um vasto patrimônio genético da qual pode ser extraído muitas plantas com potencial terapêutico para várias doenças. No Brasil, a farmacopeia popular é muito diversificada, acredita-se que isto é resultado da miscigenação cultural entre indígenas, africanos e europeus (MELO et al., 2007; ETHUR et al., 2011). Apesar dos avanços da ciência e da medicina, nos últimos anos houve um aumento expressivo do uso indiscriminado de produtos naturais pela população, ou seja, sem nenhuma recomendação médica e, muitas vezes, sem qualquer comprovação científica do uso da planta e sua toxicidade.

Diante deste cenário, em 2008 a Portaria Interministerial nº 2.960 instituiu o Programa Nacional de Plantas Medicinais e Fitoterápicos e criou o Comitê Nacional de Plantas Medicinais e Fitoterápicos, que visam a ampliação das opções terapêuticas e melhoria da atenção à saúde aos usuários do Sistema Único de Saúde – SUS; uso sustentável da biodiversidade brasileira; valorização do conhecimento tradicional das comunidades e povos tradicionais, dentre outras ações. Desta forma o SUS elencou 71 espécies vegetais com potencial terapêutico, no intuito de orientar as investigações científicas. Dentre essas plantas, ressalta-se *Arrabidaea chica* e as espécies do gênero *Vernonia* (BRASIL, 2009).

Arrabidaea chica, conhecida popularmente no Maranhão como pariri, é uma planta distribuída em quase todo o Brasil e mais frequentemente encontrada na Floresta Amazônica (VON POSER et al., 2000). É utilizada pela população para fins terapêuticos no combate as enfermidades de pele, cólicas intestinais, anemia, icterícia e processos inflamatórios (GENTRY, 1992; RÊGO, 1995; KALIL FILHO, et al., 2000). O uso popular direcionou o desenvolvimento de várias pesquisas que buscaram investigar a ação medicinal como anti-inflamatório, adstringente, antioxidante, antiulcerogênico, antimicrobiano, antifúngico, cicatrizante, diurético, antianêmico e anti-hipertensivo (BRAGA et al., 2005; ALVES et al., 2008; BARBOSA et al., 2008; JORGE et al., 2008; TAFFARELLO, 2008; CARTÁGENES, 2009; AMARAL et al., 2012).

Vernonia brasiliiana é uma planta da família Asteraceae, cuja família possui 109 espécies com propriedades medicinais, sendo 12 delas de uso etnoveterinário

(TOYANG e VERPOORTE, 2013). *V. brasiliensis* é nativa do Brasil onde é conhecida popularmente como assa-peixe, amplamente distribuída em quase todo território nacional, com exceção da região Sul (KISSMANN, 2000; RODRIGUES, 2006).

No gênero *Vernonia* são encontradas plantas ricas em compostos bioativos e óleos essenciais com ações anti-inflamatória, antimicrobiana, antifúngica e inseticida comprovadas (GARG e SIDDIQUI, 1992; FORMISANO et al., 2006; COSTA et al., 2009; LIU et al., 2009; MAIA et al., 2010). O potencial antiprotózoário também já foi avaliado em estudos *in vitro* e *in vivo* realizados por Carvalho et al. (1991) e De Almeida Alves et al. (1997) que demonstraram uma atividade antiplasmodica, sendo justificada a ação pela presença das lactonas sesquiterpênicas.

De acordo Taufner et al. (2006) a maioria da população utiliza plantas medicinais para o tratamento de doenças como uma alternativa aos medicamentos alopáticos. Dentre as doenças em que são utilizadas plantas no tratamento, citam-se as leishmanioses, cujo número de trabalhos científicos de identificação e caracterização biológica de extratos brutos, frações e óleos de plantas medicinais, em busca de agentes leishmanicidas de baixa toxicidade e alta eficácia é um desafio que tem envolvido diversos grupos de pesquisa ao redor do mundo (WENIGER et al., 2004; ROCHA et al., 2005; COSTA et al., 2006; BRAGA et al., 2007; ESTEVEZ et al., 2007; FERREIRA et al., 2010; SANTOS et al., 2012; COLARES et al., 2013; RIBEIRO et al., 2014; ROTTINI et al., 2015).

A leishmaniose é uma das endemias parasitárias de maior importância em termos de saúde pública no mundo, devido a sua ampla distribuição. É causada por diversas espécies de protozoários do gênero *Leishmania* e transmitida ao homem e animais por meio da picada de insetos flebotomíneos (BRASIL, 2011). Trata-se de uma doença espectral, que de acordo com a espécie de *Leishmania* e resposta imunológica do indivíduo pode ser classificada em quatro formas clínicas distintas: cutânea, cutânea difusa, mucosa e visceral (MCGWIRE e SATOSKAR, 2014).

A forma cutânea é a mais comum mundialmente, com cerca de 1 milhão de casos/ ano e representa 50 a 75% dos novos casos registrados (WHO, 2015). No Brasil, as principais espécies de *Leishmania*, pertencentes aos subgêneros

Leishmania ou Viannia, causadoras da leishmaniose tegumentar americana (LTA) humana são: *L. braziliensis*, *L. amazonensis*, *L. guyanensis*, *L. naiffi*, *L. lansoni*, *L. shawi* e *L. lindenbergi* (GONTIJO e CARVALHO, 2003; SILVEIRA et al., 2004; JENNINGS et al., 2014).

As regiões Norte e Nordeste do Brasil são as que apresentam a maior incidência de casos de LTA. No período de 2001 a 2010, na região Nordeste foram registrados 78.400 casos, destacando-se os estados do Maranhão (30.515 casos ou 38,92%), Ceará (14.684 casos ou 18,72%) e Bahia (25.125 casos ou 32,04%) (NEGRÃO e FERREIRA, 2014).

As drogas de eleição para o tratamento das leishmanioses são os antimoniais pentavalentes (Sb^V), seguidos da anfotericina B e as pentamidinas, que apesar da ação leishmanicida podem apresentar efeitos adversos como artralgias, mialgias, cardiotoxicidade, hepatotoxicidade, nefrotoxicidade, anorexia, náusea e vômito (HEPBURN et al., 1993; BRUMMITT et al., 1996). Além desses efeitos, o outro inconveniente é que o tratamento preconizado é injetável, causando aversão ao paciente e requerendo pessoal técnico, dificultando assim a sua aplicação, em especial nas comunidades rurais (MIOT et al., 2005).

Um ponto importante é que as drogas supracitadas são preconizadas para o tratamento em humanos e a Portaria Interministerial nº 1.426/2008 proíbe, em todo o território nacional, o tratamento da leishmaniose em cães infectados ou doentes, com produtos de uso humano ou produtos não-registrados no Ministério da Agricultura, Pecuária e Abastecimento. Portanto, é premente a obtenção de novas drogas que sejam eficazes, de baixa toxicidade, fácil acesso e utilização pela população, fundamentando a viabilidade do tratamento em animais, uma vez que o conhecimento sobre plantas medicinais simboliza muitas vezes o único recurso terapêutico de muitas comunidades rurais e grupos étnicos (MACIEL et al., 2002).

Desta forma, sendo a leishmaniose uma doença de grande relevância para a saúde pública é que esta pesquisa foi proposta, no intuito de avaliar as propriedades fitoquímicas da *Arrabidaea chica* e *Vernonia brasiliana* no tratamento da leishmaniose tegumentar, por meio da elaboração de uma preparação tópica que atue como leishmanicida e cicatrizante cutâneo.

3 REVISÃO DE LITERATURA

3.1 Plantas Medicinais

3.1.1 Histórico

Desde o princípio, as primeiras civilizações, Egito, Grécia, Índia e China (KLAYMAN, 1985; LI e WU, 2003) perceberam que algumas plantas continham princípios ativos, que ao serem experimentados no combate às doenças revelavam empiricamente um poder curativo. Estas então foram denominadas de plantas medicinais que durante muito tempo foram os principais recursos terapêuticos utilizados para tratar a saúde das pessoas (BADKE et al., 2011).

Existem evidências históricas e arqueológicas de que as propriedades curativas das plantas já eram conhecidas desde o período Neolítico (aproximadamente 10.000 anos atrás). Acredita-se que os ancestrais, por meio da observação da natureza tenham constatado o valor terapêutico das plantas (BHATTARAM et al., 2002).

Na China, há relatos do uso medicinal da planta *Artemisia annua*, para tratamento de pessoas com malária, durante a Dinastia de Mawangdui Han, que reinou entre 206 a.C. a 220 d.C. (KLAYMAN, 1985; LI e WU, 2003).

Importantes descobertas também foram realizadas pelos egípcios, que aprenderam a preparar diuréticos, vermífugos, purgantes e antissépticos de modo natural utilizando plantas. Os povos do Egito descobriram desde muito cedo as propriedades – do ópio (*Papaver somniferum*), utilizado como sedativo e calmante; do óleo de rícino (*Ricinus communis*), da alcaravia (*Carum carvi*) e da hortelã pimenta (*Mentha piperita*) como digestivo; e da cila (*Drimys urticaria*) como estimulante cardíaco, entre outras. Ressalta-se também a Índia, que teve um papel muito importante na história do uso de plantas medicinais por meio da elaboração de um tratado médico, intitulado “Caraka”, que descrevia a utilização de mais de 500 plantas, por volta do século I a.C. (BHATTARAM et al., 2002).

No Brasil, também é notável a importância histórica do uso de plantas medicinais. Desde a época da colônia, quando Pero Vaz de Caminha escreveu a “Carta do Achamento”, destinada ao Rei de Portugal, Dom Manuel, já se falava das

riquezas nacionais. Nesta, ele retrata que “Não podia fazer qualquer afirmação sobre a existência de ouro, prata, nem coisa alguma de metal ou ferro, mas as águas são muito infindas, graciosas; querendo-a aproveitar, dar-se-á nela tudo, por bem das águas que têm”. Isso retrata o grande potencial em biodiversidade do Brasil. Fato este, propiciado pelo potencial hídrico, pelas condições climáticas e edáficas, sendo possível a adaptação e desenvolvimento de inúmeras espécies de vegetais (ALVES et al., 2008; ZUANAZZI e MAYORGA, 2010).

Nos séculos de colonização, a utilização de plantas para tratamento das patologias era patrimônio somente dos índios (ELDIN e DUNFORD, 2001). Configurando assim, a evolução do uso das plantas juntamente com os hábitos culturais. A população em geral utilizava medicamentos importados, em especial da Europa. Além disso, não existia um conhecimento em relação ao correto armazenamento das plantas, a fim de preservar suas propriedades medicinais, ou seja, seus princípios ativos (MARTINS et al., 2000). Nesse contexto histórico, muito tempo se passou, para que as plantas medicinais do território brasileiro, que já estavam sendo utilizadas há bastante tempo pelos estrangeiros, no tratamento de diversas patologias, fossem conhecidas mundialmente (BRUNING et al., 2012).

Em meados de 1930 e 1940, a descoberta de princípios ativos, passíveis de serem isolados, possibilitou uso no tratamento de doenças específicas. Estes princípios ativos, configurados por todo um processo evolutivo, que no decorrer de milhões de anos, as plantas foram desenvolvendo suas próprias defesas químicas, biossintetizando compostos que agem em alvos moleculares específicos, sendo estes, necessários para sobrevivência das espécies vegetais, no intuito de se defender do ataque de herbívoros e patógenos em geral (FERREIRA e PINTO, 2010).

Nos países em desenvolvimento, dentre eles o Brasil, bem como nos países desenvolvidos, a partir da segunda metade das décadas de 70 e 80, ocorreu um crescimento das “medicinas alternativas”, em especial na fitoterapia, demonstrado este campo ser um estudo promissor (LUZ, 1997). Contudo, apesar do aumento da importância, que está diretamente relacionado a busca por melhor qualidade de vida da população, fazendo uso de métodos mais naturais e saudáveis para manutenção da saúde, associado ao alto custo de medicamentos industrializados, bem como a

dificuldade de acesso a assistência médica e farmacêutica. Muitos estudos ainda são necessários para a comprovação científica da eficácia e segurança da utilização de determinadas plantas, configurando ainda, um uso indiscriminado com base no conhecimento popular (SIMÕES, 1988; MATSUDA, 2002; BRUNING et al., 2012).

3.1.2 Utilização

Planta medicinal é toda aquela que administrada ao homem ou animal, por qualquer via ou forma, exerça alguma ação terapêutica (ALMASSY JÚNIOR et al., 2005). Os medicamentos tradicionais abrangem uma variedade de práticas de saúde, abordagens, conhecimentos e crenças (ABBASI et al., 2015). As plantas são utilizadas como recursos para novos compostos medicinais ao longo da história da humanidade e, ainda hoje, servem como base de muitos medicamentos empregados na rotina médica (CRAGG et al., 2009).

O homem busca na natureza uma forma de ajudar o organismo a normalizar funções fisiológicas prejudicadas, restaurar a imunidade, promover a desintoxicação e o rejuvenescimento (FRANÇA et al., 2008).

O uso de plantas medicinais, tornou-se bastante comum no Brasil como uma alternativa aos serviços de saúde tradicionais (FORMAGIO et al., 2013), seja em áreas rurais desprovidas de recursos de saúde pública, ou em áreas urbanas, como opção ou complemento à medicação alopática. Essa tradição tem sido repassada às populações a cada geração, e se configura como uma nova ciência, a Fitoterapia (LIMA e DIMESNTEIN, 2006). O Ministério da Saúde brasileiro instituiu a fitoterapia como prática oficial da medicina e definiu como prioridade a sua investigação (SILVA et. al., 2006). A farmacologia das plantas e o desenvolvimento de suas características químicas tem sido alvo de pesquisas no Brasil (YUNES et al., 2001).

O Brasil é detentor da maior diversidade de flora no mundo (MYERS et al., 2000), o que evidencia o seu grande potencial para exploração racional dos recursos vegetais e para difusão das práticas fitoterápicas. Tais práticas são capazes de gerar benefícios tanto no âmbito cultural, de valorização dos costumes populares, como contribuições para validação científica do uso das espécies vegetais (KÜLKAMP et al., 2007; MENEZES et al., 2012), uma vez que várias destas são consumidas sem que suas propriedades farmacológicas sejam, de fato, conhecidas

(SANTOS e TORRES, 2012). Existe a necessidade de desmistificar a falsa ideia de que as plantas não podem causar nenhum mal à saúde, e cabe às pesquisas e aos profissionais de saúde esclarecer para a comunidade de usuários que muitas plantas com uso terapêutico apresentam algum tipo de toxicidade ou contraindicação de uso (TOMAZZONI et al., 2006).

No Estado do Maranhão, o consumo de plantas com finalidades terapêuticas é bastante elevado e difundido, sendo facilmente encontradas em mercados, feiras livres, viveiros e nos quintais das residências. Todas as partes das plantas são utilizadas como tratamento em diversas afecções (Tabela 1), sob várias formas de apresentação, tais como chás, formulações tópicas, infusões etc (MADALENO, 2011).

Tabela 1 – Plantas e suas indicações terapêuticas utilizadas na medicina tradicional no Estado do Maranhão

Nome popular	Nome científico	Referência	Usos na medicina popular
Abacateiro	<i>Persea americana</i>	Monteles e Pinheiro (2007)	Afecções dos rins (folha)
Açoita cavalo	<i>Luehea divaricata</i> <i>Luehea grandiflora</i>	Monteles e Pinheiro (2007); Madaleno (2011).	Afecções nos rins e próstata
Alecrim	<i>Rosmarinus officinalis</i>	Madaleno (2011).	Analgésico
Alfavaca	<i>Ocimum basilicum</i> <i>Ocimum gratissimum</i>	Madaleno (2011).	Casos de gripe, diurético
Algodão	<i>Gossypium arboreum</i>	Madaleno (2011).	Anti-inflamatório
Amburana	<i>Amburana cearensis</i>	Madaleno (2011).	Analgésico, sinusite
Ameixa	<i>Syzygium cumini</i>	Madaleno (2011).	Diabetes, úlcera, gastrite
Anador	<i>Plectranthus barbatus</i>	Madaleno (2011).	Analgésico
Andiroba	<i>Carapa guianensis</i>	Madaleno (2011)	Queda de cabelo
Aroeira	<i>Myracrodruon urundeuva</i>	Madaleno (2011).	Anti-inflamatório
Arruda	<i>Ruta graveolens</i>	Madaleno (2011).	Analgésico
Babosa	<i>Aloe vera</i>	Monteles e Pinheiro (2007); Madaleno (2011).	Queimaduras, afecções de pele, laxativo, gastrite, cicatrizante
Batata de Purga	<i>Ipomoea dumosa</i>	Monteles e Pinheiro (2007).	Derrame, abortive
Boldo	<i>Plectranthus barbatus</i>	Monteles e Pinheiro (2007).	Má digestão, problemas hepáticos.

Cabacinha	<i>Luffa operculata</i>	Monteles e Pinheiro (2007); Madaleno (2011).	Abortiva, sinusite, dor de estômago.
Caju	<i>Anacardium occidentale</i>	Monteles e Pinheiro (2007); Madaleno (2011); Nascimento e Conceição (2011).	Analgésico
Camomila	<i>Matricaria chamomila</i>	Madaleno (2011).	Calmante
Camapu	<i>Physalis angulata</i>	Monteles e Pinheiro (2007); Madaleno (2011).	Hepático, regulação do ácido úrico em casos de gota.
Carambola	<i>Averrhoa carambola</i>	Monteles e Pinheiro (2007).	Doenças do trato genito-urinário
Carqueja	<i>Baccharis crispa</i>	Madaleno (2011).	Obesidade
Catuaba	<i>Anemopaegma arvense</i>	Madaleno (2011).	Afrodisíaco
Chanana	<i>Turnera guianensis</i>	Monteles e Pinheiro (2007); Madaleno (2011); Nascimento e Conceição (2011).	Hepática, anti-inflamatória
Cidreira	<i>Melissa officinalis</i>	Madaleno (2011); Nascimento e Conceição (2011).	Calmante
Copaíba	<i>Copaifera langsdorffii</i> <i>Copaifera reticulata</i>	Monteles e Pinheiro (2007); Nascimento e Conceição (2011).	Anti-inflamatório, afecções da garganta.
Cordão de São Francisco	<i>Leonotis nepetifolia</i>	Madaleno (2011).	Afecções locomotoras, cálculo renal.
Erva doce	<i>Pimpinella anisum</i>	Madaleno (2011).	Auxilia na digestão
Espinheira santa	<i>Maytenus ilicifolia</i>	Madaleno (2011).	Gastrite e câncer
Fedegoso	<i>Senna uniflora</i>	Monteles e Pinheiro (2007).	Afecções circulatórias
Gengibre	<i>Zingiber officinale</i>	Monteles e Pinheiro (2007); Madaleno (2011).	Afecções da garganta
Girassol	<i>Helianthus annuus</i>	Madaleno (2011).	Casos de derrame
Janaúba	<i>Himatanthus sucubus</i>	Monteles e Pinheiro (2007).	Afecções do trato gastrointestinal
Jatobá	<i>Hymenaea courbaril</i>	Monteles e Pinheiro (2007); Madaleno (2011).	Afecções urinárias, anemias e câncer.
Jerimum	<i>Cucurbita pepo</i>	Madaleno (2011).	Casos de congestão cerebral
Jurubeba	<i>Solanum lycocarpum</i>	Monteles e Pinheiro (2007).	Afecções do trato gastrointestinal
Limão	<i>Citrus medica</i>	Monteles e Pinheiro (2007).	Afecções respiratórias

Língua de Vaca	<i>Elephantopus mollis</i>	Monteles e Pinheiro (2007).	Afecções genito-urinárias
Mamoeiro	<i>Carica papaya</i>	Madaleno (2011).	Contra diarreias e má digestão
Mandacaru	<i>Opuntia ficus-indica</i>	Monteles e Pinheiro (2007).	Afecções respiratórias
Manjerição	<i>Ocimum minimum</i>	Madaleno (2011).	Afecções gastrointestinais
Manjerona	<i>Origanum majorana</i>	Monteles e Pinheiro (2007).	Afecções gastrointestinais
Mastruz	<i>Chenopodium ambrosioides</i>	Monteles e Pinheiro (2007); Madaleno (2011); Nascimento e Conceição (2011).	Anti-inflamatório
Melão de São Caetano	<i>Momordica charantia</i>	Monteles e Pinheiro (2007).	Afecções de pele
Mororó	<i>Bauhinia forficata</i>	Madaleno (2011).	Diabetes
Mutamba	<i>Guazuma ulmifolia</i>	Monteles e Pinheiro (2007).	Abortivo
Noni	<i>Morinda citrifolia</i>	Madaleno (2011).	Artrite, anti-inflamatório, gastrite, preventivo de câncer.
Pariri	<i>Arrabidaea chica</i>	Madaleno (2011).	Afecções urinárias, gastrointestinais, do aparelho reprodutor feminino, anemia, diabetes.
Picão	<i>Bidens pilosa</i>	Nascimento e Conceição (2011).	Afecções do fígado
Piqui	<i>Caryocar brasiliense</i>	Monteles e Pinheiro (2007); Madaleno (2011); Nascimento e Conceição (2011).	Afecções respiratórias, queda de cabelo, dores reumáticas.
Quebra pedra	<i>Phyllanthus niruri</i>	Monteles e Pinheiro (2007); Madaleno (2011); Nascimento e Conceição (2011).	Cálculo renal
Quiabo	<i>Abelmoschus esculentus</i>	Monteles e Pinheiro (2007).	Afecções locomotoras
Quina	<i>Cinchona officinalis</i> <i>Quassia amara</i>	Monteles e Pinheiro (2007); Madaleno (2011).	Afecções do sistema reprodutor feminino, afecções infecciosas
Romã	<i>Punica granatum</i>	Madaleno (2011); Nascimento e Conceição (2011).	Afecções da garganta
Sucupira	<i>Bowdichia nitida</i>	Madaleno (2011).	Afecções da garganta, anti-inflamatório.
Tomate	<i>Physalis philadelphica</i>	Madaleno (2011).	Anemia
Trapiá	<i>Crataeva tapia</i>	Monteles e Pinheiro (2007).	Afecções locomotoras
Unha de gato	<i>Uncaria tomentosa</i>	Madaleno (2011).	Analgésico

Urucum	<i>Bixa orellana</i>	Monteles e Pinheiro (2007); Nascimento e Conceição (2011).	Afecções respiratórias
Vassourinha	<i>Scoparia dulcis</i>	Nascimento e Conceição (2011).	Afecções do aparelho auditivo
Vique	<i>Mentha spicata</i>	Monteles e Pinheiro (2007); Madaleno (2011).	Analgésico, antigripal.

Fonte: Adaptado de MADALENO (2011) e CAVALCANTI e ALBUQUERQUE (2013).

A comunidade científica revela um interesse cada vez maior sobre este campo, ao reconhecer o verdadeiro benefício para a saúde que as plantas proporcionam (MIGUEL e MIGUEL, 2004). A própria Organização Mundial da Saúde reconhece que a solução para o combate a inúmeras doenças, especialmente as chamadas “doenças negligenciadas”, reside no conhecimento tradicional e na elaboração de novos fármacos derivados dos produtos da biodiversidade (WHO, 2002).

3.2 *Arrabidaea chica* Verlot.

A espécie *A. chica* (Humb. & Bonpl.) B. Verlot., está classificada taxonomicamente, segundo Cronquist (1981), em:

- ✓ Divisão: Magnoliophyta
- ✓ Classe: Magnoliopsida
- ✓ Subclasse: Asteridae
- ✓ Ordem: Scrophulariales
- ✓ Família: Bignoniaceae
- ✓ Gênero: *Arrabidaea*

A família Bignoniaceae compreende 120 gêneros, com aproximadamente 800 espécies, que estão distribuídas nas regiões tropicais da América Central e do Sul, bem como na África (VON POSER et al., 2000).

A. chica é uma planta nativa em quase todo o Brasil, sendo muito comum na Floresta Amazônica. Possui vários nomes populares, dentre eles, pariri, crajiru, carajuru, cipó cruz, coá-guajuru, guajuru-piranga, oajuru e pyranga (BORRÁS, 2003). Caracteriza-se por ser uma planta arbustiva, em média com 2,5 cm de altura,

de ramos cilíndricos e globosos na fase inicial e depois tetrágonos, lenticelado-verrucosos e estriados. Suas folhas são pecioladas, compostas por dois ou três folíolos. Os folíolos podem ser oblongos, oblongos-lanceolados ou oval-lanceolados, contudo raramente ovalados e quase sempre curto-agudo-acuminados, obtuso na base, glabros nas duas páginas, coriáceos, discolores ou concolores. Pode apresentar folhas modificadas em gavinhas, o que facilita a fixação ao substrato. As flores são campanuladas, róseas ou violáceas, aveludadas, dispostas em panícula terminal piramidal, frouxa com até 22 cm de comprimento. O fruto é capsular, linear, alongado, afilado nas extremidades, com nervura mediana, de cor castanho-ferrugem e sementes ovoides (LORENZI e MATOS, 2002; COSTA et al., 2001).

Na medicina tradicional, as espécies do gênero *Arrabidaea* são utilizadas em quadros de cólicas intestinais, enterocolites, dermatopatias (psoríase, dermatites, piodermite, úlceras), anemia, conjuntivite, icterícia, prevenção de cáries dentárias, hepatopatias, transtornos reprodutivos, assepsia em feridas e como cosmético. De modo geral, ela apresenta um amplo espectro de ação em especial como adstringente e anti-inflamatória. Vale ressaltar também, sua utilização em casos de câncer de boca, de útero e leucemia (ALBUQUERQUE, 1989; GENTRY, 1992; RÊGO, 1995; KALIL FILHO et al., 2000).

Na Amazônia, *A. chica* é largamente utilizada como anti-inflamatório, cicatrizante e antianêmico; preparada na forma de chá, proveniente das folhas, para administração por via oral ou lavagens vaginais (BORRÁS, 2003). Além disso, faz-se uso na forma de tinturas sob as feridas na pele ou como tatuagens pelos índios, ou também, na forma de pasta como repelente (CORRÊA, 1926; ALBUQUERQUE, 1989). No Maranhão, há relatos dessa espécie no tratamento de cálculos renais e hipertensão arterial (RÊGO, 1995).

Entretanto, apesar da ampla utilização popular, muitos estudos ainda são necessários para avaliar o potencial medicinal dessa planta. Dentre os trabalhos já desenvolvidos, relata-se as avaliações de atividade anti-inflamatória, mediante pesquisas *in vitro* e *in vivo*, testando o extrato de *A. chica* em casos de edema induzido por formalina em pata de camundongo, onde apresentou significativa redução do edema na dose de 70 mg/kg (ANDRADE et al., 1997); casos de pleurisia e ativação de linfócitos em camundongos, onde houve redução na produção de

óxido nítrico e diminuição no número de neutrófilos pleurais na dose de 200 mg/kg (SAMPAIO et al., 1998) e modelos experimentais de alergia, com inibição do acúmulo de leucócitos totais, neutrófilos e eosinófilos (MELLO et al., 2002). Demonstrado assim, uma potencial atividade anti-inflamatória e imunomoduladora da *A. chica*.

Adicionalmente, a ação anti-inflamatória e cicatrizante foi avaliada em pacientes com queimadura cutânea de primeiro e segundo grau, demonstrando resultados satisfatórios (LIMA et al., 1995). Entretanto, Jorge et al. (2008), ao avaliar os efeitos cicatrizante, anti-inflamatório, antiulcerogênico e antioxidante do extrato bruto metanólico de *A. chica*, constatou que não houve redução do edema de pata induzido por carragenina, nem o edema de orelha induzido por óleo de crôton em ratos, todavia de modo positivo observou a indução de proliferação de fibroblastos *in vitro*, estímulo da síntese de colágeno *in vivo* e *in vitro*, atividade antiulcerogênica e moderada capacidade antioxidante.

Aro et al. (2013) investigaram o efeito tópico cicatrizante do extrato de *A. chica* em secção de tendão de ratos Wistar e verificaram uma melhor organização do colágeno nos grupos tratados em relação ao grupo controle, além de um aumento na quantidade de sulfato de dermatan no 14º dia de tratamento.

O potencial antianêmico da *A. chica* também já foi avaliado por alguns pesquisadores, porém este efeito ainda é questionado. Oliveira et al. (1996), em estudos com ratos Wistar, durante 60 dias de tratamento via oral com extrato de *A. chica* verificaram um aumento nos níveis de hematócrito, hemoglobina, hematimetria e leucometria, porém após a interrupção do protocolo terapêutico esses valores reduziram. Além disso, Arakian et al. (1998) relatam que o ferro existente nas folhas da planta não é biodisponível.

Cartágenes (2009) avaliou o extrato hidroalcóolico das folhas de *A. chica*, fazendo estudos de composição química, toxicidade e atividade no sistema cardiovascular de ratos normotensos e hipertensos; onde verificou um alto percentual de flavononóis, flavonononas e compostos esteroidais e, em menor quantidade, compostos taninos flobafênicos. Além disso, constatou que a administração prolongada do extrato induziu um efeito hipotensor, com diminuição

da pressão arterial sistólica, provavelmente pela inibição do influxo de cálcio intracelular, associado a ausência de efeitos toxicológicos.

Estudos fitoquímicos em extratos hidroalcólico e aquoso de *A. chica* demonstraram a presença de flavonoides, taninos e saponinas, com ausência de antraquinonas e alcaloides (OLIVEIRA et al., 1996; COSTA et al., 2001). Vale ressaltar, que o primeiro estudo químico com as folhas de *A. chica* foi realizado por Chapman et al. (1927), que relatam o isolamento da substância química 3-deoxiantocianidina, mais especificamente a 1,6,7-dihidroxi-5,4'-dimetoxi-flavylium (carajurin) e a 6,7,4'-trihidroxi-5',-metoxi-flavylium (carajurone), responsáveis pela cor avermelhada do extrato. Estudos posteriores, isolaram outros compostos, como a flavona acacetin, ácido triterpeno oleanólico e mais duas deoxiantocianidinas (6,7,3'-4'-tetrahydroxy-5-methoxy-flavylium / 6,7,3'-trihidroxi-5,4'-dimetoxi-flavylium). (TAKEMURA et al., 1995; ZORN et al., 2001; DEVIA et al., 2002).

Alves et al. (2008) em testes farmacológicos determinaram a prospecção química na tintura de *A. chica*, verificando a presença de: açúcares redutores, alcaloides, antocianidinas, antocianinas, antraquinonas, esteroides/triterpenoides, fenóis, flavanonois, flavanois, flavanonas, saponinas e taninos catéquicos.

Williams e Grayer (2004) descrevem que mais de 50 novas antocianinas foram isoladas das flores, frutos, sementes e folhas das plantas. Elas são substâncias químicas pertencentes ao grupo dos flavonoides glicosilados, formados por antocianidinas e açúcares (MAZZA e MINIATI, 1993), sendo fundamentais para planta em vários aspectos, como no mecanismo de polinização de flores, dispersão de sementes por insetos e defesa contra insetos predadores (KONG et al., 2003).

Estudos farmacológicos com antocianinas remetem sobre o alto poder antioxidante em doenças metabólicas (ZAFRA-STONE et al., 2007). Nessa condição, trabalhos foram realizados, como por exemplo os de Amaral et al. (2012) que avaliaram o potencial antioxidante, citotóxico, antimicrobiano e diurético do extrato etanólico e frações de *A. chica*, os quais comprovaram a ação antioxidante e diurética, além da ausência de resposta antimicrobiana e citotóxica. Siraichi et al. (2013) avaliaram a composição fenólica e a atividade antioxidante das folhas de *A. chica*, verificando por cromatografia líquida acoplada à espectrometria de massa em

tandem com ionização eletrospray (LC-ESI-MS / MS) dois importantes flavonoides, scutellarein e apigenina, sendo estes os principais responsáveis pela expressiva ação antioxidante.

Em relação ao potencial antifúngico, antiprotzoário e antimicrobiano dessa planta, vale ressaltar os trabalhos realizados por Barbosa et al. (2008), que averiguaram a ação do extrato etanólico e frações sob formas de leveduras, fungos dermatófitos e *Trypanosoma cruzi*, onde constataram a inibição do crescimento do *Trichophyton mentagrophytes* e um significativo efeito tripanocida. Ferreira et al. (2013) avaliaram o potencial antimicrobiano *in vitro* do extrato etanólico de *A. chica* sob as cepas de *Escherichia coli*, *Salmonella*, *Shigella* e *Lactobacillus* e, verificaram uma concentração inibitória mínima (CIM) variando de 15,62 a 62,5 mg/ml conforme o tipo de cepa.

Rodrigues et al. (2014) avaliaram o efeito leishmanicida de cinco frações obtidas a partir do extrato bruto em hexano de *A. chica*, sob as formas promastigotas de *L. amazonensis* e *L. infantum*, determinando a concentração inibitória mínima (CIM), que foi de 37,2 e 18,6 µg/ml, respectivamente. Esse resultado, foi referente a uma das frações avaliadas, que possuía como componentes químicos principais: ácido linoléico metil éster (25,38%), ácido n-hexadecanóico (19,61%), ácido octadecanóico (14,10%) e gamma-sitosterol (12,85%). Eles também avaliaram alterações ultraestruturais do parasito sob efeito dessa fração, verificando inchaço mitocondrial, ruptura de membrana mitocondrial, presença de vesículas no citoplasma, na mitocôndria e na bolsa flagelar e alterações no complexo de Golgi.

3.3 *Vernonia brasiliiana* Less.

O gênero *Vernonia*, está inserido na tribo Vernonieae e família Asteraceae, também denominada Compositae. Essa família é constituída de 3 subfamílias, 17 tribos (BREMER, 1994) e possui aproximadamente 25.000 espécies elencadas em 1.600 gêneros, correspondendo a maior família de angiospermas conhecida. (HATTORI e NAKAJIMA, 2008). Esse gênero é um dos maiores e mais importante dentro dessa família, agrupando 1.500 espécies distribuídas nas regiões tropicais e sub-tropicais, principalmente na África e América do Sul (MISEREZ et al., 1996).

A família Asteraceae vem sendo muito estudada, sob o ponto de vista da morfologia, anatomia, ontogenia, citogenética, ecologia, fitoquímica e estrutura molecular (HOLMES, 1996). A maioria dos estudos visam contribuir para o aumento no conhecimento sobre a classificação dentro da família. Todavia, ainda se tem muitas discussões em torno da análise filogenética da tribo Vernonieae, onde diversos pesquisadores estudaram as relações sub-tribais e características morfológicas, no intuito de melhor delimitar taxonomicamente os gêneros e montar um adequado cladograma (JONES, 1979; KEELEY e TURNER, 1990; ROBINSON, 1999; NAKAJIMA e SEMIR, 2001). Sendo assim, na América do Sul, para Jones (1979) o termo utilizado é *Vernonia*, em contrapartida a sinonímia proposta por Robinson (1999) é *Vernonanthura*.

No Brasil, a família Asteraceae apresenta boa dispersão, com a existência aproximada de 1.900 espécies com 196 gêneros (HATTORI e NAKAJIMA, 2008), sendo que 900 espécies compreendem ao gênero *Vernonia*. Dentro desse gênero, ressalta-se a espécie *V. brasiliiana*, conhecida por diferentes nomes populares, como assa-peixe, assa-peixe preto, assa-peixe roxo, pau de múquem, pau de muqueca e manjerição de cavalo (XAVIER et al., 2003).

O gênero *Vernonia* abrange ervas, arbustos e árvores. Apresenta capítulos agrupados de várias formas, escorpioides, trisóides, em panículos, corimbos, solitários. Aquênios angulosos ou costados, glabros ou seríceos. O papus é quase sempre duplo, onde a fileira externa possui cerdas curtas e a fileira interna cerdas plumosas, barbeladas ou escabrosas. As flores podem ser brancas, rosas, violetas, vermelhas, azuis ou roxas (HATTORI e NAKAJIMA, 2008).

Esse gênero é conhecido por ter várias espécies de uso: alimentar, *Vernonia amygdalina* e *Vernonia colorata* (BURKILL, 1985; IWU, 1993); medicinal, *V. amygdalina*, *Vernonia condensata*, *Vernonia cineria*, *Vernonia guineensis* e *Vernonia conferta*; e industrial, *Vernonia galamensis*, para extração de óleo (TOYANG e VERPOORTE, 2013).

Na medicina popular brasileira, as espécies de *Vernonia* possuem ampla utilização, em especial as folhas e raiz, com relatos de uso em quadros de gripe, resfriados, tosse, bronquite, contusões, hemorroidas, reumatismo, cólicas hepáticas,

hemorragia e infecções uterina (ANDRADE et al., 2000; FILIZOLA, 2003; CORRÊA et al., 2004). Em países da África, muitas espécies de *Vernonia* também são utilizadas medicinalmente no tratamento de infecções, amebíase, malária e doenças sexualmente transmissíveis; além de relatos no combate ao estresse, estimulante e afrodisíaco (IWU, 1993; KAMBIZI e AFOLAYAN, 2001; TONA et al., 2004).

Algumas dessas utilizações já foram comprovadas cientificamente, como por exemplo os estudos realizados por Toyang et al. (2012a) com a espécie *V. guineenses*, que demonstrou ação anti-helmíntica contra o *Trichuris muris* e antimicrobiana em bactérias Gram-negativas (*Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa* e *Salmonella typhimurium*) e Gram-positivas (*Staphylococcus aureus* e *Staphylococcus epidermidis*). Toyang et al. (2012b) constataram *in vitro*, com a mesma espécie, atividade antiangiogênica e inibição de crescimento celular em várias linhagens, como por exemplo, A375, A549 e MCF-7, provenientes de melanoma, câncer pulmonar e câncer de mama em humanos, respectivamente. Os autores atribuíram essa atividade antitumoral a presença de duas lactonas sesquiterpênicas, vernopicrin e vernomelitsin, identificadas por espectroscopia e ressonância magnética nuclear (RMN).

Ressalta-se a *V. amygdalina* como uma das espécies mais estudadas dentro do gênero com estudos leishmanicida. Tadesse et al. (1993) ao avaliarem os extratos clorofórmicos (EC) e metanólicos (EM) sob formas promastigotas e amastigotas de *Leishmania aethiopica* encontraram IC₅₀ de 18,5 µg/ml e 13,3 µg/ml (EC); IC₅₀ de 74,4 µg/ml e 45,8 µg/ml (EM), respectivamente.

Posteriormente, Alawa et al. (2012), também avaliaram o potencial leishmanicida de *V. amygdalina*, *in vitro* e *in vivo* com *Leishmania major*, obtendo redução da viabilidade do parasito, relatando que a planta remete estudos promissores no tratamento da leishmaniose.

Atividades antioxidante e anti-inflamatória também foram investigadas no gênero *Vernonia*, como exemplo o trabalho de Hira et al. (2013) que avaliaram essas propriedades utilizando o extrato etanólico da folha de *Vernonia patula*; verificando uma redução significativa no edema induzido por carragenina e histamina, apresentando o extrato portanto, uma atividade anti-inflamatória aguda, bem como

antioxidante em função de alguns componentes da planta como flavonoides, taninos e compostos fenólicos. Os polifenóis são os principais responsáveis pela atividade antioxidante, em virtude das suas propriedades redutoras, que é diretamente proporcional ao número e posição de hidroxilas na molécula (MELO et al., 2008).

Em se tratando da espécie *V. brasiliiana*, relatam-se os trabalhos desenvolvidos por Carvalho et al. (1991) que avaliaram o potencial antimalárico do extrato bruto dessa espécie utilizando diferentes solventes, em testes *in vivo* com *Plasmodium berghei* e *Plasmodium falciparum*, constatando assim, uma redução entre 40-50% da capacidade de multiplicação do parasito em ambos os casos, mesmo sob doses relativamente baixas. Posteriormente, Arias et al. (1995) corroboraram os resultados acima, com atividade do extrato bruto das folhas contra *P. berghei* e *Trypanosoma cruzi*.

De Almeida Alves et al (1997) relatam também o isolamento de triterpenos com atividade antiplasmódica em estudos *in vitro* e *in vivo*, com redução de 45% da parasitemia do *P. berghei* na concentração de 1.000 mg/kg *in vivo* e, redução de 45% *in vitro* para o *P. falciparum* na concentração de 25 µg/ml.

Filizola (2003) ao estudar o extrato metanólico das folhas da *V. brasiliiana* identificou duas moléculas polifenólicas com atividade antimicrobiana em cepas de *Micrococcus flavus*, *Staphylococcus aureus*, *Candida albicans* e *Candida krusei*; outras duas moléculas polifenólicas com atividade antifúngica. Adicionalmente, identificou terpenóides e açúcares redutores e, constatou ausência de cumarinas, fenil-propanoglicosídeos, taninos, saponósidos e alcaloides.

Óleos essenciais obtidos das folhas de *V. remotiflora* e *V. brasiliiana* também apresentaram atividade antimicrobiana de largo espectro, inibindo o crescimento de bactérias Gram-negativas (*Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Salmonella choleraesuis*, *Klebsiella pneumoniae*) e Gram-positivas (*Staphylococcus aureus*, *Bacillus subtilis*) (MAIA et al., 2010). Efeito este atribuído provavelmente, a presença de sesquiterpenos, em especial (E)-cariofileno/biciclogermacreno ou (E)-cariofileno/germacreno D, os quais já se tem relatos na literatura demonstrando sua potente ação antibacteriana (FORMISANO et al., 2006; COSTA et al., 2009) e antifúngica (GARG e SIDDIQUI, 1992).

Em relação ao óleo essencial de *V. brasiliiana*, as análises químicas obtidas por cromatografia gasosa acoplada à espectrometria de massa (CG-EM), demonstraram que essa espécie possui 93,4% de sesquiterpenos, sendo os mais expressivos, (E)-cariofileno correspondendo a 36,7% e o germacreno-D com 35,5% (MAIA et al., 2010).

Martins et al. (2014) em estudo com extratos etanólicos e óleos essenciais obtidos das folhas, flores e raiz de *V. brasiliiana* identificou que o maior percentual de fenóis totais e taninos estão presentes na folha ao comparar com flor e raiz. Em relação a composição do óleo essencial, os compostos com maior percentual foram: modheph-2-eno, alfa-isocomeno, cipereno, beta-isocomeno e beta-cariofileno na raiz; enquanto que na flor foi ácido palmítico, hex-2-en-1-ol, hexan-1-ol e septacosano; e na folha, trans-cariofileno, germacreno-D, biciclogermacreno, escaptulenol e ácido palmítico. Além das análises fitoquímicas, o pesquisador constatou que os extratos foram tóxicos nas concentrações em que apresentaram ação contra *T. cruzi* e *L. amazonensis*. Entretanto, os óleos não demonstraram toxicidade celular e tiveram ação tripanossomida e leishmanicida.

3.4 Legislação de Fitoterápicos

A fitoterapia corresponde o tratamento feito com o uso de plantas medicinais, ou seus derivados, sem a utilização de forma direta dos princípios ativos isolados (SCHENKEL et al., 2000). Segundo a RDC nº 14 de 2010, medicamento fitoterápico é aquele obtido exclusivamente de matérias-primas ativas vegetais, cuja eficácia e segurança são validadas por meio de levantamentos etnofarmacológicos, de utilização ou evidências clínicas. Desta forma, não podem ser incluídos no medicamento fitoterápico substâncias ativas isoladas, de qualquer origem, nem as associações destas com extratos vegetais (BRASIL, 2010).

Calixto (2001) relata que cerca de 40% dos fármacos disponíveis foram desenvolvidos de fontes naturais, sendo 25% de plantas, 12% de microrganismos e 3% de animais. Além disso, das 252 drogas que a Organização Mundial de Saúde considera como essenciais, 11% foram obtidas de plantas.

Em 1978 a Organização Mundial de Saúde reconheceu oficialmente a utilização de fitoterápicos ou plantas medicinais com fins profilático, curativo,

paliativo ou com finalidade diagnóstica. Fato este de grande relevância, visto que 80% da população dos países em desenvolvimento fazem uso de plantas medicinais para cuidados básicos em saúde (GURIB-FAKIM, 2006; LIMA e DIMESNTEIN, 2006).

A partir desse reconhecimento, surge então uma série de legislações, dentre elas a Portaria Ministerial nº 212 de 1982, que fundamenta a investigação clínica de plantas de uso medicinal; bem como o Decreto nº 5.813 de 2006, que institui a Política Nacional de Plantas Medicinais, estabelecendo diretrizes e linhas prioritárias para o desenvolvimento de ações com o objetivo do acesso seguro e uso racional de plantas medicinais, com enfoque no fortalecimento das cadeias produtivas e no uso sustentável da biodiversidade brasileira. Posteriormente, em 2010, a Portaria nº 886 que regulamenta o programa Farmácia Viva no Sistema Único de Saúde (SUS). Vale ressaltar também, a Instrução Normativa nº 4 de 2014, que institui um guia de orientação para registro de medicamento e, registro e notificação de produto tradicional fitoterápico (BRASIL, 2010; BRASIL, 2011).

Neste guia, aborda-se a diferença entre medicamento fitoterápico (MF) e produto tradicional fitoterápico (PTF) (Quadro 1). O ponto principal é que no MF a segurança e eficácia são comprovadas por estudos clínicos, enquanto no PTF a segurança e efetividade são comprovadas pelo tempo de uso na literatura técnico-científica (BRASIL, 2014).

Quadro 1 – Diferenças entre os fitoterápicos tratados pela RDC nº 26/2014.

Diferenças	Medicamento Fitoterápico (MF)	Produto Tradicional Fitoterápico (PTF)
Comprovação de segurança e eficácia/efetividade (SE)	Por estudos clínicos.	Por demonstração de tempo de uso.
Boas Práticas de Fabricação (BPF)	Segue a RDC nº 17/2010.	Segue a RDC nº 13/2013.
Informações do fitoterápico para o consumidor final	Disponibilizadas na bula.	Disponibilizadas no folheto informativo.
Formas de obter a autorização junto à ANVISA	Registro ou registro simplificado.	Registro, registro simplificado ou notificação.

Fonte: BRASIL, 2014.

O desenvolvimento de medicamentos a partir de extratos vegetais padronizados mediante pesquisas, que usam os mesmos padrões estabelecidos para medicamentos sintéticos, seguem critérios estabelecidos pela Agência Nacional de Vigilância Sanitária (ANVISA) (BRASIL, 2009).

A regularização sobre o uso da biodiversidade brasileira, no intuito de garantir acesso seguro e uso racional de plantas medicinais e fitoterápicos, também está sob o âmbito do Conselho de Gestão do Patrimônio Genético (CGEN), que é responsável por emitir as autorizações de acesso ao patrimônio genético de espécies animais e vegetais, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico.

Ressalta-se nesse contexto, o Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA, o qual através da Instrução Normativa nº 154 de 2007, institui o Sistema de Autorização e Informação em Biodiversidade – SISBIO. Este é um sistema de atendimento à distância que permite os pesquisadores solicitarem autorização, por meio de registro voluntário, para coleta de material biológico no território nacional, objetivando entre outras ações, atividade científica ou didática de ensino superior.

Nas últimas décadas, houve um aumento considerável no interesse pela fitoterapia entre usuários, pesquisadores e serviços de saúde, ocorrendo uma revalorização no emprego destes medicamentos, além do aprimoramento e a produção em escala industrial, diferentemente das formas artesanais que

caracterizaram os estágios iniciais da utilização (TUROLLA e NASCIMENTO, 2006; ROSA et al., 2011).

Proporcional ao interesse, o comércio de medicamentos fitoterápicos vem crescendo a uma taxa anual média de 15%, sendo mais evidente na Alemanha, Ásia e Estados Unidos da América (EUA). Estima-se que o mercado mundial de fitoterápicos movimente aproximadamente US\$ 43 bilhões/ano, e apenas nos Estados Unidos este mercado represente US\$ 5 bilhões anuais (TUROLLA e NASCIMENTO, 2006; BRITO, 2010).

3.5 Leishmaniose

As leishmanioses correspondem um complexo de doenças que afetam o homem e outros mamíferos nas regiões tropicais e subtropicais, causadas por mais de 20 tipos de protozoários do gênero *Leishmania*, com diversas manifestações clínicas, cujo espectro varia desde a forma cutânea branda até a forma visceral grave (BELLO et al., 2011; PLANO et al., 2011), as quais diferem nos aspectos imunopatológicos e graus de morbidade e mortalidade (KHEIRANDISH et al., 2013).

3.5.1 O parasito

Leishmania está classificada taxonomicamente, segundo Lainson e Shaw (1987) da seguinte forma:

- ✓ Reino: Protista (Haeckel, 1886);
- ✓ Sub-reino: Protozoa (Goldfuss, 1817);
- ✓ Filo: Sarcomastigophora (Honiberg e Balamuth, 1963);
- ✓ Subfilo: Mastigophora (Diesing, 1866);
- ✓ Classe: Zoomastigophora (Calkins, 1909);
- ✓ Ordem: Kinetoplastida (VickKerman, 1976);
- ✓ Subordem: Trypanosomatina (Kent, 1880);
- ✓ Família: Trypanosomatidae (Grobbs, 1905);
- ✓ Gênero: *Leishmania* (Ross, 1903).

Nesse gênero estão inseridos três subgêneros (*Leishmania*, *Viannia* e *Sauroleishmania*), cuja classificação é baseada no local de colonização do parasito no segmento intestinal do flebotomíneo, com confirmação por análises moleculares

filogenéticas. O subgênero *Viannia*, é formado por espécies que se desenvolvem na porção posterior do intestino do hospedeiro invertebrado, enquanto que o subgênero *Leishmania*, corresponde as espécies que colonizam porção anterior e média do intestino (SAF'JANOVA, 1982; LAINSON e SHAW, 1987). Já o subgênero *Sauroleishmania*, compreende as espécies do parasito encontradas em répteis, não é patogênico aos seres humanos, e por várias vezes foi incluído e excluído do gênero. No entanto, análises filogenéticas de DNA indicaram que este é um grupo de desenvolvimento secundário, derivado das espécies de mamíferos (ORLANDO et al., 2002; BATES, 2007).

O gênero *Leishmania* corresponde parasitas unicelulares e heteroxenos, que no decorrer do ciclo biológico apresentam duas formas evolutivas distintas: amastigota e promastigota. Amastigota é a forma imóvel, intracelular obrigatória das células fagocitárias mononucleares no hospedeiro mamífero; caracterizada como um corpo ovóide, medindo entre 2,1 a 3,2 μm ; possui uma única mitocôndria, na forma de bastonete, bem desenvolvida próxima ao flagelo, denominada cinetoplasto, que contém DNA circular (kDNA); o núcleo é grande arredondado, ocupando normalmente a maior parte da célula; na região anterior do corpo, possui invaginações da membrana que formam a bolsa flagelar, onde fica alojado o flagelo, que não é livre nessa fase. A bolsa flagelar é o local onde ocorrem os mecanismos de endocitose e exocitose. Além dessas ultraestruturas, verifica-se também microtúbulos, complexo de Golgi, retículo endoplasmático, entre outras pouco diferenciadas. A forma amastigota se desenvolve poucas horas após ser internalizado pelas células-alvo do hospedeiro vertebrado (WEBSTER e RUSSEL, 1993; LAUFS et al., 2002; BATES, 2007).

A promastigota é a forma extracelular e móvel do parasita, encontrada no tubo digestivo do hospedeiro invertebrado. Apresenta corpo alongado e fusiforme, medindo aproximadamente entre 14 e 20 μm , com um flagelo livre que emerge da porção anterior do parasito, com medida igual ou superior ao maior diâmetro do corpo. Comparada a forma amastigota, as promastigotas possuem núcleo maior, mitocôndria, complexo de Golgi e retículo endoplasmático mais evidentes. Durante o desenvolvimento das formas promastigotas no intestino do inseto vetor, estas passam por uma série de modificações, sendo classificadas em cinco estágios: 1º procíclicas, formas ovóides recém ingeridas; 2º nectomonas, formas mais delgadas

encontradas no intestino médio; 3º leptomonas, formas menores; 4º haptomonas, imóveis, encontradas na válvula estomacal, possuem flagelo curto; 5º metacíclicas, pequenas, grande mobilidade, flagelo longo, muito resistente, corresponde o estágio infectante (GOSSAGE et al., 2003; KAMHAWI et al., 2006; BATES, 2007).

3.5.2 Ciclo Biológico

Independente da espécie de *Leishmania*, o ciclo biológico é do tipo heteroxeno, ou seja, para o seu desenvolvimento necessitam de dois hospedeiros: um invertebrado, que são dípteros hematófagos do gênero *Phlebotomus* no Velho Mundo e *Lutzomyia* no Novo Mundo e, um vertebrado que de acordo com a espécie de *Leishmania* pode ser canídeo, roedor ou marsupial (MAGILL et al., 1993).

Ao picar um hospedeiro vertebrado parasitado, o flebotomíneo fêmea se infecta com as formas amastigotas, as quais se transformam nas primeiras 24 horas em promastigotas, no interior do trato digestório do inseto, mediante divisão por fissão binária longitudinal, sofrendo transformações até o estágio infectante e, depois desse estágio, locomovem-se para porção anterior do tubo digestivo. Durante um novo repasto sanguíneo, as formas promastigotas metacíclicas são regurgitadas e infectam o hospedeiro vertebrado (SEHGAL et al., 2012). Essas formas infectantes rapidamente se diferenciam em amastigotas e assim escapam dos mecanismos de defesa do hospedeiro, principalmente porque se ligam aos receptores de fibronectina, receptor do complemento C3 e receptores de açúcares, como o receptor manose-fucose, dentre outros (KANE e MOSSER, 2000; AKILOV et al., 2007; SHARMA e SINGH, 2009).

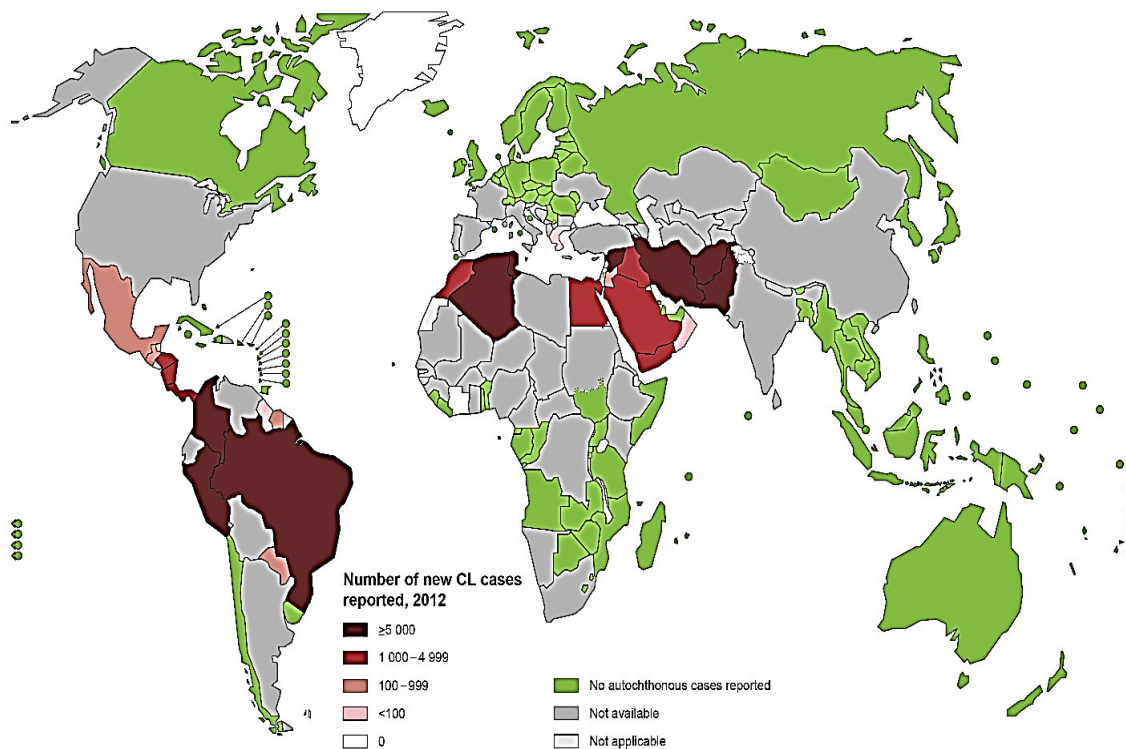
De modo geral, as amastigotas no interior do vacúolo parasitóforo, inibem os diversos mecanismos de defesa do hospedeiro, como fusão fagossomo-endossomo, liberação de enzimas hidrolíticas, mecanismos de sinalização celular, produção de citocinas e óxido nítrico, impedindo assim a lise e mantendo a multiplicidade. Após intensa replicação ocorre ruptura das células do hospedeiro o que permite a re-infecção de outros fagócitos, que uma vez infectados migram para os linfonodos drenantes e, em seguida, para outros órgãos como o baço, fígado e a medula óssea (ZILBERSTEIN e SHAPIRA, 1994; SEHGAL et al., 2012).

3.5.3 Epidemiologia e Aspectos Clínicos

As leishmanioses são consideradas pela Organização Mundial da Saúde como doenças negligenciadas por estarem associadas a baixos indicadores sociais. Por conseguinte, essas doenças são mais prevalentes em países em desenvolvimento, cujos recursos aplicados no controle, tratamento e pesquisas são insuficientes (MASHAYEKHI-GHOYONLO et al., 2014). Entretanto, após o surgimento do HIV, houve uma mudança do perfil epidemiológico (SINGH, 2014) e as leishmanioses passaram a ser endêmicas em mais de 98 países dos 05 continentes (ALVAR et al., 2012).

De acordo com a Organização Mundial de Saúde, são registrados 1,3 milhão casos novos por ano, sendo 300 mil casos de leishmaniose visceral (LV) e 1 milhão de leishmaniose tegumentar (LT) (WHO, 2015). Apesar da ampla distribuição das leishmanioses, 90% dos casos de LV ocorrem em Bangladesh, Brasil, Etiópia, Índia, Nepal e Sudão, enquanto que a maioria dos casos de LT (Figura 1) são registrados no Afeganistão, Algéria, Brasil, Colômbia, Irã, Paquistão, Peru, Arábia Saudita, Síria e Tunísia (SINGH, 2014).

Figura 1 – Mapa de endemicidade da leishmaniose cutânea no mundo.



Fonte: WHO, 2013.

No Brasil, em 2010, foram registrados 22.397 casos de LT e 3.536 de LV, com 219 óbitos confirmados. Segundo o Ministério da Saúde, o Nordeste é a região brasileira com maior número de casos, em especial nos estados do Ceará e Maranhão (BRASIL, 2011). No cenário mundial, aproximadamente 350 milhões de pessoas estão expostas à leishmaniose, com média de 12 milhões de infectados e 60 mil óbitos (JORDA et al., 2011; PLANO et al., 2011).

Neste trabalho abordaremos apenas a LT. Ela é causada por variedades dermatrópicas de *Leishmania*, com grande diversidade de parasitos sendo encontrados na Região Amazônica (RANGEL e LAINSON, 2009). A manutenção do parasito em partes cutâneas ou sua subsequente dispersão para os órgãos internos é o que proporciona as diferentes patologias e manifestações clínicas, como cutânea, muco-cutânea, cutânea difusa ou visceral (PLANO et al., 2011). O quadro clínico está diretamente relacionado com a espécie do parasita, a interação parasita-hospedeiro, a presença de coinfeções e o estado imunológico do hospedeiro (MEDEIROS et al., 2011).

As principais espécies, conforme o subgênero, tropismo e ocorrência no Velho e Novo Mundo, relacionadas à saúde pública estão listadas na Tabela 2.

Tabela 2 – Espécies de *Leishmania* de importância à saúde pública

Subgênero	<i>L. (Leishmania)</i>	<i>L. (Leishmania)</i>	<i>L. (Viannia)</i>	<i>L. (Viannia)</i>
Velho Mundo	<i>L. donovani</i> <i>L. infantum</i>	<i>L. major</i> <i>L. tropica</i> <i>L. killicki</i> ^a <i>L. aethiopica</i> <i>L. infantum</i>		
Novo Mundo	<i>L. infantum</i>	<i>L. infantum</i>	<i>L. braziliensis</i>	<i>L. braziliensis</i>
		<i>L. mexicana</i>	<i>L. guyanensis</i>	<i>L. panamensis</i>
		<i>L. pifanoi</i> ^a	<i>L. panamensis</i>	
		<i>L. venezuelensis</i>	<i>L. shawi</i>	
		<i>L. gamhami</i> ^a <i>L. amazonensis</i>	<i>L. naiffi</i> <i>L. lainsoni</i> <i>L. lindenbergi</i> <i>L. peruviana</i> <i>L. colombiensis</i> ^b	
Tropismo principal	Viscerotrópica	Dermotrópica	Dermotrópica	Mucotrópica

^a Status da espécie está sob discussão; ^b Posição taxonômica está sob discussão.

Fonte: Adaptado de WHO, 2010.

No Brasil, a leishmaniose tegumentar também conhecida como úlcera de Bauru é causada por diversas espécies de *Leishmania*. Na região Amazônica foram identificadas as seguintes espécies causadoras de LT: *L. braziliensis*, *L. amazonensis*, *L. guyanensis*, *L. naiffi*, *L. shawi*, *L. lindenbergi* (JENNINGS et al., 2014). A forma cutânea é a mais comum e provoca lesões de pele e úlceras, principalmente nas partes expostas do corpo, deixando cicatrizes e deficiências graves (WHO, 2015), podendo ter cura espontânea em alguns meses ou, dependendo da espécie de *Leishmania*, progredir para a forma cutânea difusa ou muco-cutânea (TELES et al., 2011).

O quadro clínico dessas duas formas é caracterizado por desfiguramento dos pacientes, com lesões ulcerativas na pele e destruição parcial ou total da mucosa do

nariz, boca e garganta. Essas deformações, proporcionam o ostracismo social do paciente (BARBOSA et al., 2011; WHO, 2015).

3.5.3.1 Leishmaniose Cutânea (LC)

Várias espécies de *Leishmania* podem causar a forma cutânea em humanos, que, na maioria dos casos, permanecem assintomáticos (MURRAY et al., 2005). O primeiro sinal da doença é uma pequena área de eritema no local da picada do flebotomíneo, que se desenvolve para uma pápula e posteriormente para um nódulo, que ulcera progressivamente por um período de duas semanas a seis meses, até formar a lesão característica da LC (REITHINGER et al., 2007). Há uma tendência para a regressão espontânea destas lesões, o que geralmente resulta em proteção para toda a vida; que pode ser restrita ou não à mesma espécie de *Leishmania*. A resolução da doença geralmente resulta em uma cicatriz que, dependendo do tamanho e localização, pode se tornar um estigma para os indivíduos afetados (YANIK et al., 2004).

Em cães, a infecção manifesta-se principalmente nas orelhas, boca, focinho e bolsa escrotal, e na maioria dos casos, não afeta o estado geral de saúde do animal (DE OLIVEIRA et al., 2013).

3.5.3.2 Leishmaniose Mucocutânea

Também conhecida como “espundia”, a leishmaniose mucocutânea (LMC) pode ser causada por várias espécies de *Leishmania*, sendo mais associada à *L. braziliensis*; por isso, geralmente seus casos estão limitados à América do Sul (DAVIES et al., 2000). Apesar de ser menos frequente que a forma cutânea, o envolvimento da mucosa nasal e oral é mais severo, levando a óbito caso não tratada (PASSOS et al., 2001).

Na LMC, o parasito se dissemina para os tecidos mucosos por via linfática ou hematogênica (REITHINGER et al., 2007). O estágio inicial da doença é caracterizado por eritema, ulceração e congestão nasal persistente. Erosões e úlceras ao redor das narinas e dos lábios são sinais da progressão do quadro, e são comumente confundidos com impetigo contagioso (DAVID e CRAFT, 2009).

Já na fase final, os indivíduos podem apresentar ulceração no palato, edema nas narinas e gengivas e periodontite. Linfadenopatia pode estar presente em infecções por *L. braziliensis*, acompanhada por sinais sistêmicos, como febre e hepatomegalia (SCOPE et al., 2003). Eventualmente há a destruição da mucosa oronasofaríngea e das cartilagens das vias aéreas superiores, causando uma desfiguração facial, e levando à infecções secundárias e obstrução aérea (DAVID e CRAFT, 2009).

3.5.3.3 Leishmaniose Cutânea Difusa

A leishmaniose cutânea difusa (LCD) é uma manifestação clínica rara, considerada uma forma anérgica da leishmaniose tegumentar (GOTO e LAULETTA-LINDOSO, 2010). É caracterizada pelo aparecimento de várias lesões de aspecto nodular em vários pontos do corpo, além de áreas não ulcerativas de infiltração difusa, com proliferação descontrolada de parasitos (HOOJA et al., 2014).

A ausência ou reduzida resposta imune celular do hospedeiro e a resistência à maior parte dos protocolos terapêuticos tornam a LDC uma enfermidade de difícil controle e tratamento (ZERPA et al., 2007).

Vários trabalhos têm relatado o aparecimento da LCD em pacientes infectados pelo HIV (CHAUDHARY et al., 2008; MEHTA et al., 2009; KHANDELWAL et al., 2011; PUROHIT et al., 2012; HOOJA et al., 2014). Nestes casos, a imunossupressão causada pelo vírus pode ser um fator importante para a apresentação atípica e progressão generalizada da doença (ASADI-KANI et al., 2010).

3.5.4 Resposta Imune

Desde a inoculação das formas promastigotas pelo flebotômíneo até a sua transformação em amastigotas no interior das células-alvo, a batalha entre o estabelecimento ou não da enfermidade vai depender da habilidade do parasito em evadir-se da resposta imune do hospedeiro, que incluem elementos da defesa inata e adaptativa (GUPTA et al., 2013).

Antes de abordar a resposta imune do hospedeiro vertebrado, vale ressaltar a importância do inseto na transmissão do protozoário, visto que existem

componentes na saliva do flebotomíneo que protegem o parasito e aumentam a sua infectividade. Como exemplo, cita-se o maxadilán, um peptídeo com ação vasodilatadora, presente na saliva do *Lutzomyia longipalpis*, que age sobre os macrófagos, exacerbando a infecção e prolongando a sobrevivência da *Leishmania*, através da inibição da produção de óxido nítrico, H₂O₂ e fator de necrose tumoral alfa (TNF- α), bem como aumentando os níveis IL-10 e IL-6 (GOMES e OLIVEIRA, 2012).

As espécies de *Leishmania* são patógenos intracelulares obrigatórios, sendo assim, os macrófagos são indispensáveis para sua sobrevivência, replicação e diferenciação. Após a infecção inicial, neutrófilos e macrófagos são recrutados para o sítio de inoculação, e a interação entre estas células influencia significativamente na progressão da doença (RIBEIRO-GOMES et al., 2007).

Estudos sugerem que os neutrófilos estão presentes em maiores quantidades, especialmente quando a infecção é iniciada pela picada do inseto vetor, e que são muito eficientes na fagocitose das formas promastigotas (PETERS et al., 2008; PETERS e SACKS, 2009). Como os neutrófilos são fagócitos de curta duração, acredita-se que estas sejam células hospedeiras intermediárias, e que atuem como "cavalos de Tróia", albergando os parasitos e causando uma infecção silenciosa dos macrófagos, evitando assim a ativação celular (LAUFS et al., 2002; VAN ZANDBERGEN et al., 2004).

Outros grupamentos celulares, como as células dendríticas (CDs) e células natural killers (NK) também exercem papel na resposta imune inata do hospedeiro. As CDs são um grupo heterogêneo de células derivadas da medula óssea, especializadas no reconhecimento, apreensão e transporte de antígenos patogênicos, além de ativarem as células NK (FREITAS e PINHEIRO, 2010). Estas, por sua vez, são as fontes primárias de INF- γ , que favorecem a diferenciação das células TCD4⁺ e restringem a disseminação do parasito, além de mediar a lise destes através da sua atividade citotóxica (LIEKE et al., 2011).

A captação de promastigotas pelas células hospedeiras é um processo mediado por receptores, que iniciam a fagocitose. Os receptores do complemento C1 e C3, de fibronectina e de manose-fucose presentes nos macrófagos

desempenham papéis importantes na fixação com as formas promastigotas (KANE e MOSSER, 2000). Curiosamente, um estudo anterior demonstrou que a ligação do parasito em um único receptor não desencadeia a ativação dos macrófagos da mesma forma que a ligação em múltiplos receptores, sugerindo a importância deste fato para o estabelecimento de uma resposta protetora adequada (ADEREM e UNDERHILL, 1999).

Da mesma forma, a superfície celular do protozoário possui substâncias que são determinantes para o início da fagocitose, e consequente sobrevivência intracelular, como o lipofosfoglicano, proteofosfoglicano e a glicoproteína GP63, uma vez que são moléculas-alvo para várias opsoninas (YAO et al., 2003; NADERER e McCONVILLE, 2008).

Os macrófagos são as principais células efetoras responsáveis pela destruição dos parasitos. Podem ser ativados de duas formas funcionalmente distintas, a forma clássica e a alternativa, cada uma delas desencadeando resultados diferentes quanto ao desenvolvimento da doença (LIU e UZONNA, 2012).

A ativação clássica é mediada pelas células do perfil Th1 e suas citocinas (INF- γ , TNF- α , IL-1 e IL-2), que estimulam a produção da enzima óxido nítrico sintase induzida (iNOS), que catalisa o aminoácido L-arginina em óxido nítrico (NO), uma molécula tóxica que desempenha um importante papel microbicida (LIEW et al., 1990).

Em contraste, a ativação alternativa é induzida pelas citocinas do perfil Th2 (IL-4, IL-10 e IL-13), que induzem à síntese de poliaminas através do metabolismo da L-arginina pela enzima arginase. Esta constatação reforça a ideia de que a arginase funciona como um fator de virulência à infecção, visto que as poliaminas são essenciais para o crescimento do parasito e sua sobrevivência no interior dos macrófagos (KROPF et al., 2005; MORENO et al., 2012).

Vale ressaltar também, os estudos realizados com as células Th17. Elas estão associadas a proteção das mucosas, doenças autoimunes e quadros alérgicos, sendo caracterizadas pela produção de IL-17, IL-22 e IL-21. Além disso, é importante frisar a produção de IL-17, que desempenha um papel importante no recrutamento, migração e ativação de neutrófilos de forma rápida ao sítio de

inoculação da *Leishmania*, iniciando o processo inflamatório e o estabelecimento da resposta imune (CHARMOY et al., 2010). Entretanto, trabalhos realizados com diferentes espécies, *L. donovani* e *L. major*, já demonstraram respostas diferentes das células TH17 em camundongos Balb/c. Isso comprova, que a atividade dessas células está diretamente relacionada a espécie infectante e ao hospedeiro (ALEXANDER e BROMBACHER, 2012).

3.5.5 Tratamento

No Brasil, é preconizado pelo Ministério da Saúde, o tratamento de humanos com antimoníato de N-metil glucamina, o desoxicolato de anfotericina B e a anfotericina B lisossomal. A prescrição dos fármacos deve ser feita considerando a faixa etária do paciente, presença de gravidez, co-morbidades e o perfil de toxicidade dos mesmos (PELISSARI et al., 2011).

Vale ressaltar, que o tratamento para animais é proibido, conforme a Portaria Interministerial nº 1.426, de 11 de julho de 2008, determinada pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA), em ação conjunta com o Ministério da Saúde, que proíbe o tratamento da leishmaniose visceral canina com produtos de uso humano ou não registrados no MAPA. Para obtenção de registro no Ministério, o medicamento de uso veterinário não pode conter em sua formulação drogas destinadas ao tratamento de seres humanos (BRASIL, 2008). Todavia, é importante citar a decisão do Tribunal Regional Federal da 3ª região, em São Paulo, que declarou a ilegalidade desta portaria, autorizando desta forma o tratamento dos cães. Apesar disso, o Conselho Federal de Medicina Veterinária (CFMV) e os Conselhos Regionais (CRMVs) mantêm seu posicionamento institucional contra esta medida (CFMV, 2013).

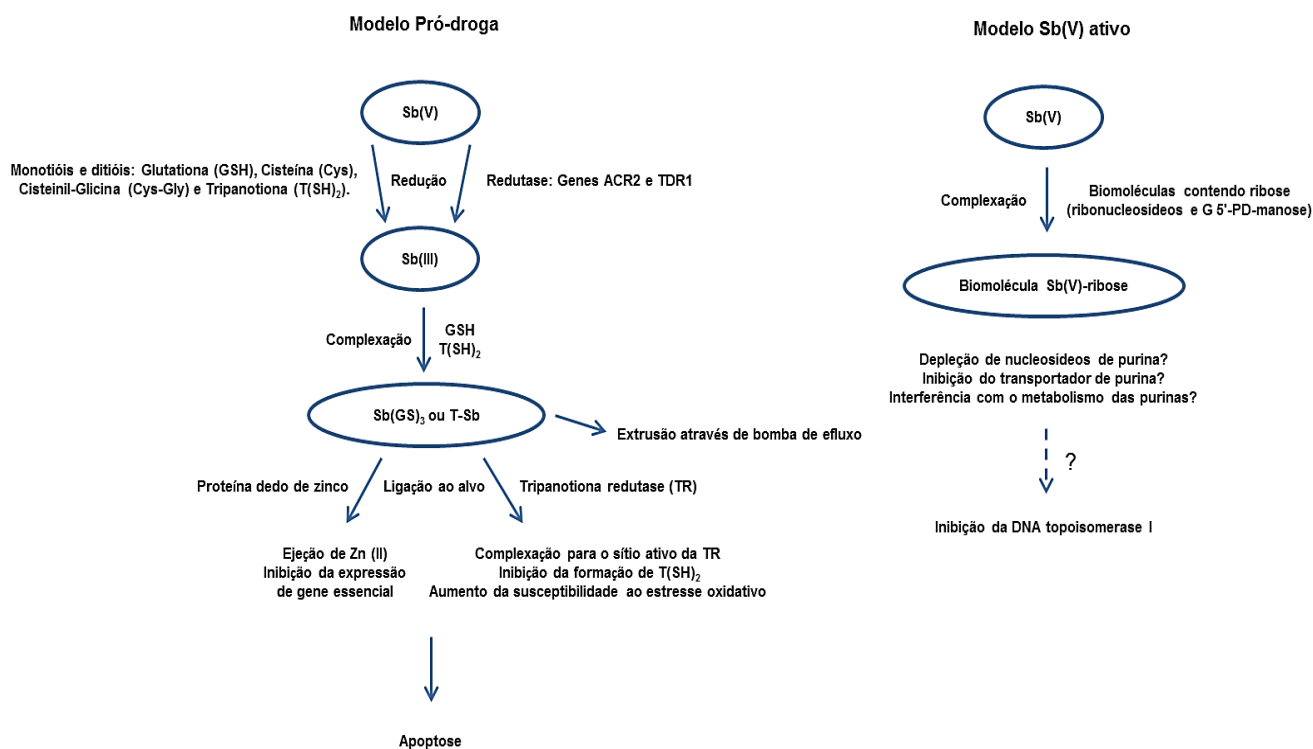
3.5.5.1 Antimoníato pentavalente

A leishmaniose cutânea é popularmente conhecida por ferida brava em virtude de sua característica em não responder aos tratamentos usuais, como pomada cicatrizante, anti-inflamatórios e antibióticos, sendo necessário o uso dos antimoníatos pentavalentes. Apesar de serem drogas utilizadas há mais de cem anos, tanto o mecanismo de ação e a fórmula estrutural são pouco conhecidos (FRÉZARD et al., 2009).

Não está claro se a forma ativa final dos antimoniais pentavalentes é Sb (V) ou Sb (III), desta forma há dois modelos propostos para explicar esse mecanismo de ação (Figura 2). O primeiro aborda que o Sb (V) se comporta como um pró-fármaco, que é reduzido no interior do organismo em Sb (III) que é mais tóxico e ativo. Este modelo é apoiado pela observação de estudos in vivo com essa conversão (SHAKED-MISHAN et al., 2001), bem como pelas constatações que os tióis podem atuar como um agente de redução (FRÉZARD et al., 2009; FERREIRA et al., 2003; YAN et al., 2003).

Quatro diferentes tióis já foram avaliados: a glutathiona (GSH) é o principal encontrado no citosol de mamíferos; a cisteína (Cis) e cisteinil-glicina (Cys-Gly), que são os tióis predominantes em lisossomos e; o conjugado de glutathiona-espermina, denominado tripanotiona [T(SH)₂], que é o tiol predominante no interior do parasita. Há relatos que esses 3 últimos tióis são os responsáveis por promover a redução de Sb (V) em Sb (III) a 37°C, com favorecimento da reação em pH ácido. Desta forma, a redução é mais acentuada nas formas amastigotas que tem menor pH intracelular e temperatura mais elevada que as promastigotas (FRÉZARD et al., 2009).

Figura 2 – Modelos propostos (Pró-droga e SbV ativado) para explicar o mecanismo de ação dos antimoniatos pentavalentes nas leishmanioses.



Fonte: Adaptado de FRÉZARD et al., 2009.

Adicionalmente aos estudos do modelo pró-fármaco, descobriu-se a participação de enzimas específicas contra o parasita no processo de redução de Sb (V) em Sb (III), são elas dependentes de tiol redutase (TDR1) e/ou redutase antimoniato (ACR2) (DENTON et al., 2004; ZHOU et al., 2004).

O outro modelo aborda que Sb (V) apresenta atividade anti-leishmania intrínseca, com inibição de uma topoisomerase I do DNA do parasita, formando um complexo Sb (V) - ribonucleosídeo que interfere no metabolismo das purinas e consequente depleção de ATP e GTP (FRÉZARD et al., 2009).

Existem comercialmente dois antimoniatos pentavalente: o antimoniato de N-metilglucamina (Glucantime®) disponibilizado na América e África e o estibogluconato de sódio (Pentostam®), nos EUA e Europa (LIMA et al., 2007).

Apesar de ainda ser a base da terapêutica da doença, seu uso na prática clínica tem várias limitações. Estes compostos são administrados por via parenteral,

diariamente (Quadro 2) durante três semanas ou mais, conforme o tipo de leishmaniose (cutânea, difusa ou mucosa) o que pode acarretar em dor local durante as injeções intramusculares e em efeitos colaterais sistêmicos, exigindo, desta forma, supervisão médica (BRASIL, 2007). Os efeitos colaterais mais comuns incluem náuseas, vômitos, fraqueza e mialgia, cólica abdominal, diarreia, pruridos, hepatotoxicidade e cardiotoxicidade (SUNDAR e CHAKRAVARTY, 2010). O aparecimento de resistência do parasito à droga é outro problema importante a ser controlado, sendo complexo e multifatorial, com abordagens sobre o uso correto e o surgimento de cepas resistentes (GUERIN et al., 2002; JEDDI et al., 2011).

Quadro 2 – Protocolo terapêutico preconizado para as LTAs, segundo a Organização Mundial da Saúde e o Ministério da Saúde.

Forma clínica	Dose	Tempo de tratamento mínimo
Leishmaniose cutânea	10-20 mg/Sb ⁺⁵ /kg/dia Recomenda-se 15 mg/Sb ⁺⁵ /kg/dia	20 dias
Leishmaniose difusa	20 mg/Sb ⁺⁵ /kg/dia	20 dias
Leishmaniose mucosa	20 mg/Sb ⁺⁵ /kg/dia	30 dias

Fonte: BRASIL, 2007.

3.5.5.2 Anfotericina B

Outro fármaco utilizado no tratamento das leishmanioses é a anfotericina B. Este é um antifúngico poliênico extraído de culturas da bactéria *Streptomyces nodosus*. É utilizada quando não há resposta satisfatória ao tratamento convencional, sendo considerado um fármaco de segunda escolha. Contudo, para gestantes é o fármaco de primeira escolha. Está disponível em duas formas: a convencional e a lipossomal, sendo esta última melhor tolerada e menos tóxica (SUNDAR et al., 2000; BRASIL, 2007). Seu mecanismo de ação é multifacetado: liga-se com os esteróis da membrana da célula hospedeira, formando poros e causando alteração da permeabilidade e do equilíbrio osmótico do parasita, com extravasamento do conteúdo celular. Também auxilia na geração de espécies reativas de oxigênio, que podem causar danos à célula, levando à morte celular (PURKAIT et al., 2012).

Apesar da sua eficácia, possui efeitos colaterais frequentes, como febre, trombocitopenia, convulsões, flebite, anemia, diminuição da função tubular renal e hipocalcemia (LIMA et al., 2007), criando a necessidade da hospitalização do paciente.

3.5.5.3 Pentamidina

A pentamidina, uma diamina aromática, é um medicamento eficaz contra vários protozoários patogênicos, incluindo as espécies de *Leishmania*. É utilizada na forma de sais, principalmente o isotionato de pentamidina, geralmente nos casos em que há resistência aos antimoniais ou em que não seja possível utilizá-lo (NEVES et al., 2011). Quanto ao seu mecanismo de ação, a teoria mais aceita tem sido a da inibição da topoisomerase II mitocondrial, que interfere no potencial da membrana da mitocôndria, no transporte de cálcio e causa modificações no cinetoplasto do protozoário (BASSELIN et al., 1996; KRAMP et al., 2005). É um fármaco em potencial para o tratamento de manutenção em pacientes imunocomprometidos, com risco elevado de recidiva (PATEL e LOCKWOOD, 2009), sendo recomendado pelo Ministério da Saúde para pacientes portadores da co-infecção *Leishmania*-HIV (BRASIL, 2007).

Embora sua eficácia seja elevada, podem ocorrer alguns efeitos colaterais, sendo os mais comuns: hipotensão, náuseas, vômitos, síncope, alterações renais, além de efeitos sistêmicos no metabolismo da glicose, como hipoglicemia e efeito diabetogênico (BRASIL, 2007).

Estudo avaliando a eficácia do isotionato de pentamidina no tratamento da leishmaniose cutânea, causada pela *L. guyanensis* demonstrou a eficácia da droga em menor tempo de tratamento e uma menor toxicidade, quando comparado ao glucantime®. O protocolo terapêutico utilizado foi de 4mg/Kg/dia, com 3 aplicações, intramuscular, durante uma semana (PAULA et al., 2003).

3.5.5.4 Miltefosina

A miltefosina é um alquilfosfolipídeo, foi primariamente desenvolvida como antineoplásico de uso oral no tratamento de metástases cutâneas em carcinomas mamários (LEONARD et al., 2001). Após estudos clínicos que identificaram seu

potencial leishmanicida, passou a ser utilizada como agente terapêutico para a doença, tornando-se o primeiro medicamento de uso oral em alguns países (CROFT e ENGEL, 2006; SINDERMANN e ENGEL, 2006). Na Índia, tem sido empregada para o tratamento de leishmaniose visceral desde 1998 (VÉLEZ et al., 2010).

A acumulação intracelular da droga parece ser o passo fundamental para sua ação. Ela interfere na membrana celular do parasita, sem interagir com o DNA, modulando a composição lipídica, a permeabilidade e a fluidez da membrana, assim como alterando o metabolismo de fosfolipídeos, induzindo a morte celular (PARIS et al., 2004; VERMA e DEY, 2004).

Estudos demonstraram que a miltefosina induz a uma morte celular semelhante a apoptose em *Leishmania donovani* (PÉREZ-VICTORIA et al., 2006). Verificou-se também que a droga foi capaz de aumentar o número de receptores de IFN- γ em macrófagos infectados *in vitro* com *L. donovani*, sendo capaz de induzir a uma resposta Th1 (WADHONE et al., 2009).

É eficaz no tratamento das formas cutânea e visceral da doença, inclusive nos casos de resistência aos antimoniais. No entanto, a necessidade de um tratamento longo (28 dias) exige a formulação de estratégias para um uso mais racional da droga, a fim de impedir o desenvolvimento de resistência em pacientes que a utilizam (PÉREZ-VICTORIA et al., 2006). Também se deve evitar a administração em mulheres gestantes, uma vez que possui potencial teratogênico (SUNDAR e OLLIARO, 2007).

3.5.5.5 Paramomicina

A paramomicina é um antibiótico aminoglicosídeo derivado da bactéria *Streptomyces nodosus* var. *paramomicinus*. Por ser ativa contra diversos tipos de parasitos, passou a ser utilizada no tratamento da leishmaniose, na forma de sulfato de paramomicina (WILLIAMS et al., 1998).

Atua no parasito através da inibição da síntese de proteínas, através da ligação às proteínas ribossômicas, induzindo uma leitura equivocada do mRNA, que ocasionará alteração no complexo de formação de peptídeos, com consequências na fluidez e permeabilidade da membrana (SOARES-BEZERRA et al., 2004).

Estudo *in vitro* mostrou que, em formas promastigotas e amastigotas de *L. donovani* expostas à paramomicina por 72 horas, houve uma diminuição do potencial mitocondrial, sendo esta organela um dos alvos potenciais da droga (JHINGRAN et al., 2009).

Por ser um medicamento de baixo custo, ampla eficácia e com curto tratamento, em alguns países, como a Índia, seu uso é bastante popular, sendo considerada droga de eleição para pacientes com leishmaniose visceral nestas localidades (SUNDAR et al., 2009). Apresenta poucos efeitos colaterais, entre eles ototoxicidade, dor no local da aplicação e aumento das transaminases hepáticas (SUNDAR et al., 2007).

3.6 Validação científica de plantas com ação leishmanicida

Vários estudos já foram realizados para identificar e caracterizar extratos brutos, frações e óleos essenciais de plantas com atividade leishmanicida. Substâncias, em especial, da classe dos alcaloides, terpenos, benzopiranos flavonoides, outros compostos fenólicos e lactonas sesquiterpênicas, vem sendo identificadas com atividade anti-leishmania (WENIGER et al., 2004; ROCHA et al., 2005; COSTA et al., 2006; BRAGA et al., 2007; FERREIRA et al., 2010; SANTOS et al., 2012; ROTTINI et al., 2015). A triagem desses compostos naturais é feita com culturas de *Leishmania* spp., em virtude da facilidade de manutenção e produção (FUMAROLA et al., 2004), com posterior avaliação em animais de laboratório.

Extratos vegetais ou compostos de origem vegetal podem fornecer uma fonte valiosa de novos agentes medicinais e a necessidade de tratamentos alternativos, eficazes e com menor toxicidade em relação as drogas convencionais, tem levado a uma busca de produtos naturais com potencial ação leishmanicida. Além disso, as vantagens obtidas a partir da busca de produtos naturais com atividade leishmanicida dão um novo impulso para a obtenção de compostos sintéticos valiosos (ROCHA et al., 2005).

Em resumo, listou-se na tabela 3, alguns trabalhos realizados nos últimos dez anos (2005 a 2015) que avaliaram a ação de plantas medicinais (extratos brutos, frações, óleos, compostos isolados) sob a espécie *Leishmania amazonensis*.

Tabela 3 – Avaliação fitoquímica e atividade leishmanicida *in vitro* de óleos, extratos, frações e compostos isolados de determinadas plantas medicinais sob formas promastigotas ou amastigotas* de *Leishmania amazonensis*

Planta	Extrato, Frações ou compostos	Fitocomponentes	IC ₅₀ (µg/ml) promastigotas	Referências
<i>Achillea millefolium</i>	Óleo essencial	-	7,8	SANTOS et al., 2010
<i>Anacardium occidentale</i>	Casca do caule	Al; Tr; Ta; Fl; Na	>250	BRAGA et al., 2007
<i>Annona muricata</i>	Extrato acetato de etila	-	25	OSORIO et al., 2007
<i>Bixa orellana</i>	Sementes	Al; Tr; Fl	22	BRAGA et al., 2007
<i>Bixa orellana</i>	Fruto	Al; Tr; Ta; Fl; Na	>250	BRAGA et al., 2007
<i>Bowdichia virgiloides</i>	Extrato etanólico (folhas)	-	>200	RIBEIRO et al., 2014
<i>Bowdichia virgiloides</i>	Extrato hexânico (folhas)	-	>200	RIBEIRO et al., 2014
<i>Cajanus cajan</i>	Extrato metanólico (folhas)	Al; St; Ta; Sa; Cu; Fl; An	62	BRAGA et al., 2007
<i>Cajanus cajan</i>	Sementes	Al; Tr; Ta; Sa; Fl	>250	BRAGA et al., 2007
<i>Calea montana</i>	Extrato etanólico	-	11*	VALADEAU et al., 2009
<i>Calophyllum brasiliense</i>	Derivados cumarínicos (Mammea A/BB)	-	3	BRENZAN et al., 2007
<i>Campomanesia lineatifolia</i>	Extrato etanólico (folhas)	-	103,27	RIBEIRO et al., 2014
<i>Campomanesia lineatifolia</i>	Fração diclorometano (folhas)	-	>200	RIBEIRO et al., 2014
<i>Campomanesia lineatifolia</i>	Fração acetato de etila (folhas)	-	96,13	RIBEIRO et al., 2014
<i>Campomanesia lineatifolia</i>	Fração hexânica (folhas)	-	147,70	RIBEIRO et al., 2014
<i>Carica papaya</i>	Extrato etanólico	-	11*	VALADEAU et al., 2009
<i>Chrysobalanus icaco</i>	Extrato etanólico (folhas)	-	61,47	RIBEIRO et al., 2014
<i>Chrysobalanus icaco</i>	Extrato hexânico (folhas)	-	62,28	RIBEIRO et al., 2014
<i>Chrysobalanus icaco</i>	Fração diclorometano (folhas)	-	91,64	RIBEIRO et al., 2014
<i>Chrysobalanus icaco</i>	Fração acetato de etila (folhas)	-	77,30	RIBEIRO et al., 2014
<i>Chrysobalanus icaco</i>	Fração hexânica (folhas)	-	130,20	RIBEIRO et al., 2014
<i>Copaifera reticulata</i>	Oleoresin	-	5	SANTOS et al., 2008
<i>Copaifera reticulata</i>	Óleo essencial	-	15*	SANTOS et al., 2012
<i>Cordia verbenácea</i>	Extrato metanólico (folhas)	Tr; Ta	120	BRAGA et al., 2007
<i>Costus spiralis</i>	Extrato metanólico (folhas)	Al; St; Fl	>250	BRAGA et al., 2007
<i>Cymbopogon citratus</i>	Óleo essencial	-	1,7	SANTIN et al., 2009
<i>Cymbopogon citratus</i>	Citral	-	8	SANTIN et al., 2009

<i>Dictyota mertensii</i>	Extrato diclorometano metanol		71,60	ALIANÇA et al., 2014
<i>Dipteryx alata</i>	Extrato etanólico (folhas)	-	51,46	RIBEIRO et al., 2014
<i>Dipteryx alata</i>	Extrato hexânico (folhas)	-	0,08	RIBEIRO et al., 2014
<i>Eugenia uniflora</i>	Extrato metanólico (folhas)	Al; Tr; Ta; Fl; Na	>250	BRAGA et al., 2007
<i>Ginkgo biloba</i>	Flavonoide (Isoginkgetin)	-	1,9*	WENIGER et al., 2006
<i>Hedychium coronarium</i>	Extrato etanólico	-	10*	VALADEAU et al., 2009
<i>Himatanthus sucuuba</i>	Extrato etanólico	-	20	CASTILLO et al., 2007
<i>Hymenaea courbaril</i>	Extrato etanólico (folhas)	-	44,10	RIBEIRO et al., 2014
<i>Hymenaea courbaril</i>	Extrato hexânico (folhas)	-	35,84	RIBEIRO et al., 2014
<i>Hymenaea stagnocarpa</i>	Extrato etanólico (folhas)	-	4,69	RIBEIRO et al., 2014
<i>Hymenaea stagnocarpa</i>	Extrato hexânico (folhas)	-	199,40	RIBEIRO et al., 2014
<i>Hypericum</i>	Extratos lipofílicos		36,1	DAGNINO et al., 2015
<i>Hyptis lacustres</i>	Extrato etanólico	-	10*	VALADEAU et al., 2009
<i>Jacaranda caroba</i>	Extrato etanólico (folhas)	-	13,22	RIBEIRO et al., 2014
<i>Jacaranda cuspidifolia</i>	Extrato etanólico (folhas)	-	10,96	RIBEIRO et al., 2014
<i>Jacaranda cuspidifolia</i>	Fração clorofórmica (folhas)	-	7,43	RIBEIRO et al., 2014
<i>Kalanchoe pinnata</i>	Flavonóide (Quercetina diglicoside)	-	45*	MUZITANO et al., 2006
<i>Laetia procera</i>	Diterpeno (Casearilucine A)	-	11,1	JULLIAN et al., 2005
<i>Laetia procera</i>	Diterpeno (Caseamembrol A)	-	11,0	JULLIAN et al., 2005
<i>Laetia procera</i>	Diterpeno (Laetiaprocerine A)	-	10,9	JULLIAN et al., 2005
<i>Laetia procera</i>	Diterpeno (Laetiaprocerine B)	-	50,9	JULLIAN et al., 2005
<i>Laetia procera</i>	Lactona (Butanolide)	-	111	JULLIAN et al., 2005
<i>Lantana camara</i>	Extrato metanólico (folhas)	Al; St; Sa; Fl	14	BRAGA et al., 2007
<i>Lantana sp.</i>	Extrato etanólico	-	10*	VALADEAU et al., 2009
<i>Lophanthera lactescens</i>	Triterpeno (LLD3)	-	0,41*	DANELLI et al., 2009
<i>Maytenus ilicifolia</i>	Extrato metanólico (folhas)	Al; Tr; Ta; Fl; Na	>250	BRAGA et al., 2007
<i>Melastomum campestre</i>	Extrato etanólico (folhas)	-	120,37	RIBEIRO et al., 2014

<i>Melacium campestre</i>	Extrato hexânico (casca do caule)	-	160,93	RIBEIRO et al., 2014
<i>Melacium campestre</i>	Extrato etanólico (raiz)	-	92,65	RIBEIRO et al., 2014
<i>Ocimum gratissimum</i>	Extrato metanólico (folhas)	Al; St; Ta; Sa; Fl	>250	BRAGA et al., 2007
<i>Ocimum gratissimum</i>	Extrato metanólico (flores)	Al; Tr; Ta; Fl	>250	BRAGA et al., 2007
<i>Ocimum gratissimum</i>	Óleo essencial	-	135	UEDA-NAKAMURA et al., 2006
<i>Ocimum gratissimum</i>	Eugenol	-	80	UEDA-NAKAMURA et al., 2006
<i>Pagianta cerifera</i>	Extrato diclorometano	-	25	BILLO et al., 2005
<i>Piper aduncum</i>	Extrato metanólico (folhas)	Al; St; Ta; Fl; An	29	BRAGA et al., 2007
<i>Piper dennisii</i>	Extrato etanólico	-	10*	VALADEAU et al., 2009
<i>Piper hispidum</i>	Extrato etanólico	-	69	ESTEVEZ et al., 2007
<i>Piper regnellii</i>	Eupomatenoid-5	-	9	VENDRAMETTO et al., 2010
<i>Piper strigosum</i>	Extrato etanólico	-	>100	ESTEVEZ et al., 2007
<i>Plantago lanceolata</i>	Extrato metanólico (folhas)	Al; Tr; Ta; Sa; Cu; Fl	>250	BRAGA et al., 2007
<i>Pluchea carolinensis</i>	Extrato fenólico		0.2-0.9	MONTRIEUX et al., 2014
<i>Polygonum hydropiperoides</i>	Extrato metanólico (folhas)	Al; Tr; Ta; Fl; An	>250	BRAGA et al., 2007
<i>Polygonum hydropiperoides</i>	Extrato metanólico (flores)	Tr; Ta; Fl	73	BRAGA et al., 2007
<i>Pothomorphe umbellata</i>	Extrato metanólico (folhas)	Al; St; Ta; Fl	39	BRAGA et al., 2007
<i>Premna serratifolia</i>	Extrato diclorometano	-	4,4	DESRIVOT et al., 2007
<i>Pseudomalmea boyacana</i>	Extrato acetato de etila	-	48,9	OSORIO et al., 2007
<i>Rollinia exsua</i>	Extrato hexânico	-	20,8	OSORIO et al., 2007
<i>Rollinia pittieri</i>	Extrato hexânico	-	12,6	OSORIO et al., 2007
<i>Rosmarinus officinalis</i>	Extrato metanólico (folhas)	Al; Tr; Ta; Sa; Fl; An	44	BRAGA et al., 2007
<i>Scaevola balansae</i>	Extrato diclorometano	-	8,7	DESRIVOT et al., 2007
<i>Schinus terebinthifolius</i>	Extrato metanólico (folhas)	Al; Tr; Ta; Fl; An	55	BRAGA et al., 2007
<i>Solanum americanum</i>	Extrato metanólico (folhas)	Al; St; Ta	40	BRAGA et al., 2007
<i>Solanum concinnum</i>	Extrato metanólico (folhas)	Al; St; Ta; Fl	>250	BRAGA et al., 2007
<i>Stachytarpheta cayanensis</i>	Partes aéreas	Al; Tr; Cu; Fl	>250	BRAGA et al., 2007
<i>Stryphnodendron obovatum</i>	Extrato etanólico (casca do caule)	-	60.0	RIBEIRO et al., 2014

<i>Syzygium cumini</i>	Extrato metanólico (folhas)	Tr; Ta; Fl; An	>250	BRAGA et al., 2007
<i>Syzygium cumini</i>	Extrato hexânico (folhas)	-	31,64	RIBEIRO et al., 2014
<i>Tanacetum parthenium</i>	Pó da planta	-	490	TIUMAN et al., 2005
<i>Tanacetum parthenium</i>	Extrato diclorometano	-	3,6	TIUMAN et al., 2005
<i>Tanacetum parthenium</i>	Parthenolide	-	0,37	TIUMAN et al., 2005
<i>Tanacetum parthenium</i>	Guaianolide	-	2,6	TIUMAN et al., 2005
<i>Tanacetum parthenium</i>	Fração diclorometano		2,40	RABITO et al., 2014
<i>Vanillosmopsis arborea</i>	Óleo essencial (α -bisabolol)	-	7,35	COLARES et al., 2013
<i>Vernonia polianthes</i>	Extrato metanólico (folhas)	Al; Tr; Cu; Fl	4	BRAGA et al., 2007
<i>Xylopia aromática</i>	Extrato metanólico	-	20,8	OSORIO et al., 2007

Fitocomponentes: Al (alcaloides), Tr (triterpenos), ST (esteróis), Ta (taninos), Sa (saponinas), Cu (cumarinas), Fl (flavonoides), An (antraquinonas); (-) não avaliado; **IC₅₀** - inibição de 50% das formas promastigotas de *L. amazonensis*; * formas amastigotas. Fonte: Adaptado de Tiuman et al. (2005); Braga et al. (2007); Ribeiro et al. (2014).

3.7 Cicatrização

A pele é o órgão mais extenso do organismo e representa a sua primeira barreira de defesa, desempenhando papel fundamental na proteção contra microrganismos (ZHANG e MICHNIAK-KOHN, 2012). Além disso, atua na manutenção da temperatura corpórea e detecção de informações sensoriais do ambiente externo (WOSGRAU et al., 2015).

Por definição, é considerado como ferida a perda da continuidade celular e anatômica de determinado tecido, podendo ter como causas traumas físicos, químicos, térmicos, infecciosos e imunológicos (BENNETT, 1988). Quando isso ocorre na pele, faz-se necessário um processo rápido e eficiente de reparação tecidual, para que haja o reestabelecimento das suas funções (LAVERDET et al., 2015).

A cicatrização é um processo complexo e dinâmico de substituição de um tecido desvitalizado por um novo tecido, que ocorre através de uma série de eventos bioquímicos em cascata; geralmente divididos em três fases: fase inflamatória, fase proliferativa e de remodelação (YOUNG e MCNAUGHT, 2011).

A fase inflamatória inicia-se imediatamente após o dano, com duração variando entre horas e dias, de acordo com a extensão da lesão. É caracterizada por vasoconstricção e agregação plaquetária (GANTWERKER e HOM, 2012).

A lesão dos vasos sanguíneos presentes no local leva ao extravasamento do sangue e seus constituintes, com posterior formação de coágulos e de uma matriz provisória, composta principalmente por fibrina e fibronectina (ROBSON et al., 2001). As plaquetas, envolvidas na formação dos coágulos, liberam citocinas, fatores de crescimento e de diferenciação celular, que contribuem para o recrutamento de leucócitos e para o início da resposta inflamatória (GAWAZ e VOGEL, 2013).

Neutrófilos, monócitos e linfócitos migram rapidamente para o local da ferida e também secretam citocinas e fatores de crescimento, atraindo maior quantidade de células envolvidas na cicatrização e estimulando sua proliferação e sobrevivência. Os macrófagos presentes produzem espécies reativas de oxigênio, que possuem função microbicida, e ajudam a prevenir possíveis infecções, através da sua ação fagocítica (STEED, 1997). Mastócitos também estão envolvidos na fase inflamatória da cicatrização, através da secreção de histamina e de mediadores pro-inflamatórios, que agem sobre a permeabilidade vascular e recrutamento celular (WULFF e WILGUS, 2013).

A fase proliferativa é a segunda fase do processo cicatricial e ocorre entre 2 a 10 dias após a lesão. É caracterizada por neoformação tecidual, com proliferação e migração de diferentes tipos celulares. Primeiramente, há a migração de queratinócitos sobre a derme lesada, seguido da formação de novos vasos sanguíneos, em um processo denominado angiogênese. Os brotamentos dos capilares formados, juntamente com fibroblastos e macrófagos, substituem a matriz de fibrina por um tecido de granulação, que forma um novo substrato para a migração de queratinócitos, em fases posteriores do processo de cicatrização (GURTNER et al., 2008).

Os queratinócitos presentes nos bordos da ferida se proliferam e atingem um estado de maturação, restaurando, desta forma, a função de proteção do epitélio. Os reguladores mais importantes da angiogênese são o fator de crescimento do endotélio vascular A (VEGFA) e o fator de crescimento de fibroblastos 2 (FGF2)

(WERNER e GROSE, 2003). A angiogênese também pode ser resultado do recrutamento de percursoros de células endoteliais derivados da medula óssea, embora esta represente apenas uma pequena contribuição neste processo (BLUFF et al., 2007).

Na parte final dessa etapa, os fibroblastos são estimulados por macrófagos e acabam se diferenciando em miofibroblastos, células contráteis que, com o passar do tempo, aproximam os bordos da ferida (OPALENIK E DAVIDSON, 2005). Fibroblastos e miofibroblastos interagem entre si e produzem matriz extracelular, composta principalmente por colágeno do tipo III (WERNER et al., 2007).

A terceira e última fase da cicatrização consiste na remodelação do tecido de granulação e na formação do tecido cicatricial. Este processo inicia-se entre 2 a 3 semanas após a injúria e pode prolongar-se até um ano ou mais (GURTNER et al., 2008). Dentre as várias proteínas essenciais para o restabelecimento da integridade tecidual, estão presentes as metaloproteases da matriz, que desempenham papel importante na modulação da matriz extracelular e na migração celular (MARTINS et al., 2013).

A síntese de matriz extracelular pelos miofibroblastos é gradativamente reduzida e o colágeno tipo III é progressivamente substituído por colágeno tipo I, que é o principal componente da derme intacta. À medida que há a progressão do processo de remodelação, a maior parte das células endoteliais, macrófagos e fibroblastos sofre apoptose, e a pele tende a recuperar sua composição normal (DESMOULIÈRE et al., 1995).

Diversas plantas são utilizadas pela medicina popular no tratamento de afecções da pele. Dentre as pesquisas nacionais envolvendo fitoterapia e cicatrização, algumas merecem destaque, como o uso da *Aloe vera* (babosa), *Schinus terebenthifolius* (aroeira), *Stryphnodendrom barbatiman* (barbatimão), *Calendula officinallis* (calêndula) e *Triticum vulgare* (trigo); pelo número de estudos utilizando fitofármacos baseados nestas espécies (DORNELES et al., 2003; MARTINS et al., 2003; BRANCO NETO et al., 2006; SOUZA et al., 2006).

A comprovação científica dos efeitos terapêuticos de plantas utilizadas na medicina tradicional tem levado a um maior número de estudos, visando avaliar o

potencial, preventivo ou curativo, especialmente dos seus compostos isolados. Esta combinação entre conhecimento empírico e científico pode produzir novos medicamentos cicatrizantes com menos efeitos colaterais indesejáveis (MAVER et al., 2015).

4 JUSTIFICATIVA

A leishmaniose cutânea é uma importante doença infecto-parasitária com altos números de novos casos anualmente, em especial nas duas últimas décadas, representando uma das antropozoonoses de maior problema para a saúde pública. As quimioterapias utilizadas já apresentaram casos de resistência, além disso, ressalta-se os agressivos efeitos colaterais propiciados por esses fármacos, os quais na maioria das vezes são cardio, nefro e/ou hepatotóxicos e, quando utilizados em sub-doses não apresentam o efeito desejado ou favorecem o percentual de resistência do parasito.

Desta forma, em face a diversidade da flora brasileira, as deficiências terapêuticas atualmente existentes para humanos com drogas convencionais, associada a ausência de fármacos específicos para animais, que são importantes reservatórios no ciclo biológico do parasito, bem como a necessidade de produtos de baixo custo, fácil obtenção e aplicação é que se fundamenta o desenvolvimento desta pesquisa na busca de substâncias naturais.

Ressalta-se ainda, que as plantas utilizadas neste estudo, *A. chica* e *Vernonia* sp., fazem parte das 71 espécies com potencial terapêutico que compõem a Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS), que visa orientar o desenvolvimento de pesquisas com as mesmas e, consequentemente, ampliar a lista de fármacos fitoterápicos disponíveis a assistência básica do SUS em todo o país.

Nesse contexto, a busca por recursos terapêuticos leishmanicidas, provenientes de plantas medicinais, de fácil obtenção, disponibilizado no serviço básico do SUS e fácil administração (via tópica), para populações de menor poder aquisitivo e/ou distante dos centros urbanos foi o que instigou o desenvolvimento desta pesquisa.

5 HIPÓTESE

Arrabidaea chica Verlot. e *Vernonia brasiliiana* Less. possuem atividade leishmanicida e cicatrizante cutâneo?

6 OBJETIVOS

6.1 Geral

- Realizar estudo fitoquímico e avaliar a capacidade leishmanicida e cicatrizante cutâneo das espécies *Arrabidaea chica* e *Vernonia brasiliiana*.

6.2 Específicos

- Identificar os constituintes químicos por meio de análise fitoquímica e CG-MS presentes no óleo da *V. brasiliiana* e nas frações clorofórmica, metanólica e acetato de etila das espécies *A. chica* e *V. brasiliiana*;
- Avaliar a toxicidade *in vitro* e *in vivo* dos extratos brutos, frações e óleo essencial das espécies vegetais estudadas;
- Avaliar a atividade leishmanicida *in vitro*, sob formas promastigotas, tratados com óleo, extratos brutos e frações das espécies vegetais escolhidas;
- Avaliar a ultra-estrutura das fomas promastigotas após tratamento com as espécies vegetais em estudo;
- Avaliar macroscopicamente e histologicamente o processo cicatricial cutâneo após tratamento com as espécies vegetais escolhidas.

Parte II

7 CAPÍTULO I

Artigo “Leishmanicid potential, cytotoxicity and wound healing of

***Arrabidaea chica* Verlot.”**

Submetido ao periódico *BMC Complementary and Alternative Medicine*, cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior é B1, fator de impacto 1.88.

O artigo foi elaborado e formatado conforme as normas do periódico supracitado, que seguem anexas ao trabalho juntamente com o comprovante de submissão.

Leishmanicid potential, cytotoxicity and wound healing of *Arrabidaea chica* Verlot.

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ABSTRACT

BACKGROUND - Leishmaniasis includes a wide complex of diseases that affect humans and other mammals, ranging from a mild cutaneous form to a severe visceral form. The standard treatment, with pentavalent antimony, is concerning due to its toxic effects. The search for alternative, effective and less toxic treatments has led to the search for natural products. This study aimed to evaluate cytotoxic, leishmanicidal and healing potential of *Arrabidaea chica*. **METHODS** - The crude ethanolic extract, as well as the chloroform, methanol and ethyl acetate fractions of *A. chica* were made, followed by phytochemical analysis. Cytotoxic evaluation was performed through MTT colorimetric assay, and the CC₅₀ was determined. After that, was analyzed the effect of the extract and fractions against *Leishmania amazonensis* promastigotes, at the intervals of 24, 48 and 72 hours, and determination of IC₅₀. The healing effect of the plant was also tested. **RESULTS** - Phytochemical screening showed that the crude extracts have flavonoids, tannins, anthocyanins and chalcones. The leishmanicidal potential of *A. chica* showed satisfactory results in concentrations between 60 to 155.9 µg/mL. The cytotoxic assay exhibited a

reduction of 50% of viable cells at the concentration of 189.9 µg/mL. The healing results indicated that the treated group exhibited more pronounced signs of resolution of the lesion in the early days, but this pattern did not persist throughout the treatment. **CONCLUSION** - These results demonstrates that *A. chica* has cytotoxic and leishmanicidal potential but its healing effect must be better studied.

Key-Words: *Arrabidaea chica*; Leishmanicid; Cytotoxicity; Wound healing.

1 BACKGROUND

Leishmaniasis is a group of disease that affect several species of mammals that live in tropical and subtropical regions. In accordance of literature more than 20 species of the protozoan *Leishmania* cause leishmaniasis, including cutaneous and visceral leishmaniasis [1, 2]. These clinical forms differs among them by clinical presentations, morbidity and mortality [3].

For World Health Organization, neglected disease are those disease associated to low social indicators, thus, this kind of diseases are more frequent in underdevelopment countries whereas there is scarcity of resource destined to control and treatment and there is few applied researches in order to appoint a good solution [4].

Among clinical form, the cutaneous is the most commom, which is characterized by skin lesions chiefly in exposed body parts [5]. Depending the specie of *Leishmania*, some patients, the leishmaniasis could heal without treatment; other, could develop cutaneous diffuse or mucocutaneous form [6].

In all clinical form pentavalent antimonials have been used as the mainstays therapy: sodium stibogluconate (Pentostam) is the available drug for the treatment of Leishmaniasis in United State of America and Europe; on the other hand, meglumine antimonate (Glucantime®) is more use in South America and Africa [7]. Despite

this therapy have been widely employed since 1940, the mechanism of actions is poorly understood and adverse effects are severe, including renal and hepatic toxicity [8]. Other limitations of this therapy is necessity of daily parenteral administration and drug resistance [2].

These limitations, vast Brazilian biodiversity and traditional knowledge lead the establishment of research networks in order to evaluate the leishmanicidal effect of plants of diverse biomes, such as Cerrado and Amazon. Thus, it is very important to draw experiments using natural products in order to disclose new compounds, which be less toxic and could be oral route. Besides that, Brazilian government elaborated the RENISUS (Rede Nacional de Plantas Medicinais de interesse do SUS), a list of plants that could be used in the Unified Health Service (SUS). *Arrabidaea chica* was included in this list.

Arrabidaea chica Verlot belongs to the Bignoniaceae Family. It is endemic in almost Brazilian territory; however, it is more frequent in Amazon Forest [9] where it used to treat skin disease, mulligrubs, anemia, jaundice and inflammatory reaction [10 - 12]. Indiscriminate use by native population lead to researchers from different countries evaluate medicinal properties of this plant, such as: anti inflammatory [13]; astringent, antioxidant [14, 15]; anti ulcer [16]; anti-hypertensive [17]; antimicrobial, antifungal [18, 19] and wound healing [20, 21].

The aim of this paper was to evaluate the phytochemical analysis of *Arrabidaea chica*, wound healing capacity and cytotoxicity, as well to evaluate the action of crude extract and fractions of this plants in promastigotes of *L. amazonensis*.

2 METHODS

2.1 Ethics Committee

The experimental protocol for this study was approved by the Ethics Committee for Animal Research of Universidade Estadual do Maranhão (CEUA-UEMA protocol 26/2009) and by the Ethics Committee in Animal Experimentation of Instituto Oswaldo Cruz - FIOCRUZ/RJ (CEUA-FIOCRUZ protocol LW72/12).

2.2 Plant

Leaf and stem samples of *Arrabidaea chica* Verlot. were cultivated and collected in the Atico Seabra Herbarium, Departamento de Farmácia, Universidade Federal do Maranhão (São Luís, Maranhão, Brazil). The voucher specimen was deposited under the number 1067. The plant material was collected between August and November 2011, corresponding to the dry season, with a rainfall rate of 900 mm and an average temperature of 28°C.

2.3 Preparation of crude extract and fractions

To obtain the crude ethanolic extract, the samples were dried at 40°C, with forced air circulation. The dried material from each part of *A. chica* was macerated and immersed in ethyl alcohol, in a proportion of 3:1 (alcohol and plant material, respectively). Solvent removal was carried out by rotoevaporation, under reduced pressure at 30 to 40°C, until the desired concentrations were achieved, in order to obtain the ethanolic extracts. The samples were stored under refrigeration and protected from light until analysis.

To determine dry weight of the samples were used three aliquots of 0.5 mL of crude ethanolic extract [17]. To calculate the income of samples, were used the total weight of the powder, the dry weight obtained and the total final volume of the concentrated extract.

The crude ethanolic extracts were successively partitioned with methanol, chloroform and ethyl acetate to obtain the respective fractions. The extraction was carried out in solid phase using silica gel, grail, pistil and approximately 500 mL of each solvent. After obtaining the fractions, they were submitted to the leishmanicidal and cytotoxic tests. Those that showed satisfactory results were sent to the Analytical Center of Universidade de São Paulo (São Paulo, Brazil), to identify bioactive compounds through gas chromatography and mass spectrometry.

2.4 Phytochemical analysis

To evaluate the presence of secondary metabolites (saponins, anthocyanins, chalcones, phenols, tannins, flavonoids, steroids, triterpenoids and anthraquinones), crude ethanolic extracts of *A. chica* were solubilized in methanol (in a proportion of 100 mg/5 mL), according to Costa (1982) and Matos (1997) [22, 23].

2.5 *In vitro* cytotoxic assay

2.5.1 Obtainment of peritoneal macrophages and cell culture

Peritoneal macrophages were obtained from eight week old female BALB/c mice, from the Laboratory Animal Breeding Center (CECAL-FIOCRUZ) and kept in a vivarium of Hélio and Peggy Pereira Pavilion, located at Oswaldo Cruz Foundation (FIOCRUZ *Leishmania amazonensis*, Rio de Janeiro, Brazil).

3 mL of 3% sodium thioglycollate were inoculated into the peritoneal cavity of the animals and, after 48 hours, they were euthanized in a CO₂ chamber. For macrophages recovery, were injected 5 mL of a cold and sterile PBS 1X solution into their abdominal cavity, followed by massage and collection of lavage, which was kept in a falcon tube placed in an ice box. After collection, the lavage was analyzed under optical microscope to verify the presence of macrophages and lack of red blood cells and bacteria.

Peritoneal lavage was centrifuged at 500 G for 10 minutes at 4°C. The supernatant was separated and the cells resuspended in 1 mL of RPMI - 1620 and counted in a Neubauer chamber. Macrophages final concentration was standardized at 5x10⁵ cells/mL. For cell culture, it was used RPMI supplemented with 10% fetal bovine serum, L-glutamine (20 mM), sodium bicarbonate solution (7.5%), penicillin (100 µg/mL) and streptomycin (50 µg/mL), incubated at 37°C with 5% CO₂.

2.5.2 CC₅₀ determination

Cytotoxicity was measured by MTT colorimetric assay, according to [57]. 100 µL of peritoneal macrophages (5x10⁵ cells/mL) were added in 96-well plates and incubated for 2 hours, at 37°C with 5% CO₂. After that, 100 µL of the crude ethanolic extracts and its fractions of *A. chica* were added to the wells in serial concentrations (600 to 0.97 µL/mL). For each concentration was maintained a negative control (with the extract and RPMI). DMSO was used as a reference drug in serial dilutions starting with 20%. After 24 hours of incubation, 5 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were added to each well, then the plates were incubated again for 2-4 hours, under the same conditions. Later, they were centrifuged at 1500 rpm and 200 µL of each well supernatant were discarded, then 100 µL of DMSO were added. The absorbance was measured in a spectrophotometer, at a wavelength of 540 nm. 50% inhibitory concentration (CC₅₀) was calculated using GraphPad Prism 5.

2.6 *In vitro* antileishmanial activity

2.6.1 Parasites

In this study was used strain (MHOM/BR/76/MA-76 – IOC/FIOCRUZ-RJ), isolated from a patient with diffuse cutaneous leishmaniasis and maintained by serial passages in BALB/c mice, and periodically re-isolated in culture medium.

Promastigotes obtained from *in vitro* cultures were grown at 26°C in Schneider's medium, supplemented with 10% fetal bovine serum, streptomycin (50 µg/mL) and penicillin (100 µg/mL). To ensure the characteristic of infectivity were used only cultures of promastigotes with a maximum of seven *in vitro* passages.

Before the evaluation of leishmanicidal activity and determination of the 50% inhibitory concentration (IC₅₀) of *A. chica*, it was performed a screening test to determine the concentration of use, with values ranging between 500 and 0.97 µg of crude ethanolic extracts and fractions. They were diluted in 1% DMSO and added in 96-well plates containing RPMI, serially and in triplicate. Wells containing only the medium and parasites, only with the medium and wells with the reference drug, Amphotericin B (2 µg/mL) were used as controls. 100 µL of *L. amazonensis* promastigotes in logarithmic phase (1x10⁶ parasites/mL) were added in each well, and the plate was incubated at 26°C. Their feasibility was measured at the end of 24, 48 and 72 hours, by observing viable parasites under an inverted microscope. These were classified according with parasites mobility on a scale of zero to ten, where zero means no viable forms and ten, viable forms.

Based on the screening results it was determined the IC₅₀, following the same aforementioned protocol, and the leishmanicidal activity was assessed by counting the viable parasites in a Neubauer chamber. Then, data were normalized using the formula: Percentual of growth inhibition = number of parasites in the control well/number of parasites in test wells; and used for statistical analysis.

2.7 Wound healing activity

2.7.1 Ointment formulation and therapeutic protocol

Two topical preparations (10 mg/g and 20 mg/g) were made, containing the crude ethanolic extract from *A. chica* leaves mixed with Lanette® cream, as a water-soluble anionic vehicle.

Forty 4-to-6 week old male Swiss Webster mice were used in this experiment, from the Central Vivarium of Universidade Estadual do Maranhão (São Luís, Maranhão, Brazil). Animals were randomly divided into two groups (n = 20 each). Control group was treated with Lanette® cream and experimental group treated with Lanette® cream containing the crude ethanolic extract of *A. chica*.

For creation of a dorsal skin wound, animals were anesthetized with an intraperitoneal injection of xylazine hydrochloride (10 to 15 mg/kg) and ketamine hydrochloride (100 to 150 mg/kg), followed by trichotomy of the dorsal region and demarcation of the skin with a punch. Resection of a circular segment of approximately 6 mm diameter was made, exposing the dorsal fascia. Hemostasis was made by digital compression with sterile gauze and the treatment was initiated immediately after hemostasis.

Therapeutic protocol was performed two times a day throughout the experimental period. Transverse and longitudinal diameters of the lesions were measured daily, using a digital caliper, and monitored through photography. Skin wound areas were calculated using the formula $A = \pi \times R \times r$, in which A represents the area, π is equal to 3.14, R represents the largest diameter and r the smallest diameter of the wound.

2.7.2. Histological analysis

After 3, 7, 14 and 21 days of treatment, mice were euthanized and the lesions were excised with a margin of 1cm of integrate skin, stored in 10% buffered formalin

solution and submitted to histological procedures. After embedding in paraffin, 5 µm thick cross sections were cut and stained with hematoxylin-eosin (HE) for morphological analysis of the tissue. Additionally, fragments of liver, spleen and kidney were collected, for evaluation of potential cytotoxic lesions.

Slides were analyzed by light microscopy and by two independent examiners, following the parameters: crust formation, presence of inflammatory infiltrate, angiogenesis, reepithelialization and collagen formation. These features were graded as absent, mild, moderate and severe, according to its appearance.

2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0.4 (GraphPad Software Inc.). IC₅₀ and CC₅₀ values were calculated from the mean percentage reduction of promastigotes and macrophages, respectively. Curves were determined by application of sigmoidal regression to logarithm concentration response data. In vitro results are representative of three different experiments, performed in triplicate, under the same temperature, CO₂, time and culture medium conditions, with similar results. Analysis of wound healing process was made by Student t test. Histological variables were analyzed by non-parametric test.

3 RESULTS

The crude ethanolic extracts of *Arrabidaea chica* leaves and stem presented an income of 59.2% and 3.33% respectively, which shows a low extraction efficiency of the stem, while using the same extraction method for the leaves.

Phytochemical screening showed both leaves and stem extracts have flavonoids, tannins, anthocyanins and chalcones. Phenolic compounds were found only on the leaves (Table 1).

In screening tests to evaluate the leishmanicidal potential in *L. amazonensis* promastigotes, *A. chica* stem extract showed no activity against the protozoan, even at the highest tested concentration (500 mg/mL), so it was not used on tests continuity.

In the screening tests of the crude ethanolic extract of *A. chica*'s leaves, there was a reduction of 50% of *L. amazonensis* promastigotes viability on the concentration of 125 µg/mL (Figure 1). In the intervals of 24, 48 and 72 hours of incubation. However, the concentration of 250 µg/mL already showed an antileishmanial activity in the first 24 hours of treatment and the total absence of viable parasites after 72 hours of incubation. The concentration of 500 µg/mL of the leaves ethanol extract was the one that showed the best leishmanicidal efficacy, in the three tested intervals.

After screening, it was determined the 50% inhibitory concentration (IC₅₀) of *L. amazonensis* promastigote forms which was 155.9 µg/mL (Figure 2).

When evaluating the leishmanicidal activity of the chloroform, methanol and ethyl acetate fractions, there was a 50% reduction of *L. amazonensis* promastigotes at concentrations above 120 µg/mL, 120 µg/mL and 60 µg/mL, respectively (Table 2).

This result demonstrated that the fractions had an action against the parasite in lower concentrations than the crude extract, and that the ethyl acetate fraction had the best result.

The cytotoxicity assay in peritoneal macrophages showed that the concentration of 189.9 µg/mL of *A. chica* leaf extract reduces by 50% the number of viable cells, corresponding to CC₅₀ (Figure 3).

Relating the CC₅₀ and IC₅₀ values of the crude extract, it is noticed a higher selectivity (Selectivity Index = 1.218) of bioactive compounds of *A. chica* by the

parasite than to the host cell, indicating a promising study in the treatment of leishmaniasis.

Regarding the evaluation of dermal irritation potential of the crude extract of *A. chica* leaves, the concentration of 20 mg/g caused skin irritation, with the presence of moderate and persistent erythema during the treatment period. Thus, the healing test was performed with the concentration of 10 mg/g.

In the macroscopic analysis of the wound healing process, it was noticed the establishment of tissue repair process, with the presence of crusts on the third day. On the seventh day it was already observed a reduction in the size of the wound and the formation of granulation tissue in both groups, treated and control (Figure 4).

In the interval of 0-3 days was observed in the group treated with *A. chica* an increase of lesion area as compared to the control group (Figure 5). In 3rd to the 10th day, there was a marked reduction in wound area, with gradual approximation of the edges in both groups, control and treated.

Between the 14th and 21th post-injury days, the retraction of the wound was almost complete, with a marked healing and tissue repair.

For histological evaluation of the healing process, were used as parameters: the presence of cellular debris, inflammatory cells, angiogenesis, collagen and epithelium formation. On the third day of treatment were observed cellular debris and moderate to intense inflammatory process in both groups. Discrete angiogenesis, collagen deposition and epithelialization were observed in the treated group (Figure 6 A-B), as well as an increased amount of mast cells.

On the seventh day of treatment, re-epithelialization and reduction of cell debris and inflammatory process was quite evident. There was no difference between the two

groups; however, the patterns of re-epithelialization, angiogenesis and collagen deposition were higher in the control group.

The healing process evaluation at the 14th to 21st day showed epithelialization and intense collagen deposition in the control group (Figure 6 C-D), while the treated group (Figure 6 E-F) showed different patterns ranging from mild to intense. In both groups, there was a mild number of inflammatory cells and mast cells. Concerning to angiogenesis, it was discreet in the treated group; in the control group there were variations from moderate to mild between the 14th and the 21st day.

The topical preparation with the crude extract of *A. chica* at a concentration of 10 mg/g had a better healing activity in the initial phase of the experiment, however, was not able to induce a stronger response throughout the 21 days of observation.

4 DISCUSSION

Plants are important sources of bioactive compounds, and represent a great potential in the research of new therapeutic agents for several diseases, among which we highlight those of interest to public health, in particular neglected diseases such as leishmaniasis.

The bioactive compounds produced by plant cells may vary depending on soil, climate and season that the plant, fruit or leaves were collected. The soil and climate conditions, along with the plant need for adaptation and defense, cause this change in chemical constituents [24].

The phytochemical analysis of *A. chica* showed the presence of flavonoids, tannins, anthocyanins, chalcones and phenolic compounds as previously described for this species [25-29].

The first chemical study with the leaves of *A. chica* was performed by [30]., who reported the isolation of the substance 3-deoxyanthocyanidin, more specifically 1,6,7-

dihydroxy-5,4'-dimethoxy-flavylium (carajurin) and 6,7,4'-trihydroxy-5', methoxy-flavylium (carajurone), responsible for the reddish color of the extract. In other studies other compounds, such as the flavone acacetin, oleanolic triterpene acid and two deoxiantocyanidins (6,7,3'-4'-tetrahydroxy-5-methoxy-flavylium and 6,7,3'-trihydroxy-5,4'-dimethoxy-flavylium) were isolated [31 - 22].

Anthocyanins are plant pigments belonging to the family of flavonoids, included in the group of phenolic compounds [34] . [35] Report that more than 50 new anthocyanins were isolated from flowers, fruits, seeds and leaves of plants. They play a pivotal role in the flower pollination mechanism, seed dispersal by insects and defense against predatory insects [36].

Pharmacological studies with anthocyanins reveal its high antioxidant power [16] in metabolic diseases [37]. [14] Evaluated the antioxidant, cytotoxic, antimicrobial and diuretic potential of ethanol extract and fractions of *A. chica*, and confirmed the antioxidant and diuretic action, and the absence of antimicrobial and cytotoxic response. The therapeutic activity of the phenolic compounds is mainly attributed to their antioxidant capacity, chelation of metal ions, modulation of gene expression and interaction with cell signaling pathways [38].

[15] Evaluated the phenolic composition and antioxidant activity of the leaves of *A. chica*, by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), and verified the presence of two important flavonoids, apigenin and scutellarein, the major responsible for the significant antioxidant action.

[39] In pharmacological tests, determined the chemical prospecting of *A. chica*'s dye, which is widely used by indigenous tribes, and verified the presence of alkaloids, anthocyanidins, anthocyanins, anthraquinone, steroids, triterpenoids, phenols, flavanonois, flavanols, flavanones, saponins and catechin tannin. This study,

presented some compounds undetected in our research, differing in the presence of anthraquinones, steroids, triterpenoids and saponins. This fact probably is due to climate differences between the two places where the plant was collected, Maranhão and Pará State, as well as how the extract was obtained. Both were obtained using the ethanol as a solvent; however, in our study it was submitted to a 72 h rotoevaporation at 40°C, while at study [39], the extract was macerated for 10 days in a capped percolator.

A pharmacological study demonstrating an in vitro antineoplastic and antioxidant activity and *A. chica* related these benefits directly to the higher content of flavonoids, especially anthocyanins, in the plant, reinforced by biotechnologies, such as treatment with xylanases [29] . [40, 41] Also evaluated the antineoplastic activity of *A. chica*, strengthening this relation with the presence of anthocyanins, flavonoids or the action of both. These and other benefits made essential the search for new sources of anthocyanins [37] .

The secondary metabolites in *A. chica*, such as flavonoids, which have an antimicrobial, antiviral, antiinflammatory, antineoplastic, antifungal and anti-protozoan action already reported in the literature [18, 41 - 43], induces many researchers to develop studies using this plant.

In our study, we evaluated the leishmanicidal potential of the crude extract and fractions of *A. chica*, which showed satisfactory results at the concentrations from 60 to 155.9 µg/ml, depending on the type fraction. The chemical constituents of the plant were more selective for the protozoan than to the host cell, with cytotoxicity effect at concentrations higher than those with the leishmanicide action.

Several studies have been performed to identify and characterize crude extracts, fractions and essential oils [44 - 51], as well as plant's isolated compounds [52, 53]

with leishmanicidal activity. The screening of these natural compounds is made with cultures of *Leishmania* spp., once it is easy to maintain and produce [54].

According to the World Health Organization, a candidate drug must undergo in vitro cytotoxicity and antiparasitic activity. After this initial screening, the results are evaluated to define the continuity of studies and the possibility of in vivo assays [55].

[56] Investigated the leishmanicidal effect of five fractions obtained from the crude hexane extract of *A. chica*, under *L. amazonensis* and *L. infantum* promastigotes forms, by determining the minimum inhibitory concentration (MIC), which was 37.2 and 18.6 µg/ml, respectively. This result is related to one of the fractions used, which had as its main chemical components: linoleic acid methyl ester (25.38%), n-hexadecanoic acid (19.61%), octadecanoic acid (14.10%) and gamma-sitosterol (12.85%). They also evaluated ultrastructural alterations of the parasite under the influence of this fraction, observing mitochondrial swelling, disruption of mitochondrial membrane, presence of vesicles in the cytoplasm, mitochondria and flagellar pocket and modifications in Golgi complex.

The significant increase in researches of plant compounds with leishmanicidal potential is due to of the major importance of this disease, which has high numbers of new cases annually. According to the World Health Organization are recorded 1.3 million new cases per year, 300,000 cases of visceral leishmaniasis and 1 million cutaneous leishmaniasis [5].

Leishmaniasis are characterized as neglected diseases since it is associated with low social indicators, being more prevalent in developing countries, whose resources applied in control, treatment and research are insufficient [4].

Additionally, plant extracts or plant compounds can provide a valuable source of novel medicinal agents and the need for alternative, effective and less toxic

treatments, compared to conventional drugs (pentavalent antimonials, amphotericin B, pentamidine) has led to a search for natural products with a potential leishmanicidal action. Moreover, the benefits obtained from the search for natural products have given a new impetus to obtain valuable synthetic compounds [45]. The crude extract from the leaves of *A. chica* showed a cytotoxic effect at the concentration of 189.9 µg/mL. However, in vivo experimental studies did not show any clinical or histopathological signs of extract toxicity, either in the gastric mucosa [58], in the pleura [59] or open wounds, sutured wound and burns [60]. The low toxicity of the aqueous extract of the plant, with no unusual signs or histopathological changes were also observed by [61].

[18] When assessing antifungal, antimicrobial and antiprotozoan potential of the ethanolic extract and fractions of *A. chica*, observed growth inhibition of *Trichophyton mentagrophytes* and a significant trypanocidal effect (*Trypanosoma cruzi*), besides the absence of relevant acute toxicity, even at the highest dose tested (1000 mg /kg).

[17] Evaluated the hydroalcoholic extract of the leaves of *A. chica*, according to its chemical composition, toxicity and activity in the cardiovascular system of normotensive and hypertensive rats, and found that chronic administration of the extract induced a hypotensive effect, with decreasing systolic blood pressure, associated with the lack of toxicological effects.

Several factors must be taken into consideration when evaluating the toxic effects of a plant extract, for example, seasonal and environmental factors, genotypic variations of the species, the part of the plant used to produce the extract, the time of collection and the age of plant. The traditional methods of extraction can also affect the income and quality (chemical composition) of the final product to be tested [29].

In traditional medicine in the Amazon region, *A. chica* is widely used as anti-inflammatory, healing and anti-anemic; prepared from the leaves as a tea, for oral or vaginal administration [16, 18, 62]. However, despite its wide popular use, several studies are still necessary to evaluate the medical potential of this plant.

In this study, we evaluated the healing potential of the crude extract of the leaves of *A. chica* in skin lesions. Initially was observed a satisfactory answer, with angiogenesis and collagen deposition, but throughout the 21-day observation period, it was found that the extract did not accelerate healing, presenting a similar profile to the control group.

Several local and systemic factors can cause variations in the healing process, leading to a delay or even prolongation of healing. Among them, local of the lesion, existence of infection, surgical technique, tissue ischemia and bandages as well as malnutrition and/or deficiency of trace minerals and vitamins [63, 64].

It is noteworthy, as cited above, that the bandages are important in the healing process. Several studies demonstrates there is a better success in healing process when the injury area is protected by specific bandages placed after the application of the test product [16, 65]. This step, not done in our study, may have influenced the retardation of healing. [66] Reports that the application of a suitable compress enables the increase of the natural debridement and simplifies the healing, because it keeps the exudate rich on mediators in the wound surface.

Wound healing is a complex and dynamic process of replacement of a devitalized tissue by a new tissue, which occurs through a series of biochemical cascade events; usually divided into three phases: inflammatory phase, proliferative and remodeling phase [67]. For a long time, wound healing treatment has been improved, in order to get always the best scarring results in the shortest possible time.

In this research, we observed a strong inflammatory response at the beginning of the healing process, with increased lesion area until the 3rd day after the surgical incision and the presence of inflammatory cells in the histological evaluation. The inflammatory phase is characterized by increased vascular permeability, immune cells chemotaxis to the site of injury, due to the release of mediators (histamine, serotonin, bradykinin, prostaglandins, thromboxanes [68].

The most pronounced reduction period in the wound area was from the 3rd to the 10th day, with retraction of the wound area, comprising the proliferative phase. On this, it is observed fibroplasia, granulation tissue formation, removal of cellular debris, with the formation of a temporary repair tissue during inflammation, and development of tissue substitutes [69].

In the healing process, fibroplasia occurs along with neovascularization [68]. With the advance of wound healing process, fibroblasts undergo phenotypic changes, with abundance of rough endoplasmic reticulum, due to intense protein synthesis; and subsequent transformation into myofibroblasts, which will help in the retraction of the lesion [70,71].

Studies performed by [16], when evaluating the healing, anti-inflammatory, antiulcerogenic and antioxidant effect of crude methanolic extract from *A. chica*, found that there was no reduction of paw edema induced by carrageenan or ear edema induced by croton oil in rats, however, they observed in vitro fibroblast proliferation induction, stimulation of collagen synthesis in vivo and in vitro, anti-ulcer activity and moderate antioxidant capacity.

It is noticed that after 10 days, [16] verified in the experimental group 96% of the wounds healed, with a prominent collagen deposition, while in the control group,

treated with saline solution, only 36% of the wounds were healed, with a mild collagen deposition.

[21] Investigated the topic healing effect of *A. chica* extract in Wistar rats' tendon section and found a better collagen organization in the treated groups when compared to the control group, as well as an increase in the amount of dermatan sulfate on the 14th day treatment.

[63] Reported that compounds exhibiting antioxidant activity can be indicative of good therapeutic agents in the healing process. Non-enzymatic antioxidant activity is promoted by flavonoids, carotenoids, ascorbic acid, glutathione, vitamin E, among others [65, 72, 73]. Thus, the healing activity of *A.chica* is attributed to the antioxidant activity of anthocyanins and to the fibroblast proliferation stimuli, and consequent collagen synthesis [16, 74].

Fibroblasts are connective tissue cells, essential for dermis formation, due to collagen production, responsible for structural firmness. After tissue injury, fibroblasts near the lesion site proliferate, migrate to the wound and produce a large amount of a matrix rich in collagen (type I and III), which helps to isolate and repair the injured tissue [70].

The proliferation of fibroblasts is directly related to the presence of macrophages in the healing process, once they release growth factors and cytokines essential for the maturation of the inflammatory phase and the beginning of the healing. Among the growth factors responsible for the proliferation, highlights are IGF-I and TGF- β . Studies have demonstrated that IGF-I increases the procollagen chain expression in cultured dermal fibroblasts [70].

In this research, control and experimental groups presented on day 14 lower healing area compared to day 07. At the end of the experiment, after 21 days, both groups

were in the final stages of the healing process, in which predominates the remodeling phase, characterized by the reduction of inflammatory cells [75], blood vessels at the lesion site [68], being the stage in which the scar acquires its maximum resistance [76].

Several plants are used in popular medicine for the treatment of skin disorders.

Among national research involving herbal medicine and healing, some are worth mentioning, such as the use of *Aloe vera* (“babosa”), *Schinus terebenthifolius* (“aroeira”), *Stryphnodendrom barbatiman* (“barbatimão”), *Calendula officinalis* (“calendula”) and *Triticum vulgare* (“trigo”); by the number of studies using phytopharmaceuticals based on these species [77 - 80].

5 CONCLUSION

Our work demonstrates that the compounds extracted from *A. chica* leaves are potential leishmanicidal agents in non-cytotoxic concentrations, requiring further researches that continue to evaluate this potential, in order to develop new drugs or be associated with drugs already used in therapeutic protocols for the disease. The wound healing potencial needs additional studies, in order to eliminate the interference of external factors and optimize the action of *Arrabidaes chica*’s extract.

LIST OF ABBREVIATIONS USED

°C – Celsius degree; µg/mL – microgram per milliliter; µL – microliter; µm – micrometer; µM – micromolar; AlCl₃ - aluminum chloride; ANOVA – Analysis of variance; CC₅₀ – 50% cellular cytotoxicity; CO₂ – carbon dioxide; DMSO – dimethyl sulfoxide; FeCl₃ - iron chloride; FGF2 - fibroblast growth factor 2; G – giros; HCl – hydrochloric acid; HE- Hematoxylin-eosin; IC₅₀ – 50% inhibitory concentration; IGF-I Insulin-like growth factor 1; mg/kg – milligrams per kilogram; mg/mL – milligrams per milliliter; mM – millimolar; MTT- (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium

bromide); NaOH – sodium hydroxide; PBS - Phosphate Buffered Saline; RPMI - Roswell Park Memorial Institute medium; TGF- β Transforming growth factor beta.

CONFLICT OF INTEREST

The authors confirm they have no conflicts of interest related to this work.

AUTHORS' CONTRIBUTIONS

JCS and FAS carried out the phytochemical analyses, leishmanicidal and cytotoxic assays, and made substantial contributions to conception, acquisition and interpretation of data. RMO, ISSO, IFBM and LL made contributions to interpretation of data, review and edition of the manuscript. AFAL and HSF collected the plant material, prepared the extracts and contributed to interpretation of data. ALAS conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Table 1 – Phytochemical prospection of crude ethanolic extract of leaves and stem of *Arrabidaea chica*, collected in Maranhão State, Brazil

Metabolites Class	Reactions	<i>Arrabidaea chica</i>	
		Leaves	Stem
Anthraquinones	NaOH	-	-
Flavonoids	AlCl ₃	+	+
Triterpenes	Anhydrous acetic	-	-
Ester	Sulphuric acid	-	-
Saponin	Foamming index	-	-
Phnenolic compound	FeCl ₃	+	-
Tannin	Gelatin	+	+
Anthocyanin	HCl / NaOH	+	+
Chalcones	HCl / NaOH	+	+

(+) presence; (-) absence

Table 2 – 50% inhibitory concentration (IC₅₀) of *Leishmania amazonensis* promastigote forms after 72 hours treatment with crude ethanolic extract and chloroform, methanolic and ethyl acetate fractions of *Arrabidaea chica* and the reference drug Amphotericin B

Treatments	IC ₅₀ (µg/mL)
Crude ethanolic fraction	155.9 ± 0.1185
Chloroform fraction	>120µg/mL
Methanolic fraction	>120µg/mL
Ethyl acetate fraction	>60µg/MI
Amphotericin B	0.76 ± 0.9218

Values represent mean ± standard deviation.

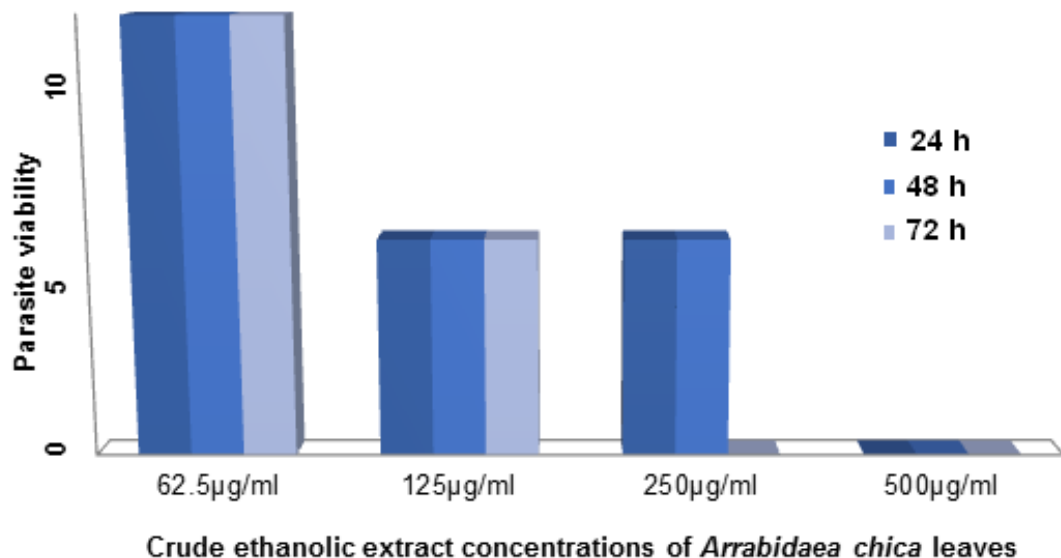


Figure 1 – Viability of *Leishmania amazonensis* promastigotes cultivated in RPMI media at 24°C and treated with crude extract of leaves of *Arrabidaea chica*, at the concentrations of 62.5 to 500 µg/mL. Score: (10) viable, (5) less viable, (0) unfeasible.

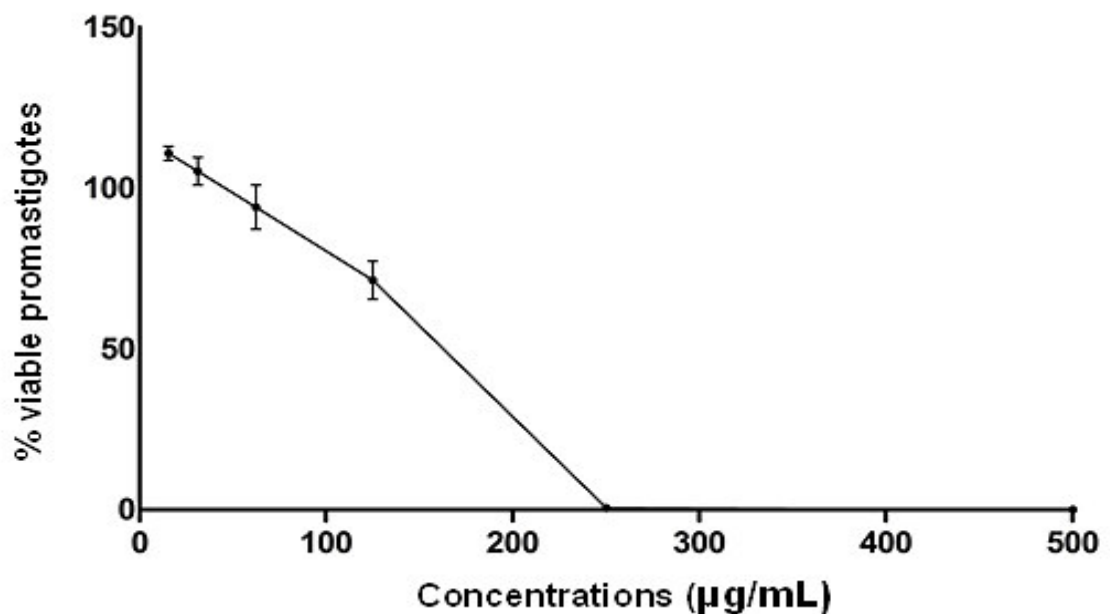


Figure 2 – In vitro leishmanicidal activity of the crude ethanolic extract of *Arrabidaea chica* against *Leishmania amazonensis* promastigotes at the concentrations of 0.97 to 500 µg/mL.

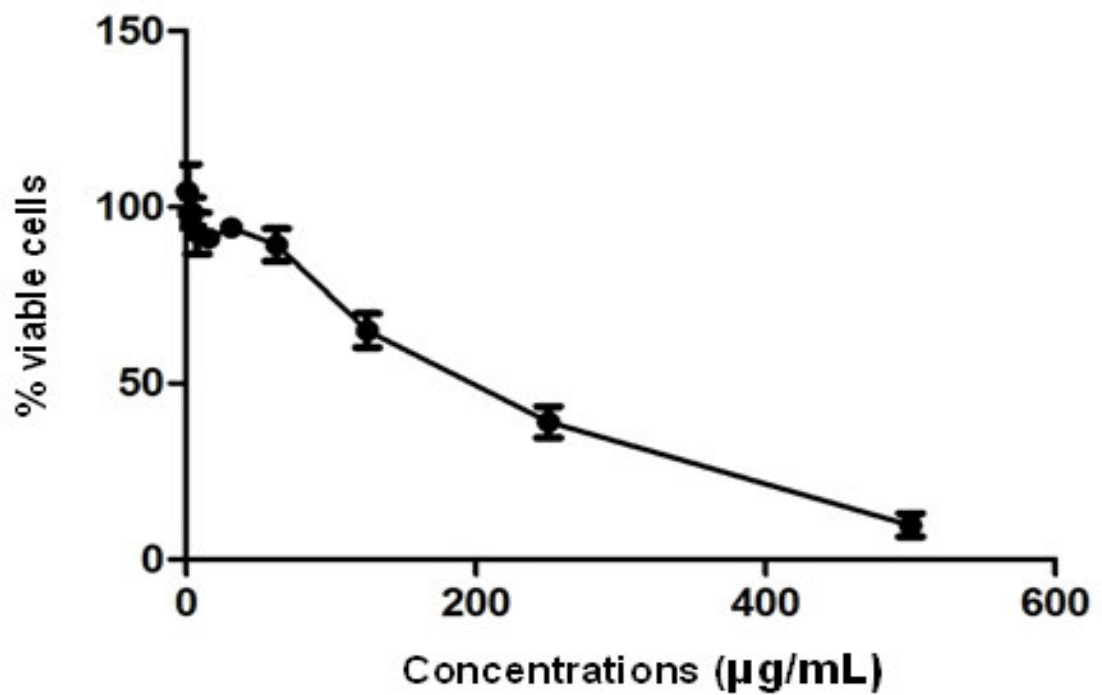


Figure 3 – Cytotoxicity evaluation in peritoneal macrophages cultured in RPMI medium, at 37°C and 5% CO₂ and treated for 24 h with ethanol extract of *Arrabidaea chica* leaves at the concentrations of 0.97 to 500 µg/mL.

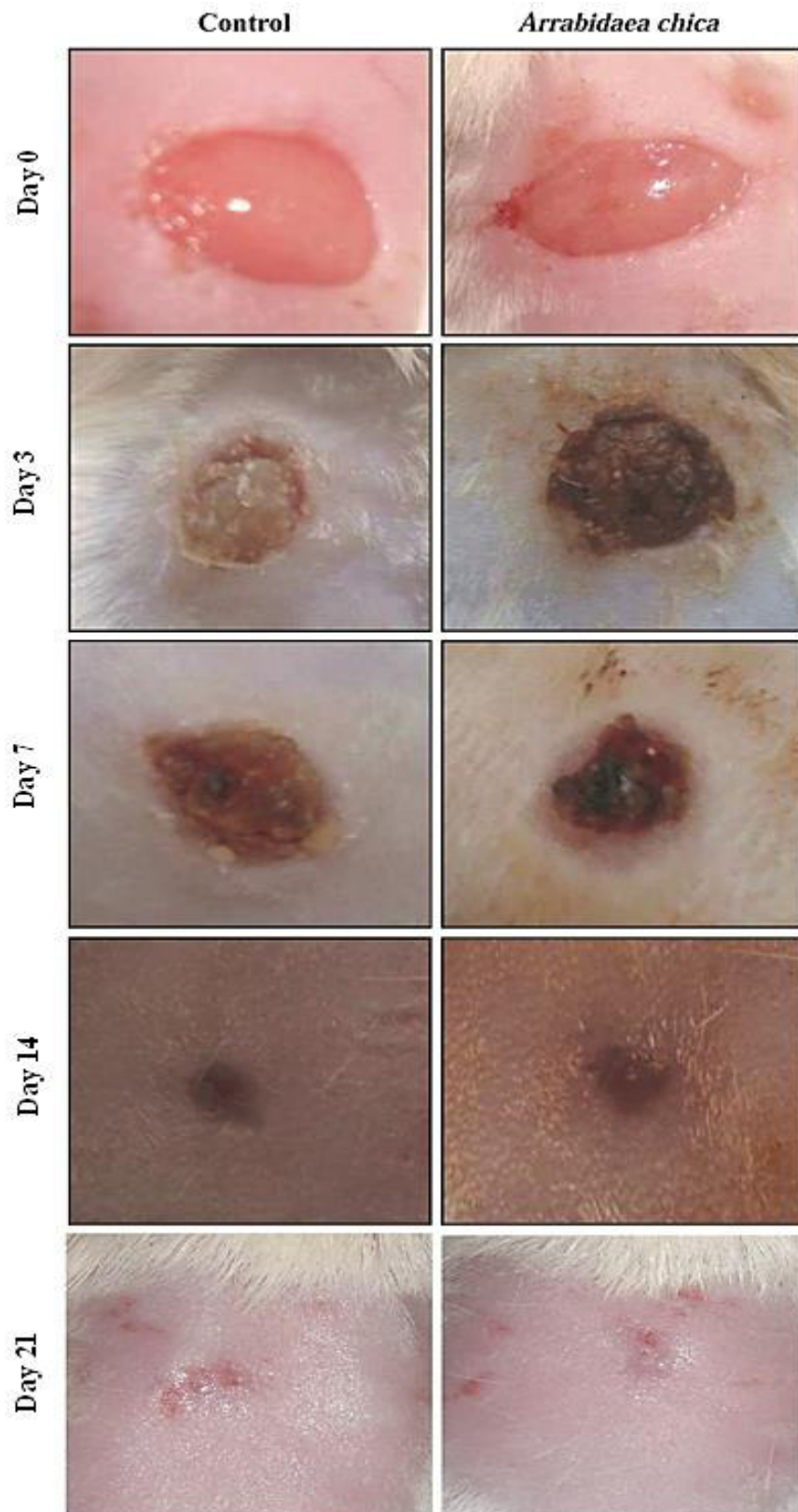


Figure 4 – Macroscopic evaluation of the healing process in Swiss mice Webster treated with LANETTE® cream (Control Group) and ethanol extract of *Arrabidaea chica* leaves (Experimental Group) over 21 days.

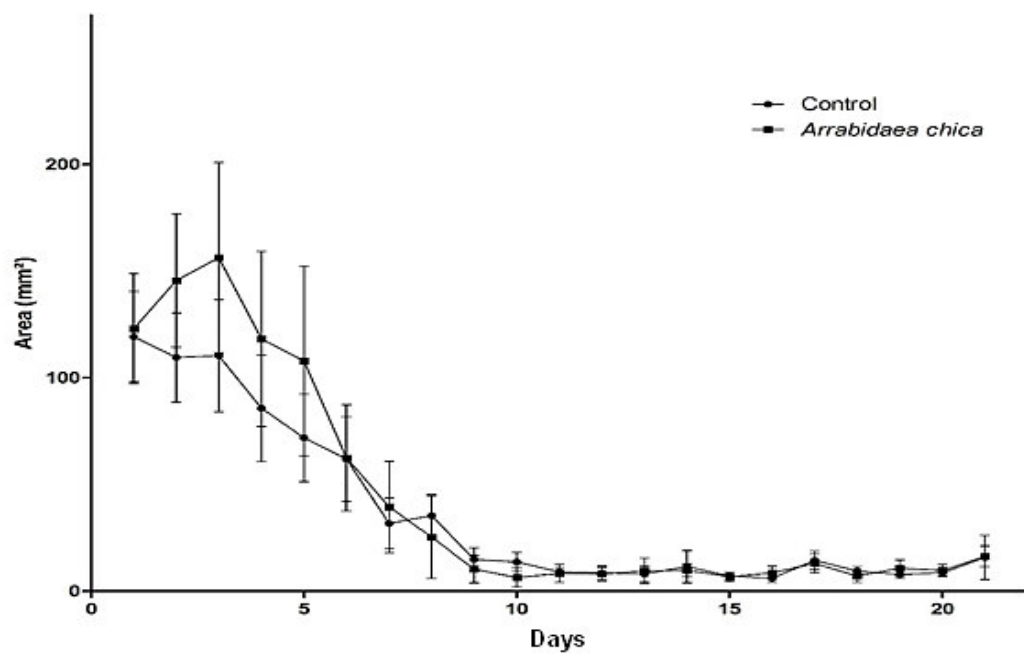


Figure 5 – Wound healing kinetics of surgical lesions in Swiss Webster mice treated with Lanette® cream (Control Group) and ethanol extract of *Arrabidaea chica* leaves (Experimental Group) over 21 days.

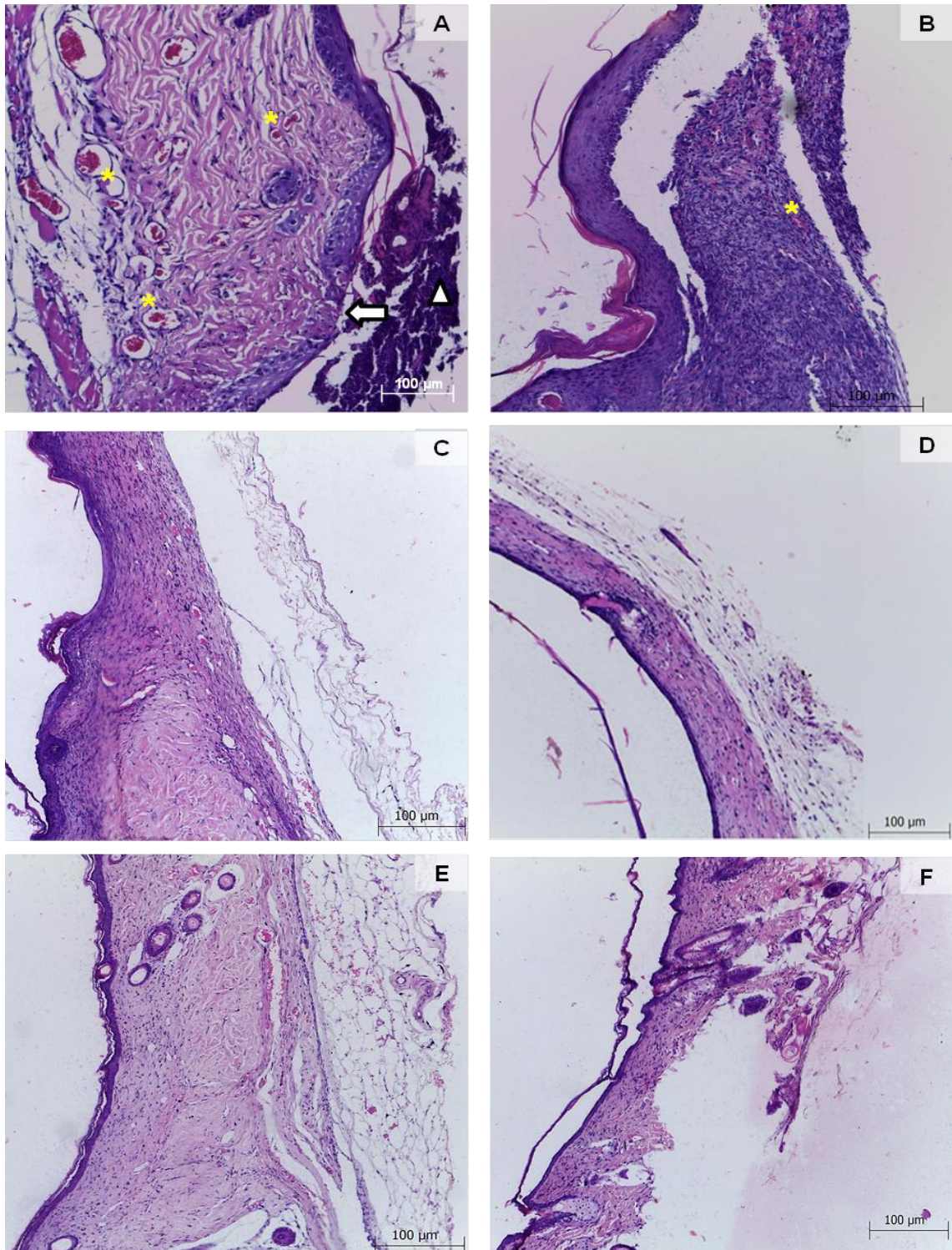


Figure 6 – Swiss Webster. Skin. Healing process through topical treatment with a cream containing the crude ethanolic extract of *A. chica* (experimental group) or only Lanette® cream (control group). A (3 days) - Experimental group: cellular debris (Δ), reepithelization (arrow), angiogenesis (*). B (7 days) - Experimental group: Absence of cellular debris and angiogenesis (*). C (14 days) and D (21 days) - Control group: Absence of cellular debris and reepithelization. E (14 days) and F (21 days) - Experimental group: Absence of cellular debris and reepithelization. HE.

Parte II

8 CAPÍTULO II

Artigo “*Vernonia brasiliiana* leaves harbour components with potential leishmanicidal action”.

Submetido ao periódico *Journal of Ethnopharmacology*, cuja classificação na área da Biotecnologia, conforme o Sistema WebQualis da plataforma da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior é B1, fator de impacto 2,939.

O artigo foi elaborado e formatado conforme as normas do periódico supracitado, que seguem anexas ao trabalho juntamente com o comprovante de submissão.

***Vernonia brasiliiana* leaves harbour components with potential leishmanicidal action**

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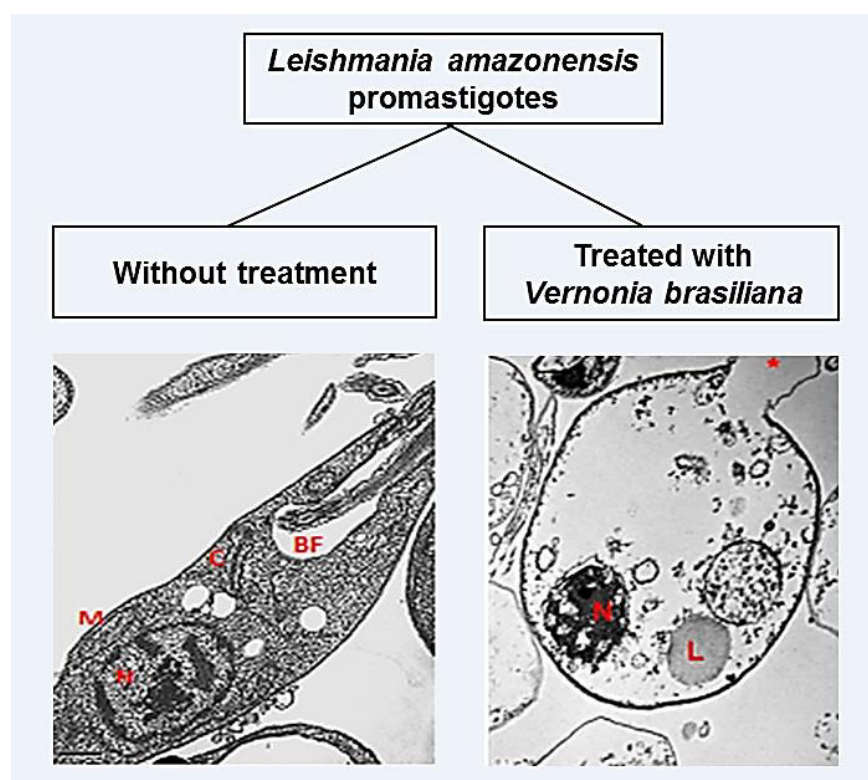
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ABSTRACT

We evaluated the phytochemical constituents and the cytotoxic and leishmanicidal potential of the crude extract, chloroform fraction and essential oil of *Vernonia brasiliiana* Less. The phytochemical analyses were performed by gas chromatography and mass spectrometry. After that, were made an *in vitro* cytotoxic assay, with the determination of cytotoxic concentration via MTT colorimetric assay; *in vitro* antileishmanial screening and determination of the 50% inhibitory capacity of *L. amazonensis* promastigotes and amastigotes forms under the effect of crude ethanolic extracts, oil and fractions of *V. brasiliiana*; and ultrastructural analysis through electronic microscopy. The phytochemical screening showed that *V. brasiliiana* major compounds are sesquiterpenes, especially caryophyllene. The crude extract, chloroform fraction and essential oil were not cytotoxic in leishmanicidal concentrations, with a selective ratio higher to the parasite than to the host cell. Electron microscopy showed significant damage was observed in the parasite's cell membranes, cytoplasm and flagellar pocket after treatment with *V. brasiliiana*, demonstrating that this species is a potential leishmanicidal agent.

Keywords: beta-caryophyllene, cutaneous leishmaniasis, *Leishmania amazonensis*, leishmanicidal potential, sesquiterpenes.



1 INTRODUCTION

Vernonia brasiliiana belongs to the Asteraceae family, which has about 109 species with medicinal properties (Toyang and Verpoorte, 2013). It is native in Brazil, popularly known as “*assa-peixe*” (Rodrigues, 2006).

The genus *Vernonia* consists of plants rich in bioactive compounds with anti-inflammatory, antimicrobial, antifungal, antiprotozoal, antineoplastic and insecticide actions (Carvalho et al., 1991; Arias et al., 1995; de Almeida Alves et al., 1997; Erasto et al., 2006; Formisano et al., 2006; Pillay et al., 2007; Costa et al., 2009; Maia et al., 2010; Alawa et al., 2012; Toyang et al., 2012; Hira et al., 2013).

The use of medicinal plants in the treatment of diseases has become an alternative to allopathic drugs (Taufner et al., 2006). Among them, we emphasize the neglected diseases, especially leishmaniasis. There is an increasing number of scientific papers of identification and biological characterization of crude extracts, fractions and oils obtained from medicinal plants with anti-*Leishmania* activity (Weniger et al., 2004; Rocha et al., 2005; Costa et al., 2006; Braga et al., 2007; Estevez et al., 2007; Ferreira et al., 2010; Santos et al., 2012; Colares et al., 2013; Ribeiro et al., 2014; Rottini et al., 2015).

These studies can provide valuable information for the development of new therapeutic agents. According to the World Health Organization, a candidate drug must undergo *in vitro* cytotoxicity and antiparasitic activity. After this initial screening, the results are evaluated to define the continuity of studies and the possibility of *in vivo* assays (Dias et al., 2009).

Leishmaniasis is one of the most important endemic parasitic diseases in terms of public health, given its wide distribution. Annually, 1.3 million new cases are recorded: 300,000 cases of visceral leishmaniasis and 1 million of cutaneous leishmaniasis (Who, 2015).

The recommended treatment for leishmaniasis are pentavalent antimonials, however, although they are used for more than 100 years, its mechanism of action is not totally known (Frézard et al., 2009). Other concerns about the therapeutic protocol are the

toxic effects of these drugs, reports of parasitic resistance, long periods of treatment and the parenteral administration route (Plano et al., 2011).

The search for therapeutic resources from medicinal plants with a leishmanicidal action, with lower toxicity, easy to obtain, provided by the basic service of the unified health system and easy administration, instigated the development of this research, which aimed to evaluate the phytochemical properties of *Vernonia brasiliiana*, besides the cytotoxic and leishmanicidal potential under the parasite *Leishmania amazonensis*.

2 MATERIALS AND METHODS

2.1 Plant

Leaves and stem of *Vernonia brasiliiana* were collected in the city of Santa Luzia (Maranhão, Brazil) between November and December 2014, which corresponds to the dry season. *V. brasiliiana* was identified at the Prisco Bezerra Herbarium, Universidade Federal do Ceará, voucher specimen number 55227.

2.2 Preparation of crude extract and fractions

To obtain the crude ethanolic extract, the samples were dried at 40°C, with forced air circulation. The dried material from each part of *V. brasiliiana* was macerated and immersed in ethyl alcohol, in a proportion of 3:1 (alcohol and plant material, respectively). Solvent removal was carried out by rotoevaporation, under reduced pressure at 30 to 40°C, until the desired concentrations were achieved, in order to obtain the ethanolic extracts. The samples were stored under refrigeration and protected from light until analysis.

The crude ethanolic extracts were successively partitioned with methanol, chloroform and ethyl acetate to obtain the respective fractions. The extraction was carried out in solid phase using silica gel, grain, pistil and approximately 500 mL of each solvent. After obtaining the fractions, they were submitted to leishmanicidal, cytotoxic and healing tests. Those that showed satisfactory results were sent to the Analytical Center of Universidade de São Paulo (São Paulo, Brazil), to identify bioactive compounds through gas chromatography and mass spectrometry.

2.3 Phytochemical analysis

To evaluate the presence of secondary metabolites (saponins, anthocyanins, chalcones, phenols, tannins, flavonoids, steroids, triterpenoids and anthraquinones), crude ethanolic extracts obtained from the leaves and stem of *V. brasiliiana* were solubilized in methanol (in a proportion of 100 mg/5 mL), according to Costa (1982) and Matos (1997).

2.4 *V. brasiliiana* essential oil extraction

The extraction of essential oil of *V. brasiliiana* leaves was performed by hydrodistillation process using a Clevenger-type apparatus. Leaves previously dried at room temperature were ground. Then, 400 g of the dried material was added with 3L of distilled water in a 6L volumetric flask, and submitted to hydrodistillation for 3 hours. The oil obtained in the process was dried using anhydrous sodium sulfate (30 mg) and stored under refrigeration until cytotoxic and leishmanicidal analyses *in vitro*. An aliquot of the oil was sent to Analytical Center at the Universidade de São Paulo (São Paulo, Brazil) to identify chemical compounds by gas chromatography and mass spectrometry.

2.5 *In vitro* cytotoxic assay

2.5.1 Obtainment of peritoneal macrophages and cell culture

Peritoneal macrophages were obtained from eight week old female Balb/c mice, from the Laboratory Animal Breeding Center (CECAL-FIOCRUZ) and kept in a vivarium of Hélio and Peggy Pereira Pavilion, located at Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, Brazil).

3 mL of 3% sodium thioglycollate were inoculated into the peritoneal cavity of the animals and, after 48 hours, they were euthanized in a CO₂ chamber. For macrophages recovery, were injected 5 mL of a cold and sterile PBS 1X solution into their abdominal cavity, followed by massage and collection of lavage, which was kept in a falcon tube placed in an ice box. After collection, the lavage was analyzed under optical microscope to verify the presence of macrophages and lack of red blood cells and bacteria.

Peritoneal lavage was centrifuged at 500 xG for 10 minutes at 4°C. The supernatant was separated and the cells resuspended in 1 mL of RPMI - 1620 and counted in a Neubauer chamber. Macrophages final concentration was standardized at 5×10^5 cells/mL. For cell culture, it was used RPMI supplemented with 10% fetal bovine serum, L-glutamine (20 mM), sodium bicarbonate solution (7.5%), penicillin (100 µg/mL) and streptomycin (50 µg/mL), incubated at 37°C with 5% CO₂.

2.5.2 CC50 determination

Cytotoxicity was measured by MTT colorimetric assay, according to Mosmann (1983). 100 µL of peritoneal macrophages (5×10^5 cells/mL) were added in 96-well plates and incubated for 2 hours, at 37°C with 5% CO₂. After that, 100 µL of the crude ethanolic extracts, its fractions and the oil of *V. brasiliensis* were added to the wells in serial concentrations (600 to 0.97 µL/mL). For each concentration was maintained a negative control (with the extract and RPMI). DMSO was used as a reference drug in serial dilutions starting with 20%.

After 24 hours of incubation, 5 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were added to each well, then the plates were incubated again for 2-4 hours, under the same conditions. Later, they were centrifuged at 1500 rpm and 200 µL of each well supernatant were discarded, then 100 µL of DMSO were added. The absorbance was measured in a spectrophotometer, at a wavelength of 540 nm. 50% inhibitory concentration (CC₅₀) was calculated using GraphPad Prism 5.

2.6 In vitro antileishmanial activity

2.6.1 Parasites

In this study was used *Leishmania amazonensis* strain (MHOM/BR/76/MA-76 – IOC/FIOCRUZ-RJ), isolated from a patient with diffuse cutaneous leishmaniasis and maintained by serial passages in Balb/c mice, and periodically re-isolated in culture medium. Promastigotes obtained from *in vitro* cultures were grown at 26°C in Schneider's medium, supplemented with 10% fetal bovine serum, streptomycin (50 µg/mL) and penicillin (100 µg/mL). To ensure the characteristic of infectivity were used only cultures of promastigotes with a maximum of seven *in vitro* passages.

2.6.2 Promastigotes assay

Before the evaluation of leishmanicidal activity and determination of the 50% inhibitory concentration (IC_{50}) of *V. brasiliensis*, it was performed a screening test to determine the concentration of use, with values ranging between 500 and 0.97 μ g of crude ethanolic extracts and fractions. They were diluted in 1% DMSO and added in 96-well plates containing RPMI, serially and in triplicate. Wells containing only the medium and parasites, only with the medium and wells with the reference drug, Amphotericin B (2 μ g/mL) were used as controls, to evaluate contamination and parasitic resistance.

100 μ L of *L. amazonensis* promastigotes in logarithmic phase (1×10^6 parasites/mL) were added in each well, and the plate was incubated at 26°C. Their feasibility was measured at the end of 24, 48 and 72 hours, by observing viable parasites under an inverted microscope. These were classified according with parasites mobility on a scale of zero to ten, where zero means no viable forms and ten, viable forms.

Based on the screening results it was determined the IC_{50} , following the same aforementioned protocol, and the leishmanicidal activity was assessed by counting the viable parasites in a Neubauer chamber. Then, data were normalized using the formula: Percentual of growth inhibition = number of parasites in the control well/number of parasites in test wells; and used for statistical analysis.

2.6.3 Electron Microscopy Analysis

In order to evaluate ultrastructural alterations by transmission electron microscopy, *L. amazonensis* promastigotes were treated with crude ethanolic extract and essential oil of *V. brasiliensis*, at the concentrations of 3, 7, 15 e 30 μ g/mL (ethanolic extract) and 1, 2, 5 e 10 μ g/mL (oil) for 24 hours. After this, were fixed in 2.5% glutaraldehyde (Sigma, USA) in a 0.1M cacodylate buffer solution, pH 7.2, overnight. Next, promastigotes were washed using this same buffer and postfixed in a solution with 1% osmium tetroxide, 0.8% potassium ferricyanide and 5mM calcium chloride, in 0.1 M cacodylate buffer, for 30 minutes. The parasites were dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in JEM-1011 transmission electron microscope (JEOL, Japan).

2.7 Ethics Committee

This study was approved by the Ethics Committee for Animal Research of Universidade Estadual do Maranhão (CEUA-UEMA), protocol 26/2009 and by the Ethics Committee in Animal Experimentation of Instituto Oswaldo Cruz - FIOCRUZ/RJ (CEUA-FIOCRUZ), protocol LW72/12.

2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0.4 (GraphPad Software Inc.). IC₅₀ and CC₅₀ values were calculated from the mean percentage reduction of promastigotes and macrophages, respectively. Curves were determined by application of sigmoidal regression to logarithm concentration response data. In vitro results are representative of three different experiments, performed in triplicate, under the same temperature, CO₂, time and culture medium conditions, with similar results.

3 RESULTS

Phytochemical screening demonstrated that the crude ethanol extracts from the leaves and stem of *V. brasiliensis* have the same classes of metabolites, which are terpenes, esters and tannins (Table 1).

Table 1 - Phytochemical screening of the crude ethanol extracts of the leaves and stems of *Vernonia brasiliensis*, collected in Maranhão State, Brazil

Metabolites class	Reactions	<i>Vernonia brasiliensis</i>	
		Leaves	Stem
Anthraquinones	NaOH	-	-
Triterpenes	Anhydrous acetic	+	+
Ester	Sulphuric acid	+	+
Saponin	Foamming index	-	-
Phenolic compound	FeCl ₃	-	-
Tannin	Gelatin	+	+
Anthocyanin	HCl / NaOH	-	-
Chalcones	HCl / NaOH	-	-

(+) presence; (-) absence;

We analyzed the chemical constituents of the chloroform fraction from the crude ethanolic extract of *V. brasiliiana* leaves, by gas chromatography–mass spectrometry technique. Twenty four components were identified, which were compared with the database of the Analytical Center library of the Chemistry Institute of University of São Paulo. Were identified as lead compounds: dihydroxypropyl octadecanoate ($C_{21}H_{42}O_4$), representing 34.21%; N-hexadecanoic acid, also known as palmitic acid ($C_{16}H_{32}O_2$), 13.12%; hexadecanoic acid dihydroxypropyl ester ($C_{19}H_{38}O_4$), 8.56%; ethyl hexadecanoate or ethyl palmitate ($C_{18}H_{36}O_2$), 6.76%; 1-chloro octadecane ($C_{18}H_{37}Cl$) 5.39%; 1,2-di-bromo dodecane ($C_{12}H_{24}Br_2$) 2.94%; octadecanol ($C_{18}H_{38}O$), 2.73%; corresponding to the peaks 20, 7, 16, 8, 21 19 and 24, respectively, indicating that this fraction has a large amount of esters, supporting the qualitative phytochemical analysis (Figure 1).

The gas chromatography–mass spectrometry performed in the essential oil sample showed as its main components: beta caryophyllene ($C_{15}H_{24}$ - 39.68%); vetivenol bicyclo ($C_{15}H_{26}O$ - 17.38%); alpha caryophyllene ($C_{15}H_{24}$ - 12.78%); caryophyllene oxide ($C_{15}H_{24}O$ - 9.78%) and alpha-copaene tricyclo ($C_{15}H_{24}$ - 7.66%), respectively (Figure 2), demonstrating that the major compounds present in the oil are sesquiterpenes, a terpene's subtype, supporting the phytochemical qualitative detection.

The essential oil extracted from *V. brasiliiana* leaves showed an income of 0.1 to 0.2%, corresponding to the hydrodistillation of leaves dried in a heater or dried at room temperature, respectively.

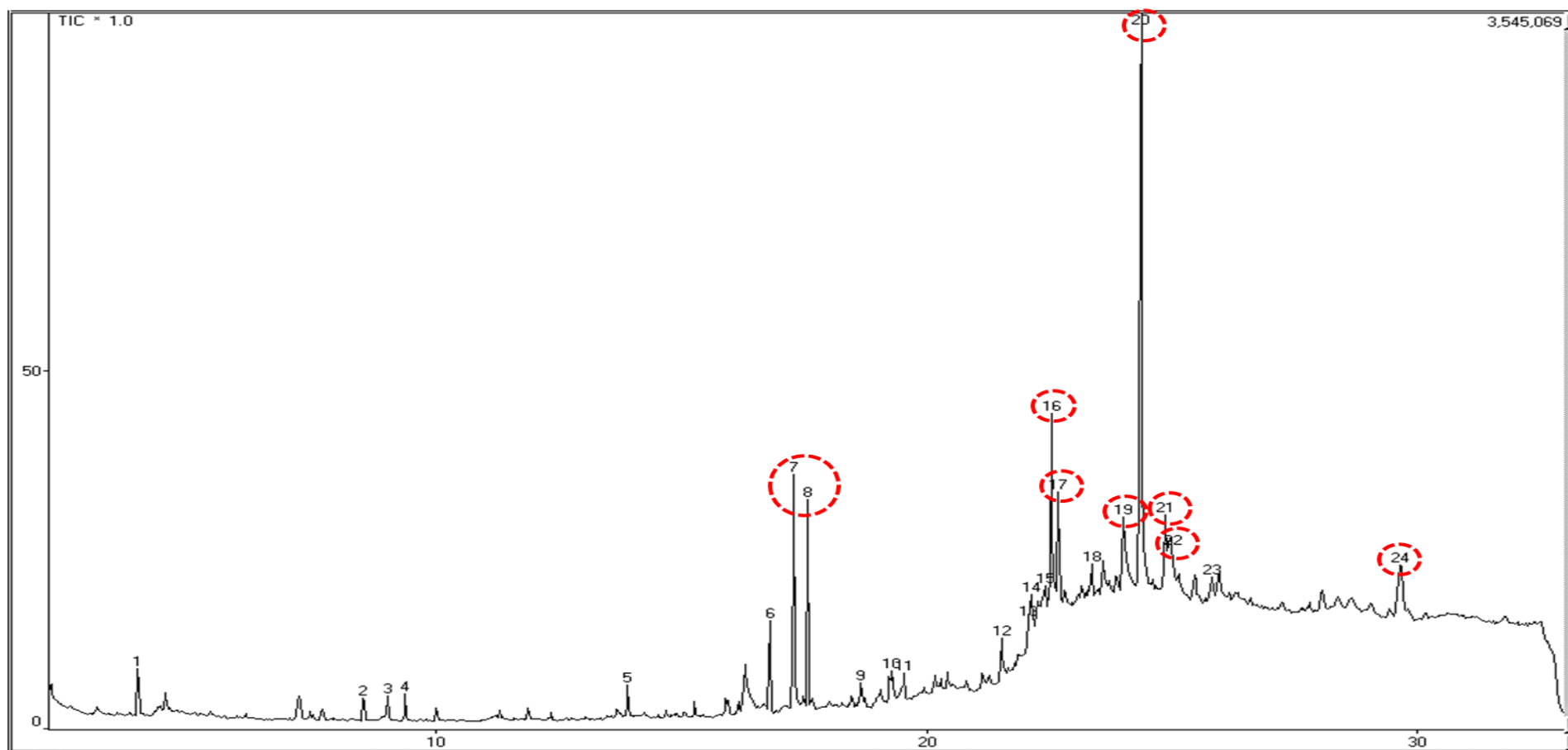


Figure 1 - Chromatogram of the chloroform fraction of *Vernonia brasiliiana* leaves through gas chromatography-mass spectrometer. Major compounds represented by peaks 7, 8, 19 16, 17, 20, 21, 22 and 24.

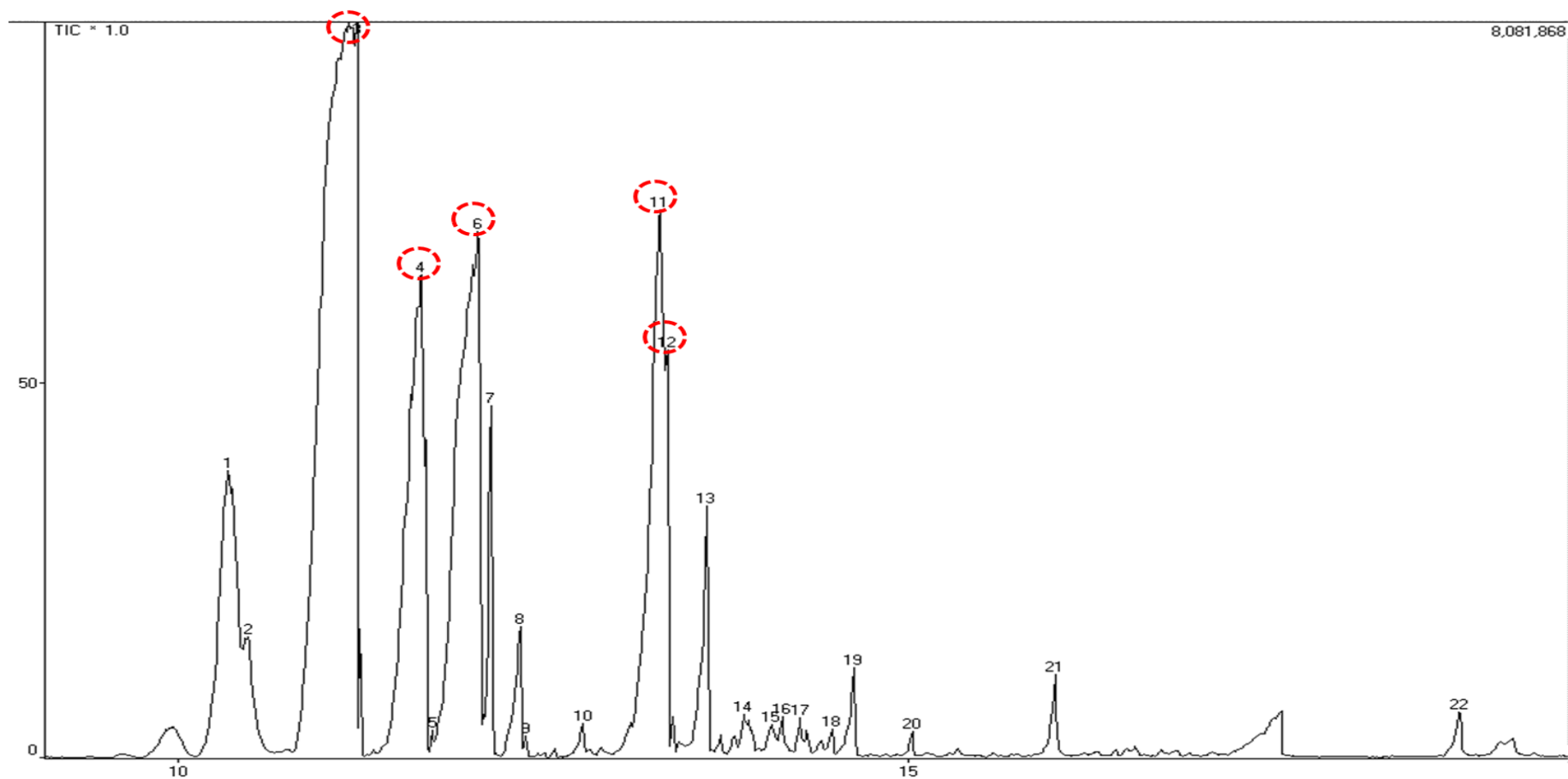


Figure 2 - Chromatogram of the essential oil extracted from the leaves of *V. brasiliiana* through gas chromatography-mass spectrometry technique. Major compounds represented by peaks 3, 4, 6, 11 e 12.

Cytotoxicity tests performed on peritoneal macrophages after 24 hours of incubation, using MTT colorimetric assay, showed that, at the concentration of 44.32 $\mu\text{g/mL}$, the crude ethanolic extract from the leaves of *V. brasiliiana* reduces by 50% the number of viable cells, corresponding to its CC_{50} (Figure 3).

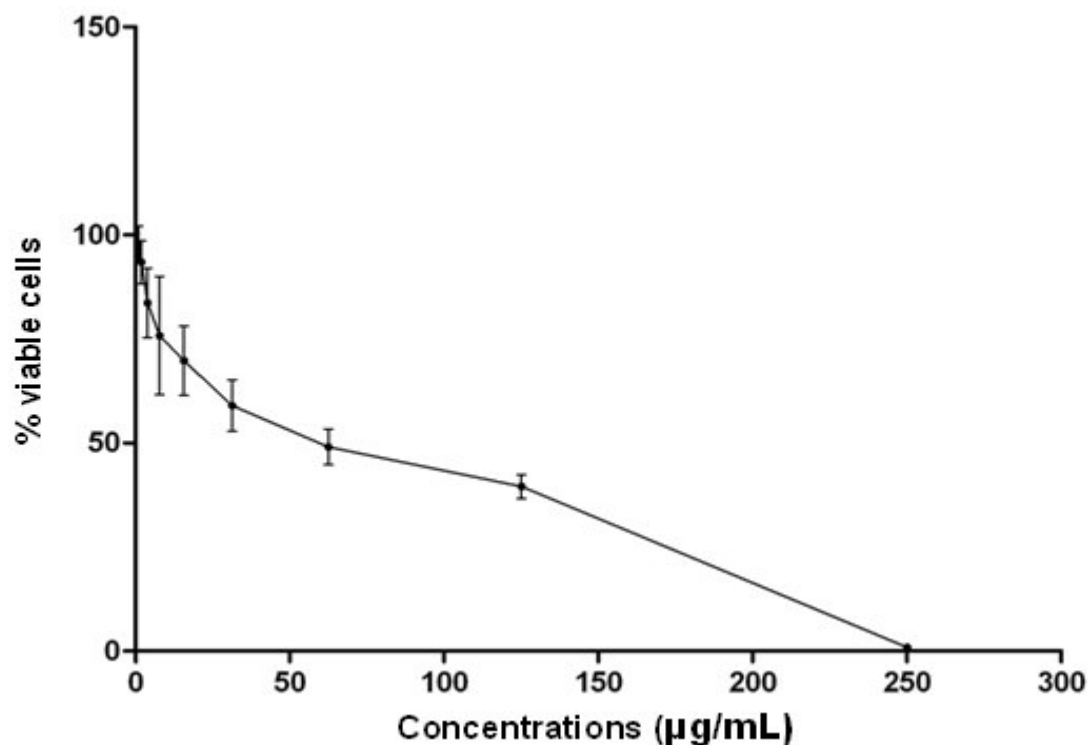


Figure 3 - Cytotoxicity evaluation of peritoneal macrophages cultured in RPMI media at 37°C with 5% CO_2 and treated for 24 h with crude ethanol extract from the leaves of *V. brasiliiana*, at the concentrations of 0.97 to 500 $\mu\text{g/mL}$.

Analyzing the cytotoxicity assay of the essential oil and chloroform, methanol and ethyl acetate fractions, it was found that, except for the ethyl acetate fraction, they all had CC_{50} higher than the crude ethanolic extract, as shown in Table 2.

Table 2 - 50% cytotoxic concentration (CC₅₀) of peritoneal macrophages 24 hours after treatment with the essential oil and fractions of *V. brasiliiana* and DMSO (reference drug)

Fractions	<i>In vitro</i> cytotoxicity	
	CC ₅₀	R ²
Essential oil	88.35 ± 0.037	0.826
Chloroform fraction	84.37 ± 0.033	0.779
Methanolic fraction	322.7 ± 0.028	0.809
Ethyl acetate fraction	41.40 ± 0.039	0.836
DMSO	7.40 ± 0.019	0.942

Values represent mean ± standard deviation. R² = correlation index (0-1).

By comparing the samples with DMSO, cytotoxic drug, it was observed that their action against cells occurred at much greater concentrations, at least six times higher, allowing the continuity of the research.

During antileishmanial screening, the crude ethanolic extract of *V. brasiliiana* leaves showed a reduction in promastigotes viability from the first 24 hours after exposure to the extract, with a total absence of parasites mobility at a concentration of 30 µg /mL. A lower anti-*Leishmania* effect was also observed in the concentration of 15 µg/mL; nevertheless, there were still viable protozoa. However, the ethanol extract from the stem showed no action against *L. amazonensis*, even at the highest concentration tested and the larger time interval, so it was not used for tests continuity.

Furthermore, the stem crude extract showed a low income and its collection promotes the destruction of the plant, which contradicts the principles of flora preservation defended in our researches.

After the screening, it was determined the 50% inhibitory concentration (IC₅₀) of *L. amazonensis* promastigotes forms, which was 9.279 µg/mL at the first 24 hours, 8.374 µg/mL (48 h) and 8.356 µg/mL (72h) (Figure 4).

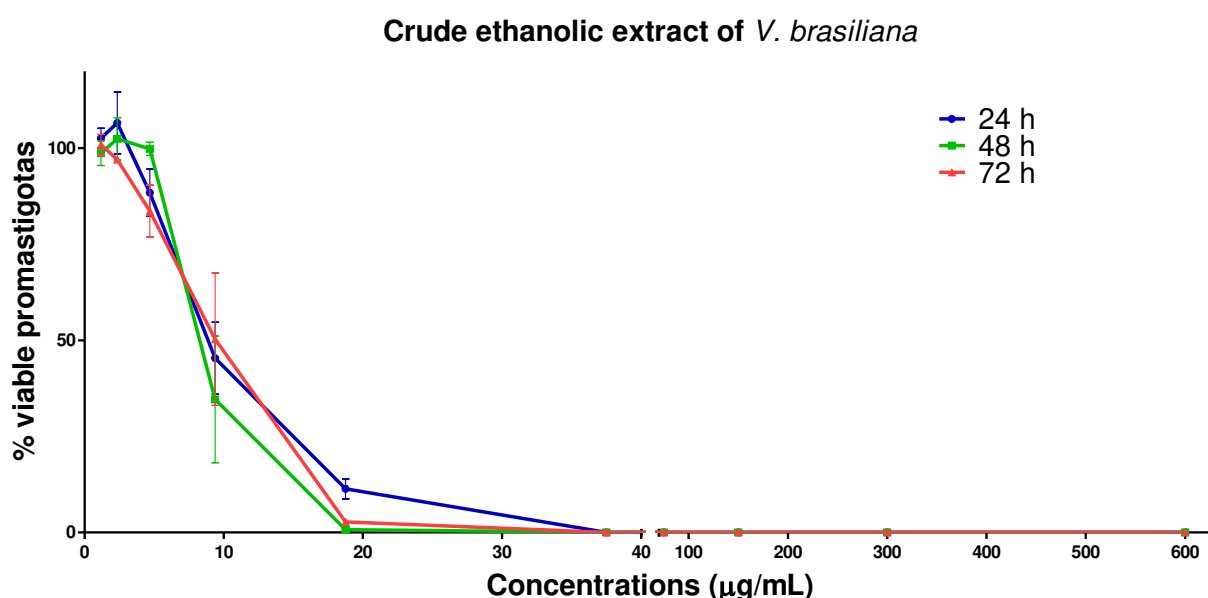


Figure 4 - *In vitro* leishmanicidal activity of the crude ethanolic extract from the leaves of *V. brasiliiana* at the concentrations of 0.97 to 600 µg/mL under *L. amazonensis* promastigotes forms (10^6 parasites/mL), in an incubation period of 24 to 72 h.

The reference drug, Amphotericin B, led to a 50% promastigotes reduction at a concentration of 0.2809 µg/mL. Regarding the leishmanicidal activity of *V. brasiliiana* fractions, the ethyl acetate fraction had a lower IC_{50} , followed by chloroform fraction, as shown in Table 3.

Table 3 - 50% inhibitory concentration (IC_{50}) of *Leishmania amazonensis* promastigotes after 24, 48 and 72 hours of treatment with crude ethanol extract, its fractions and essential oil of *V. brasiliiana*, and the reference drug, Amphotericin B

Treatment	IC_{50} (µg/mL) 24 h	IC_{50} (µg/mL) 48 h	IC_{50} (µg/mL) 72 h
Crude ethanolic extract	9,279 ±0,08428	8,374 ±0,1016	8,356 ±0,08179
Essential Oil	2,022 ±0,1122	1,930 ±0,09498	1,718 ±0,09551
Ethyl acetate fraction	2,209 ±0,06885	2,075 ±0,07261	1,907 ±0,09693
Chloroform fraction	4,341 ±0,09758	3,198 ±0,07543	3,144 ±0,08900
Ethyl acetate fraction	122,7 ±0,1256	64,71 ±0,1046	62,82 ±0,09415
Amphotericin B	0,280 ±0,1139	0,799 ±0,09113	0,899 ±0,1267

Values represent mean ± standard deviation.

In a similar way, it was analyzed the essential oil of *V. brasiliiana*, subjected to an initial viability screening and then, determining the IC₅₀. During the screening, preliminary results showed absence of viable forms at the first 24 hours of treatment, at the concentration of 15.26 µg/mL, and even at the lowest tested concentration, 0.97 µg/mL, there was a reduction in the viability of promastigotes when compared to untreated forms.

After counting the viable promastigotes forms in a Neubauer chamber, it was determined the IC₅₀ of 2.022 µg/mL, at the first 24 hours of exposure to the parasite (Figure 5), demonstrating that the oil and ethyl acetate and chloroform fractions have antileishmanial activity at concentrations lower than the crude extract.

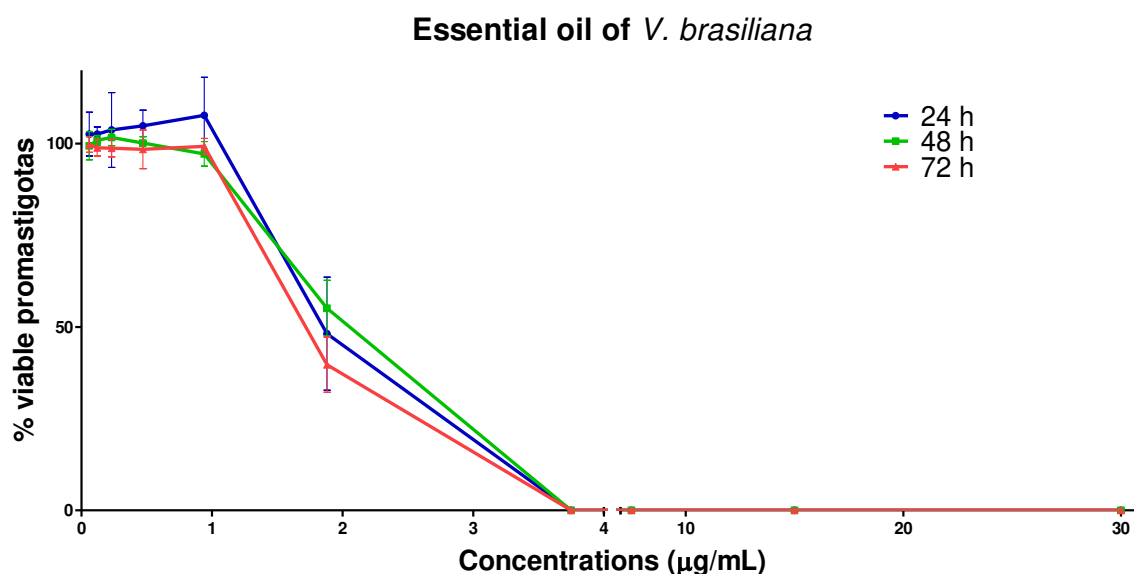


Figure 5 - *In vitro* leishmanicidal activity of the essential oil of *V. brasiliiana* at the concentrations of 0.97 to 600 µg/mL under *L. amazonensis* promastigotes forms of (10⁶ parasites/ml), at an incubation period of 24 to 72 h.

It is noteworthy that the ethanol extract, its three fractions and the essential oil of *V. brasiliiana* presented CC₅₀ values greater than the IC₅₀ values, therefore, the bioactive compounds are more selective to the parasite than to the host cells. Is worth mentioning that the essential oil has a selectivity for *L. amazonensis* more than 40 times, as presented in Table 4, indicating that *V. brasiliiana* is a promising plant in the treatment of leishmaniasis.

Table 4 - Selectivity profile of the crude ethanolic extract, essential oil and chloroform, ethyl acetate and metanol fractions of *V. brasiliensis* under promastigote forms of *Leishmania amazonensis*, relative to CC₅₀

Plant compound	SI	CC ₅₀		IC ₅₀ (24h)	
		(µg/mL)	R ²	(µg/mL)	R ²
Ethanolic crude extract	4.77	44.32	0.881	9.279	0.8677
Essential oil	43.69	88.35	0.826	2.022	0.8122
Chloroform fraction	19.43	84.37	0.779	4.341	0.7982
Ethyl acetate fraction	18.74	41.40	0.836	2.209	0.8374
Methanolic fraction	2.63	322.7	0.809	122.7	0.7348

CC₅₀ = Cytotoxic concentration for 50% of the cells; IC₅₀ = Concentration able to inhibit 50% of the promastigotes; SI = selectivity index = CC₅₀/IC₅₀. R² = correlation index (0-1).

In order to better understand the action of *V. brasiliensis* bioactive components under the parasite, it was proceeded ultrastructural analysis of *L. amazonensis* promastigotes forms after treatment with the crude ethanolic extract and chloroform fraction, at the concentrations of 3, 7, 15 and 30 µg/mL and with the essential oil, at the concentrations of 1, 2, 5 and 10 µg/mL. Electron microscopy evidenced significant alterations in cellular components of the parasite, under the effect of the crude extract, essential oil and chloroform fraction, as shown in Figures 6, 7 and 8.

Regarding the treatment with the crude ethanolic extract, it was found that at a concentration of 3 µg/mL, there were chromatin condensation and presence of intracytoplasmatic lipid droplets and vesicles, as well as the presence of vesicles inside the kinetoplast and flagellar pocket (Figure 6B). The concentration of 7 µg/mL showed intense cytoplasmatic vacuolization (Figure 6C), and at the concentration of 15 µg/mL, these atypical vacuoles had an electron-dense material in its interior (Figure 6D). Increasing the extract concentration, there was an enhancement on the alterations, and at a concentration of 30 µg/mL, there was a significant amount of cytoplasmatic lipid droplets, condensation of the mitochondrial matrix and swelling of kinetoplast (Figure 6E). The ethanol extract caused many cytoplasmatic changes, which were more pronounced in the highest concentration tested.

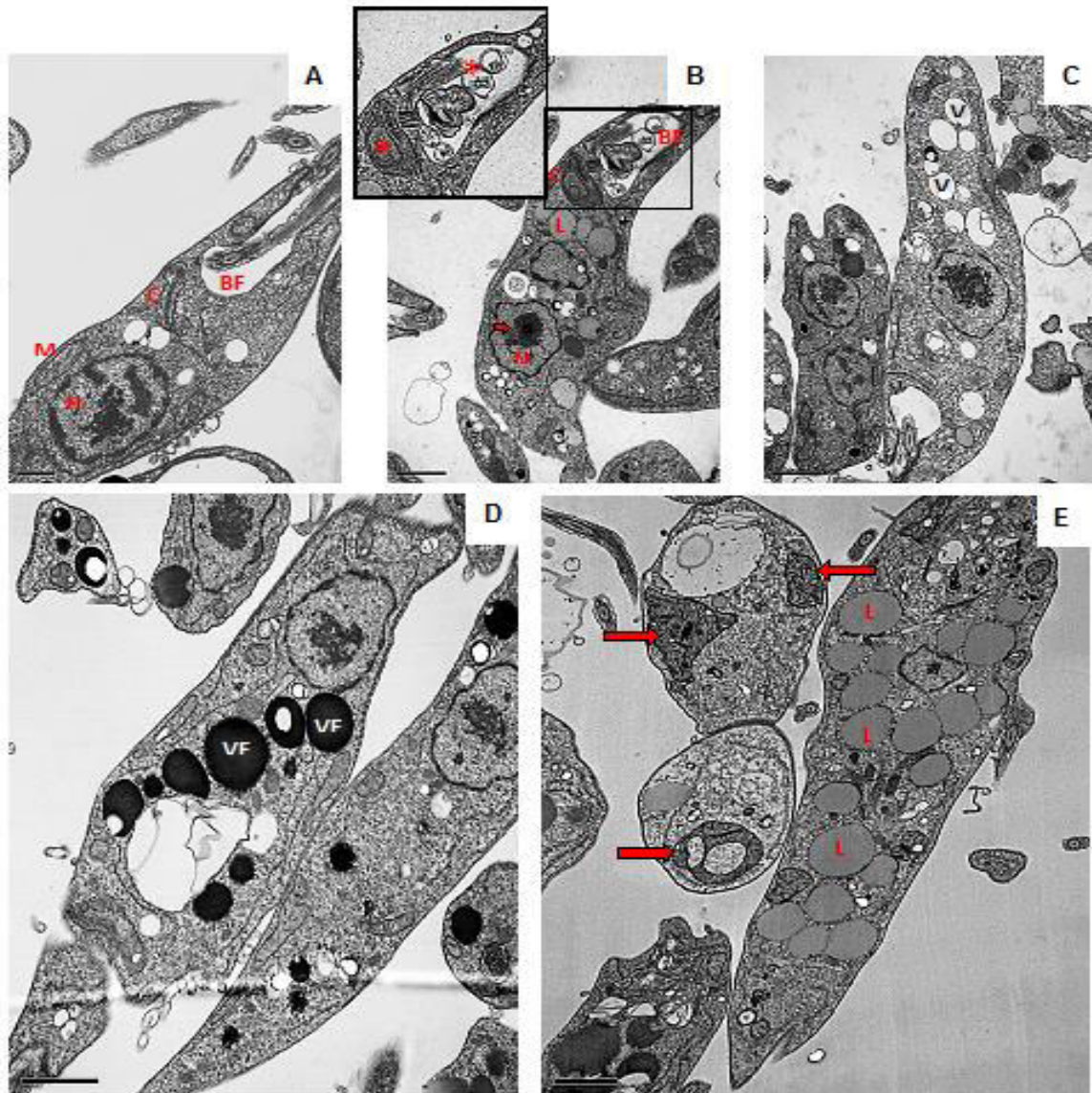


Figure 6 - Ultrastructural effect of crude ethanol extract of *V. brasiliiana* leaves under *L. amazonensis* promastigotes grown in RPMI supplemented media and incubated for 24 hours at 26°C. (A) Control group, normal morphology. (B - E) *L. amazonensis* promastigotes treated with different concentrations of *V. brasiliiana* extract. (B) 3 µg/mL: chromatin condensation (lower arrow), vesicles within the flagellar pocket and kinetoplast (*), lipid droplets in the cytoplasm (L). (C) 7 µg/mL: atypical vacuoles (V) in the cytoplasm. (D) 15 µg/mL: atypical vacuoles with electron-dense material (VE) in the cytoplasm. (E) 30 µg/mL: large amount of lipid droplets (L) in the cytoplasm, condensation of mitochondrial matrix and swelling of the kinetoplast (large arrow). BF - flagellar pocket, C - kinetoplast; M - mitochondria; N - nucleus. 1µm.

The promastigotes treated with different concentrations of the essential oil showed cytoplasmatic and nuclear modifications. At the concentration of 1 µg/mL, it was observed the presence of intranuclear electron-dense material, lipid droplets and atypical vacuoles of different sizes in the cytoplasm (Figure 7A). With a higher concentration, 2 µg/mL (Figure 7B), there was an increase in the number of vacuoles, and some electron-dense material inside; additionally, it was observed changes in flagellar pocket, fulfilled with vacuoles, and intense exocytosis activity.

At higher concentrations of 5 and 10 µg/mL (Figure 7C and D), there was an increase of the atypical vacuoles, with different sizes, increase of the electron-dense material and of the lipid droplets, and damage to the nuclear membrane, with a significant swelling. Additionally, the concentration of 10 µg/mL (Figure 7E), was also observed the condensation of kinetoplast mitochondrial matrix, besides the separation of the parasite's lipid bilayer membrane.

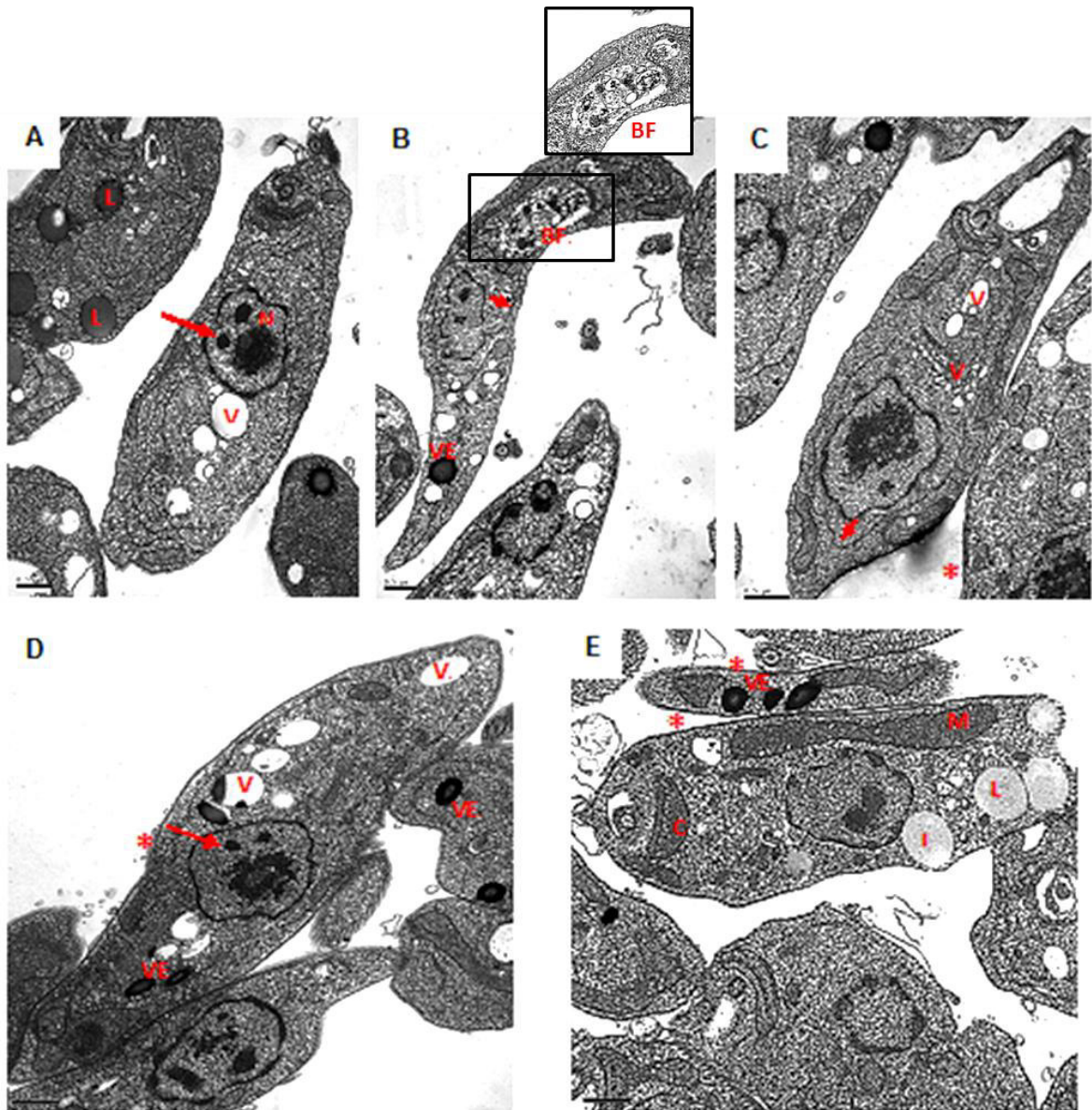


Figure 7 - Ultrastructural effect of essential oil of *V. brasiliiana* leaves under *L. amazonensis* promastigotes grown in RPMI supplemented media and incubated for 24 hours at 26°C. (A) 1 µg/mL: presence of electron-dense material in the nucleus (large arrow), lipid droplets (G) and atypical vacuoles (V) in the cytoplasm. (B) 2 µg/mL: atypical vacuoles with electron-dense material (SS) in the cytoplasm; vesicles and electron-dense material in the flagellar pocket; expansion of the nuclear membrane (lower arrow). (C) 5 µg/mL: atypical vacuoles (V) of different sizes in the cytoplasm; expansion of the nuclear membrane (lower arrow); separation of the lipid bilayer of the cell (asterisk). (D and E) 10 µg/mL: atypical vacuoles (V) with electron-dense material (LV), condensation of mitochondrial matrix and kinetoplast, presence of electron-dense material in the nucleus (large arrow), lipid droplets (G), separation of the lipid bilayer of the cell (asterisk). BF - flagellar pocket, C - kinetoplast; M - mitochondria; N - nucleus.

Regarding the ultrastructural effect of chloroform fraction, it can be seen serious damage to cytoplasmatic organelles and to the lipid membrane of the parasite (Figure 8), more significantly than the ones observed under the effect of ethanol extract, at the same concentrations. At the smallest concentration tested, 3 $\mu\text{g/mL}$, minor alterations were observed, with the presence of few intracytoplasmatic vacuoles (Figure 8B). However, at the concentration of 7 $\mu\text{g/mL}$, there was more significant changes, with the presence of intracytoplasmatic vacuoles and lipid droplets, kinetoplast swelling and mild damage of the parasite lipid bilayer (Figure 8C and D). The promastigotes treated with 15 $\mu\text{g/mL}$ showed vacuolization in the flagellar pocket, expansion of the nuclear membrane, kinetoplast swelling and vacuolization (Figure 8E). By doubling the concentration of the chloroform fraction to 30 mg/mL , it can be seen an increase of intracytoplasmatic lipid droplets, disruption of the lipid bilayer, chromatin condensation and destruction of cytoplasmic organelles (Figure 8F and G).

These cytoplasmic and/or nuclear changes compromise the parasite survival, either through directly lesions in the nuclear membrane or lipid bilayer, as well as promoting the destruction or disruption of intracytoplasmatic organelles, such as mitochondria, kinetoplast and, in particular, changes in flagellar pocket, which compromise the parasite's mechanisms of pinocytosis and exocytosis, which are fundamental for their survival

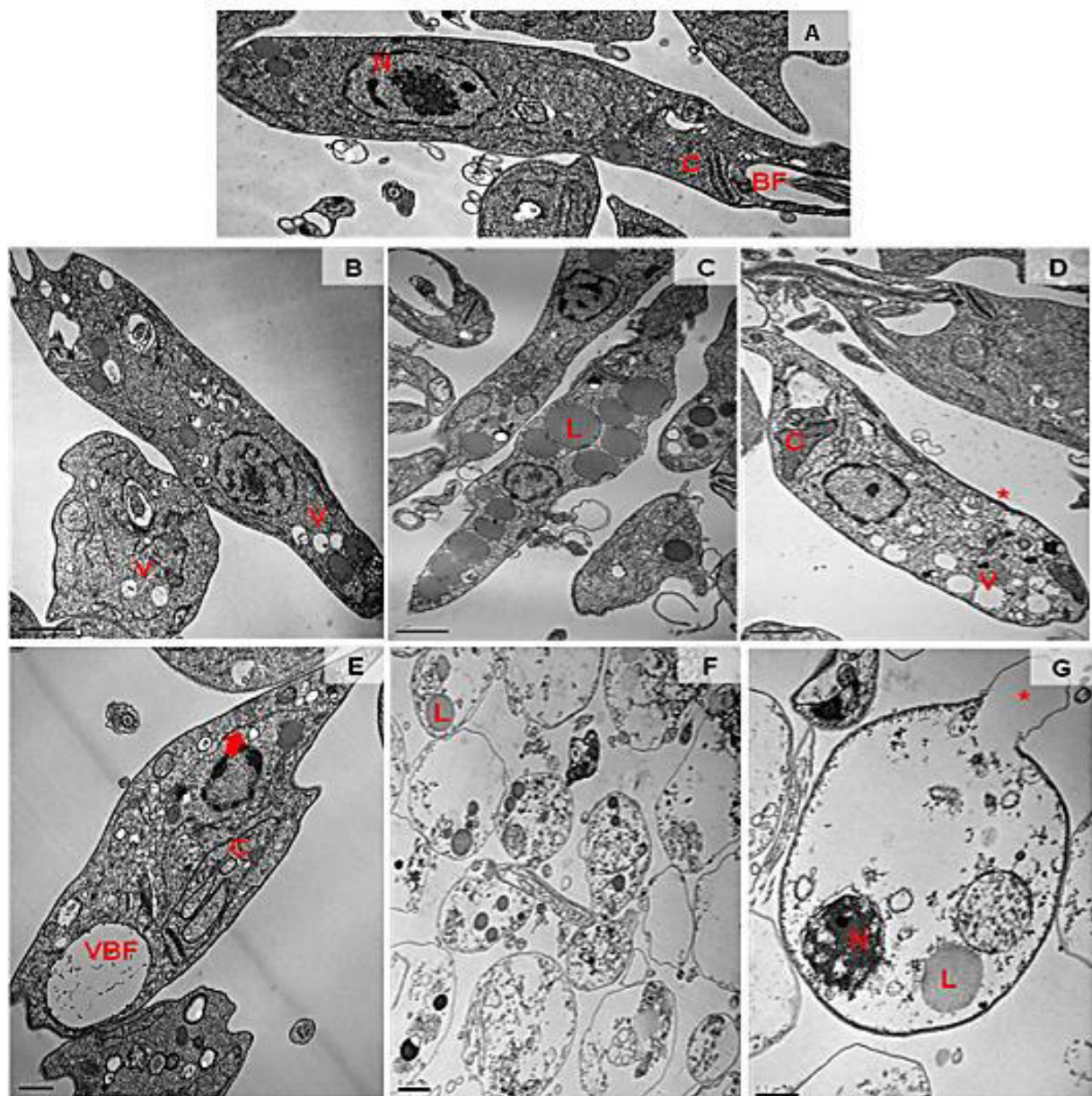


Figure 8 - Ultrastructural effect of chloroform fraction obtained from the crude ethanolic extract of *V. brasiliiana* leaves under *L. amazonensis* promastigotes grown in RPMI supplemented media and incubated for 24 hours at 26°C. (A) Control group promastigote, normal morphology. **(B - G)** *L. amazonensis* promastigotes treated with different concentrations of the chloroform fraction: **(B)** 3 µg/mL, mild alterations, intracytoplasmic vacuoles (V); **(C and D)** 7 µg/mL, vacuoles (V) and intracytoplasmic lipid droplets (L), kinetoplast tumefaction (C), discrete separation of the parasite lipid bilayer (asterisk); **(E)** 15 µg/mL, vacuolization in the flagellar pocket (VBF), expansion of the nuclear membrane (arrow), tumefaction and vacuolization in the kinetoplast (C); **(F and G)** 30 µg/mL, intracytoplasmic lipid droplets (L), disruption of the lipid bilayer (asterisk), a small amount of cytoplasmic organelles, chromatin condensation (N). BF – flagellar pocket, C – kinetoplast; N – nucleus. 0,5 to 1,0 µm.

4 DISCUSSION

The plant biodiversity is a valuable source of knowledge and development of researches, and it is an important reserve of phytochemicals compounds, that must be used consciously on the search for new therapeutic resources for many diseases; in particular those diseases where the recommended drugs are potentially toxic and causes parasitic resistance.

This way, the search for natural compounds with leishmanicidal action is rising over the past decade, with several studies being made using crude extracts, fractions and oils of leaves, fruits, seeds, roots and stems of many plants, under many species of *Leishmania* (Rocha et al., 2005; Costa et al., 2006; Braga et al., 2007; Micheletti e Beatriz, 2012; Colares et al., 2013; Ribeiro et al., 2014; Rottini et al., 2015).

The targets of the anti-parasitic drugs are mainly related to the parasite's biochemical and metabolic pathways (Mishra et al., 2013). What is intended in searching for new natural drugs is that their phytochemicals compounds have a selective inhibitory action on the enzymes in these pathways, without causing damage to host cells. Some routes are already known, among them the polyamine, purine, glycolytic, thiols and sterol pathways. The high leishmanicidal efficacy of amphotericin B is related to the drug's ability to form chemical complexes with the sterols, especially interfering with the ergosterol present in the cell membrane, leading to the appearance of intramembranous pores, ionic imbalance and death of the parasite (Singh et al., 2014).

Plants synthesize several chemical compounds, primary and secondary metabolites, according to the soil and climatic conditions; and their need for adaptation and defense against the attack by herbivores and pathogens in general (Mishra, et al., 2010). Many secondary metabolites, such as quinones, alkaloids, terpenes, sterols, saponins, phenolic compounds and their derivatives have been investigated for anti-parasite potential (Micheletti e Beatriz, 2012; Singh et al., 2014). We emphasize here the terpenes and sterols, which correspond to the major components in the specie *V. brasiliiana*.

Terpenes correspond to a wide variety of substances derived from the mevalonic acid whose classification is according to number of isoprene units (C₅), where the group of sesquiterpenes is the one with 15 carbons (Viegas Junior, 2003). The essential oil of *V. brasiliiana* showed significant amounts of sesquiterpenes, especially caryophyllene.

Essential oils, which comprise mixtures of volatile compounds generally have, as major constituents, terpenoids, in particular, mono- and sesquiterpenes (De Medeiros et al., 2011). Some research groups have evaluated the leishmanicidal potential of essential oils, such as Rottini et al. (2015), who studied the antileishmanial action of the sesquiterpene α -bisabolol under the parasite *L. amazonensis*. It was observed a 50% inhibition of the parasites at the concentrations of 4.26 μ g/ml in promastigotes and 14.82 μ g/ml in amastigotes, after 48 h of treatment.

Previous research had already shown that α -bisabolol would be a potential leishmanicidal agent. Colares et al. (2013) when investigating the anti-*Leishmania* action (against *L. amazonensis*) of the specie *Vanillosmopsis arborea* Baker, an Asteraceae, found that 97.9% of the constituents present in the essential oil extracted from this plant corresponded to α -bisabolol. This component separately showed an IC₅₀ of 4.95 μ g/ml, in contrast to the oil IC₅₀ value, which was 7.35 μ g/ml.

V. brasiliiana, as well as the aforementioned plant, belongs to the Asteraceae family, which is one of the most representative group of angiosperms (Hattori e Nakajima, 2008). Other Asteraceae, such as *Artemisia absinthium* and *Echinops kebericho*, have been evaluated regarding the leishmanicidal effect of its essential oil, under the species *L. donovani* and *L. aethiopica*, and the minimum inhibitory concentration was 0.0097 to 0.1565 μ g/ml in promastigotes forms and 0.24 to 0.42 μ g/ml in amastigotes, varying according to the type of parasite (Tariku et al., 2011). It is believed that this anti-parasitic activity is related to the presence of a sesquiterpene named lactone desodrocostus, which is the major compound of *E. kebericho* (Rondon et al., 2011).

Many sesquiterpenes from extracts and essential oils have been isolated, and their leishmanicidal action evaluated individually. It is highlighted the compound dehydrozaluzanin C, present in different weeds of Asteraceae family (Macias et al.,

2000). It was extracted from *Munnozia maronii* leaves, and tested in 12 *Leishmania* species, among them *L. amazonensis*, whose IC₅₀ in the first 24 hours was 5 µg/ml (Fournet et al., 1993).

In this study, *V. brasiliiana* essential presented a large percentage of sesquiterpenes, especially beta-caryophyllene, which corresponds to approximately 40% of the total composition. Maia et al. (2010) when studying the same species in the State of Ceará observed the presence of 36.7% of beta-caryophyllene or (E) -caryophyllene.

This compound has already been found in other species of the genus *Vernonia*, such as *V. remotiflora*, with a wide spectrum antimicrobial activity, inhibiting the growth of Gram-negative bacteria (*Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Salmonella choleraesuis*, *Klebsiella pneumoniae*) and Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) (Maia et al., 2010). The potent antimicrobial and antifungal action of this compound is well reported (Garg and Siddiqui, 1992; Formisano et al., 2006; Costa et al., 2009).

Besides these activities, the leishmanicidal potential of caryophyllene has been evaluated by Monzote et al. (2014). When verifying the effect of the essential oil and major compounds of *Chenopodium ambrosioides*, caryophyllene was isolated and tested under *L. amazonensis* promastigotes and amastigotes, which was obtained an IC₅₀ of 3.7 µg/ml for the oil and 4.9 µg/ml for the isolated compound, thus evidencing the leishmanicidal potential of caryophyllene. However, in this case, caryophyllene was not primarily responsible for antileishmanial activity, whereas the other major oil compound, ascaridole, showed an IC₅₀ of 0.1 µg/ml, and a selectivity index to the parasite four times greater when compared to caryophyllene.

Previous studies by Santos et al. (2012) using copaiba oil extracted from *Copaifera reticulata* have shown that beta-caryophyllene is the major compound of this plant, representing 40.9%. Both oil and caryophyllene showed antileishmanial activity in promastigotes, causing serious ultrastructural alterations in the parasite, in particular damage to the cytoplasm and denaturation of cell membrane.

Studies carried out by Martins et al. (2014) using *V. brasiliiana* essential oil demonstrated differences of chemical compounds according to the part of the plant

used. Were identified as main compounds: modheph-2-ene, alpha-isocomen, ciperen, beta-isocomen and beta-caryophyllene in the root; palmitic acid, hex-2-en-1-ol, hexan-1-ol and septacosan in the flowers; trans-caryophyllene, germacrene-D bicyclogermacrene, escaptulenol and palmitic acid in the leaves.

By relating the data from this survey with those present in the literature, it is clear that the production of secondary metabolites by plants suffer diverse influences, as the same fraction of the same plant species, but under different soil and climatic conditions, have some different major compounds.

Besides phytochemical analysis, Martins et al. (2014) evaluated the leishmanicidal and trypanocidal potential of essential oils and crude ethanolic extracts. It was found that the extracts were toxic at the concentrations that showed activity against *Trypanosoma cruzi* and *L. amazonensis*. However, the essential oil showed no cell toxicity and presented a tripanossomidal and leishmanicidal action, with an IC₅₀ of 150 µg/ml in *L. amazonensis* promastigotes. It is worth noting that this concentration is 75 times higher than the one found in our work, and with a lower selectivity index to the parasite. In this work, the oil presented an IC₅₀ of 2.022 µg/ml, with selectivity index above 43%. Additionally, the ethanol extract was not cytotoxic in leishmanicidal concentrations.

Another important class of secondary metabolites found in our study was esters, prevalent in *V. brasiliiana* chloroform fraction. Some studies have been made with different types of esters, evaluating their action under *Leishmania* species (Rocha et al., 2005; Pan et al., 2012), especially against promastigotes and amastigotes of *L. mexicana*, where was observed a leishmanicidal activity at very low concentrations, ranging from 1.4 to 14.5 µM in amastigotes forms (Garcia-Granados et al., 1997; Compagnone et al., 1998).

Among *V. brasiliiana* fractions studied in this research, the chloroform fraction was the one that showed better results, with a selectivity index to the parasite higher than the ethyl acetate and methanol fractions and the crude extract itself, however was lower when compared to the essential oil.

V. brasiliiana essential oil presented a significant leishmanicidal activity, at very low concentrations, with an IC₅₀ of 2.022 µg/ml under *L. amazonensis* promastigotes.

These values are close to those found by Santin et al. (2009), evaluating *Cymbopogon citratus* oil, which IC₅₀ was 1.7 µg/ml, however, were below the values of *Achillea millefolium* oil which IC₅₀, was 7.8 µg/ml (Santos et al., 2010).

The low IC₅₀ values, together with a greater selectivity to the parasite of the crude extract, chloroform fraction and essential oil, lead us to the need for ultrastructural analysis, where it was verified serious damage to *Leishmania* cellular components.

Similar ultrastructural changes in *L. amazonensis* promastigotes were already reported by Santos et al. (2012), with copaiba oil, as well as by Colares et al. (2013), with *V. arborea* essential oil and its major compound α-bisabolol. Subsequent studies by Rottini et al. (2015) confirmed the alterations promoted by α-bisabolol, culminating in severe damage to cytoplasmatic organelles, disruption of the lipid bilayer and parasite death.

The literature reports that terpenes can easily overcome the lipid bilayer and consequently enter the cell, where they will promote cytoplasmatic changes, with organelles damage as well as nuclear and mitochondrial membrane damage (Santoro et al., 2007); which confirms the mitochondrial swelling, disruption of nuclear membrane and separation of cell bilayer observed in promastigotes treated with the essential oil.

Santos et al. (2012) found that copaiba oil, whose main component is the sesquiterpene beta-caryophyllene, promote alterations in mitochondrial membrane potential, with a potential reduction according to the concentration (100 and 200 µg/ml) and the treatment course. This alteration was analyzed by fluorescence intensity using the reagent rhodamine 123. Rottini et al. (2015) also verified a reduction in mitochondrial membrane potential through flow cytometry, in 69.06% of *L. amazoensis* promastigotes after exposure to α-bisabolol IC₅₀. It is important to note that *Leishmania* have only one mitochondria, and it is essential to the parasite, due to ATP synthesis and oxidative phosphorylation (Sen et al., 2004).

Besides mitochondrial alterations, it was found in *Leishmania* exposed to the three treatments increased lipid inclusions and intracytoplasmatic vacuolation, directly proportional to the increase of the concentrations, indicating alterations in the

parasite's lipid metabolism. It is highlighted that the cell membrane has a lipid composition, and ergosterol is one of the most important lipids present in *Leishmania* and the action target of main drugs, once modifications in this component will promote instability and compromise the membrane selective permeability, with subsequent death of the parasite (Nibret and Wink, 2010; Singh et al., 2014).

The most significant ultrastructural changes were observed in parasites exposed to different concentrations of chloroform fraction, resulting in the disruption of the cell membrane at the highest concentration tested. It is believed that this action of the chloroform fraction is directly related to the chemical compounds present in the same, predominantly esters, classified as simple or neutral lipids, soluble in nonpolar organic solvents, such as chloroform (Solomons and Fryhle, 2013).

It is known that highly lipophilic compounds, such as esters, have a great ability to cross the cell membrane, which is formed by a lipid bilayer. Consequently, these compounds will easily accumulate inside the promastigotes and promotes alterations in some compounds permeability, such as intrinsic electrolytes, leading to the death of the parasite (De Medeiros et al., 2011).

This way, our work demonstrates that compounds extracted from *V. brasiliensis* leaves, in particular those present in the essential oil and chloroform fraction, are potential antileishmanial agents in non-cytotoxic concentrations, with a high selectivity to *L. amazonensis*, causing serious ultrastructural damages that compromise the parasite viability. It is necessary to continue this study in order to evaluate this potential *in vivo*, with the purpose to develop new drugs that can be used in the treatment of cutaneous leishmaniasis.

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Parte III

Considerações finais, Conclusão,

Referências e Anexos

9 CONSIDERAÇÕES FINAIS

- ✎ A prospecção fitoquímica demonstra que os extratos etanólicos brutos (EEB) da folha e caule de *Arrabidaea chica* possuem flavonoides, compostos fenólicos, taninos, antocianinas e chalconas;
- ✎ Nos EEBs de *Vernonia brasiliiana* foi identificado os metabólitos terpenos, esterres e taninos nas folhas e caule;
- ✎ O óleo essencial de *V. brasiliiana* apresentou na análise cromatográfica uma predominância por substância da classe dos sesquiterpenos;
- ✎ Os EEBs das folhas de *A. chica* e *V. brasiliiana* e, óleo essencial de *V. brasiliiana* apresentam citotoxicidade celular *in vitro* (CC₅₀ - 189,9 µg/mL, 44,32 e 88,35, respectivamente);
- ✎ O EEBs do caule de *A. chica* e *V. brasiliiana* não apresentaram atividade leishmanicida em concentrações inferiores a 500 µg/mL;
- ✎ O EEB das folhas da *Arrabidaea chica* possui atividade contra formas promastigota de *L. amazonensis*, com um IC₅₀ de 155,9 µg/mL;
- ✎ O EEB das folhas da *V. brasiliiana* possui atividade contra formas promastigota de *L. amazonensis*, com um IC₅₀ 18 vezes inferior ao da *A. chica*;
- ✎ O óleo essencial (OE) de *V. brasiliiana* apresentou atividade leishmanicida em concentrações muito inferiores aos EEBs e frações avaliadas;
- ✎ O EEB, a FC e o OE de *V. brasiliiana* promoveram importantes alterações na ultra-estrutura das formas promastigotas de *L. amazonensis*, com comprometimento da membrana celular e lesão/destruição de organelas citoplasmáticas;
- ✎ Os extratos, frações e óleo essencial das espécies vegetais estudadas apresentaram uma maior seletividade a *L. amazonensis* em comparação a célula hospedeira;
- ✎ A preparação tópica elaborada com EEB da folha de *A. chica*, apresentou inicialmente uma resposta cicatricial satisfatória, com angiogênese e colagenização, contudo que não perdurou acentuada ao longo do período de observação, não demonstrando, portanto, otimização do processo de cicatricial.

10 CONCLUSÃO

- ☞ O potencial cicatricial da *A. chica* precisa de estudos posteriores, eliminando a interferência dos fatores externos e otimizando a ação do extrato na lesão;
- ☞ Os compostos extraídos das folhas de *V. brasiliiana* e *A. chica* são potenciais agentes leishmanicida em concentrações não citotóxicas, fundamentando e impulsionando pesquisas futuras, que deem continuidade a avaliação desse potencial, no intuito de isolar os compostos bioativos, visando o desenvolvimento de novos fármacos e disponibilidade de um recurso terapêutico alternativo no tratamento da leishmaniose cutânea.

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- List of abbreviations used (if any)
- Competing interests
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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](http://www.rsc.org/dose/title%20of%20subordinate%20document). Accessed 15 Jan 1999.

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DESCRIPTION

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their **biological** and **pharmacological effects** based on the principles established through international conventions.

Early people confronted with illness and disease, discovered a wealth of useful **therapeutic agents** in the plant and animal kingdoms. The empirical knowledge of these **medicinal substances** and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of **indigenous remedies**. Chemists continue to use **plant-derived drugs** (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

In recent years the preservation of local knowledge, the promotion of indigenous medical systems in primary health care, and the conservation of biodiversity have become even more of a concern to all scientists working at the interface of social and natural sciences but especially to ethnopharmacologists. Recognizing the sovereign rights of States over their natural resources, ethnopharmacologists are particularly concerned with local people's rights to further use and develop their autochthonous resources.

Accordingly, today's ethnopharmacological research embraces the multidisciplinary effort in the:

- documentation of **indigenous medical knowledge**,
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The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals. **Please note that figures and tables should be embedded in the text as close as possible to where they are initially cited.** It is also mandatory to upload separate graphic and table files as these will be required if your manuscript is accepted for publication.

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RESEARCH ARTICLE

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Leishmanicidal, cytotoxicity and wound healing potential of *Arrabidaea chica* Verlot

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Abstract

Background: Leishmaniasis includes a wide complex of diseases that affect humans and other mammals, and can range from a mild cutaneous form to a severe visceral type. The safety of the standard treatment using pentavalent antimony is a concern due to its toxic effects. The search for alternative, effective and less toxic treatments has led to the testing of natural products. The present study aimed to evaluate the cytotoxic, leishmanicidal and healing potential of *Arrabidaea chica*.

Methods: The crude ethanolic extract, as well as the chloroform, methanol and ethyl acetate fractions of *A. chica* were prepared and phytochemical analysis was performed. Cytotoxic evaluation was carried out through MTT colorimetric assay, and the 50 % cellular cytotoxicity was determined. After that, the effect of the extract and fractions against *Leishmania amazonensis* promastigotes, at intervals of 24, 48 and 72 h, was analyzed, and 50 % inhibitory concentration was determined. The healing effect of the plant was also tested in surgical lesions in Swiss mice skin.

Results: Phytochemical screening showed that the crude extracts contained flavonoids, tannins, anthocyanidins and chalcones. The leishmanicidal potential of *A. chica* produced satisfactory results in concentrations of between 60 and 155.9 µg/mL. Cytotoxic assay revealed a 50 % reduction in viable cells at a concentration of 189.9 µg/mL. The healing results indicated that the treated group exhibited more pronounced signs of lesion resolution in the early period, but this pattern did not persist throughout the treatment.

Conclusions: The results of the present study demonstrate that *A. chica* has cytotoxic and leishmanicidal potential but its healing effect must be better studied.

Keywords: *Arrabidaea chica*, Cytotoxicity, Healing, Leishmanicidal activity

Background

Leishmaniasis is a group of diseases that affect several species of mammals that live in tropical and subtropical regions. Literature describes more than 20 species of the protozoan *Leishmania* that causes leishmaniasis, including cutaneous and visceral leishmaniasis [1, 2]. These clinical forms differ in clinical presentation, morbidity and mortality [3].

According to the World Health Organization, neglected diseases are those associated with low social indicators, and are thus more frequently found in developing countries where there is a lack of resources aimed at control and treatment and little research into finding an effective solution [4].

Among the clinical forms of the disease, the cutaneous, characterized by skin lesions, chiefly in exposed body parts, is the most common [5]. Depending on the species of *Leishmania*, in some patients leishmaniasis can heal without treatment, while in others diffuse cutaneous or mucocutaneous forms can develop [6].

In all clinical forms, pentavalent antimonials have been used as a mainstay therapy: the available drug for the treatment of leishmaniasis in the United States and Europe is sodium stibogluconate (Pentostam), while meglumine

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antimonate (Glucantime®) is more used in South America and Africa [7]. Despite this therapy having been widely employed since 1912, the mechanism of action is poorly understood and adverse effects, which include renal and hepatic toxicity, are severe [8]. Other limitations of this therapy are the necessity of daily parenteral administration and the resistance of the drug [2].

These limitations, the vast Brazilian biodiversity and traditional knowledge has led to the establishment of research networks to evaluate the leishmanicidal effect of plants from various biomes, such as the Cerrado and Amazon. It is therefore very important to perform experiments using natural products in order to identify new, less toxic, compounds which could be taken orally. In addition, the Brazilian government has created the RENISUS (Rede nacional de plantas medicinais de interesse do sistema único de saúde), a list of plants that can be used in the Unified Health Service, and *Arrabidaea chica* is included in this list.

Arrabidaea chica Verlot, popularly known as pariri, belongs to the Bignoniaceae Family, popularly known as *pariri*. It is endemic in almost all of Brazil but is most frequently found in the Amazon Forest [9] where it is used to treat skin disease, mulligrubs, anemia, jaundice and inflammatory reaction [10–12]. Widespread use by native populations lead researchers from different countries to evaluate the medicinal properties of this plant, including its anti-inflammatory [13]; astringent, antioxidant [14, 15]; anti-ulcer [16]; antimicrobial, antifungal [17, 18] and wound healing [19, 20] effects.

The aim of the present study was to evaluate the phytochemical effects, wound healing capacity and cytotoxicity of *Arrabidaea chica*, as well to evaluate the action of the crude extract and fractions of these plants on promastigotes of *L. amazonensis*.

Methods

Ethics committee

The experimental protocol for this study was approved by the ethics committee for animal research of the Universidade Estadual do Maranhão (CEUA-UEMA protocol 26/2009) and by the ethics committee in animal experimentation of Instituto Oswaldo Cruz - FIOCRUZ/RJ (CEUA-FIOCRUZ protocol LW72/12).

Plant

Leaf and stem samples of *Arrabidaea chica* Verlot. were cultivated and collected in the Atico Seabra Herbarium, Departamento de Farmácia, Universidade Federal do Maranhão (São Luís, Maranhão, Brazil). The plant material was identified by Dr. Ana Zélia Silva and a voucher specimen was deposited under the number 1067. The plant material was collected between August and November 2011, which represents the dry season in

this region, with a rainfall rate of 900 mm and an average temperature of 28 °C.

Preparation of crude extract and fractions

To obtain the crude ethanolic extract, the samples were dried at 40 °C with forced air circulation. The dried material from each part of *A. chica* was macerated and immersed in ethyl alcohol, in a proportion of 3:1 (alcohol and plant material, respectively). In order to obtain the ethanolic extracts solvent removal was carried out by rotoevaporation, under reduced pressure at 30 to 40 °C, until the desired concentrations were achieved. The samples were stored under refrigeration and protected from light until analysis.

To determine the dry weight of the samples three aliquots of 0.5 mL of crude ethanolic extract were used. The yield of samples was calculated using the total weight of the powder, the dry weight obtained and the total final volume of the concentrated extract.

Additionally, the crude ethanolic extracts were fractionated by sequential partition using solvents of increasing polarity: chloroform, ethyl acetate and methanol. Extraction was carried out in the solid phase using silica gel, grail, pistil and approximately 500 mL of each solvent. Once obtained, the fractions were submitted to leishmanicidal and cytotoxic tests. Those that produced satisfactory results were sent to the Analytical Center of the Universidade de São Paulo (São Paulo, Brazil), to identify the bioactive compounds through gas chromatography and mass spectrometry.

Phytochemical analysis

To evaluate the presence of secondary metabolites (saponins, anthocyanidins, chalcones, phenols, tannins, flavonoids, steroids, triterpenoids and anthraquinones), crude ethanolic extracts of *A. chica* were solubilized in methanol (in a concentration of 100 mg/5 mL), according to Costa and Matos [21, 22].

In vitro cytotoxic assay

Obtaining of peritoneal macrophages and cell culture

Peritoneal macrophages were obtained from 4-week-old female BALB/c mice, from the Laboratory Animal Breeding Center (CECAL-FIOCRUZ) and kept in the Hélio and Peggy Pereira Pavilion animal facility, located at the Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, Brazil).

Three mL of 3 % sodium thioglycollate was inoculated into the peritoneal cavity of the animals and, after 72 h, were euthanized in a CO₂ chamber. For macrophage recovery, 5 mL of a cold and sterile pH 7, 2 PBS solution was injected into the abdominal cavity, followed by massage and collection of lavage, which was kept in a falcon tube, placed in an icebox. After collection, the lavage was analyzed under an optical microscope to verify the

presence of macrophages and the lack of red blood cells and bacteria.

Peritoneal lavage was centrifuged at 500 xg for 10 min at 4 °C. The supernatant was discarded and the cells resuspended in 1 mL of RPMI 1640 and counted in a Neubauer chamber. Final macrophage concentration was standardized at 5×10^5 cells/well. For cell culture, RPMI supplemented with 10 % fetal bovine serum, L-glutamine (20 mM), sodium bicarbonate solution (7.5 %), penicillin (100 µg/mL) and streptomycin (50 µg/mL) was used, incubated at 37 °C with 5 % CO₂.

CC₅₀ determination

Cytotoxicity was measured by MTT colorimetric assay, in accordance with [23]. Briefly, 100 µL of peritoneal macrophages (5×10^5 cells/well) were added to 96-well plates and incubated for 2 h, at 37 °C with 5 % CO₂. After that, 100 µL of the crude ethanolic extracts or fractions of *A. chica* were added to the wells in serial concentrations (600 to 0.97 µL/mL). For each concentration a negative control was maintained. Dimethyl sulfoxide (DMSO) was used as a control drug in serial dilutions starting at 20 %. After 24 h of incubation, 5 µL of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] was added to each well, then the plates were incubated again for 2–4 h under the same conditions. Later, they were centrifuged at 1500 xg and 200 µL of each well supernatant was discarded, before 100 µL of DMSO was added. Absorbance was measured in a spectrophotometer, at a wavelength of 540 nm. 50 % cellular cytotoxicity (CC₅₀) was calculated using Graph-Pad Prism 5.

In vitro antileishmanial activity

Parasites

The present study used the *Leishmania amazonensis* (MHOM/BR/76/MA-76 – IOC/FIOCRUZ-RJ) strain, isolated from a patient with diffuse cutaneous leishmaniasis and maintained by serial passages in BALB/c mice, and periodically re-isolated in culture medium. Promastigote forms were grown at 26 °C in Schneider's medium, supplemented with 10 % fetal bovine serum, streptomycin (100 µg/mL) and penicillin (100 µg/mL). To ensure infectivity only cultures of promastigote with a maximum of seven *in vitro* passages were used.

Before evaluation of leishmanicidal activity and determination of the 50 % inhibitory concentration (IC₅₀) of *A. chica*, a screening test was performed to determine the concentration used in IC₅₀ assay. The concentration used ranged between 500 and 0.97 µg of crude ethanolic extracts or fractions, diluted in 1 % DMSO, and added to 96-well plates containing RPMI, serially and in triplicate. Wells containing medium and parasites, medium only, and the reference drug, Amphotericin B (2 µg/mL),

were used as controls. 100 µL of *L. amazonensis* promastigotes in the logarithmic phase (1×10^6 parasites/mL) were added to each well, and the plate was incubated at 26 °C. Parasite viability was measured after 24, 48 and 72 h, by observing motility under an inverted microscope. The parasites were classified according to mobility on a scale of zero to ten, where zero means no viable forms and ten, viable forms.

Based on the screening results the IC₅₀ was determined, following the same aforementioned protocol, and leishmanicidal activity was assessed by counting the viable parasites in a Neubauer chamber. The data was then normalized using the formula: Percentage of growth inhibition = number of parasites in test wells/number of parasites in the control well, multiplied by 100; and used for statistical analysis.

Wound healing activity

Cream and therapeutic protocol

Two topical preparations (10 mg/g and 20 mg/g) were prepared containing the crude ethanolic extract from *A. chica* leaves mixed with Lanette® cream, as a water-soluble anionic vehicle.

Forty 4-to-6 week old male Swiss Webster mice from the animal facility of the Universidade Estadual do Maranhão (São Luís, Maranhão, Brazil) were used in this experiment. The animals were randomly divided into two groups ($n = 20$ each). The control group was treated with Lanette® cream and the experimental group was treated with Lanette® cream containing the crude ethanolic extract of *A. chica*.

For creation of a dorsal skin wound, the animals were anesthetized with an intraperitoneal injection of xylazine hydrochloride (10 to 15 mg/kg) and ketamine hydrochloride (100 to 150 mg/kg). Trichotomy of the dorsal region and demarcation of the skin with a punch was then carried out. Resection of a circular segment of approximately 6 mm diameter was performed, exposing the dorsal fascia. Hemostasis was carried out by digital compression with sterile gauze, with treatment initiated immediately afterwards.

The therapeutic protocol was performed twice a day throughout the experimental period. Transverse and longitudinal diameters of the lesions were measured daily, using a digital caliper, and monitored through photography. Skin wound areas were calculated using the formula $A = \pi \times R \times r$, in which A represents the area, π is equal to 3.14, R represents the largest diameter and r the smallest diameter of the wound.

Histological analysis

After 3, 7, 14 and 21 days of treatment, the mice were euthanized and the lesions were excised with a margin of 1 cm of integrate skin, stored in 10 % buffered formalin

solution and submitted to histological procedures. After embedding in paraffin, 5 µm thick cross sections were cut and stained with hematoxylin-eosin (HE) for morphological analysis of the tissue. Additionally, fragments of liver, spleen and kidney were collected, for evaluation of potential cytotoxic lesions.

Slides were analyzed by light microscopy by two independent examiners, following the parameters: crust formation, presence of inflammatory infiltrate, angiogenesis, reepithelialization and collagen formation [24]. These features were graded as absent, mild, moderate and severe, according to their appearance.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0.4 (GraphPad Software Inc.). IC₅₀ and CC₅₀ values were calculated from the mean percentage reduction of promastigotes and macrophages, respectively. Curves were determined by application of sigmoidal regression to logarithm concentration response data. *In vitro* results are representative of three different experiments, performed in triplicate, under the same temperature, CO₂, time and culture medium conditions, with similar results. Analysis of wound healing was performed using the Student's t-test. Histological variables were analyzed by non-parametric test. Differences were considered significant at $P < 0.05$.

Results

Yield of extraction and phytochemical analysis

The crude ethanolic extracts of *Arrabidaea chica* leaves and stem presented a yield of 59.2 and 3.33 % respectively, which shows that the stem has a low extraction efficiency, as the same extraction method was used for both stem and leaves.

Phytochemical screening showed that both leaves and stem extracts contain flavonoids, tannins, anthocyanidins and chalcones. Phenolic compounds were found only in the leaves (Table 1).

Table 1 Phytochemical prospection of crude ethanolic extract of leaves and stem of *Arrabidaea chica*, collected in Maranhão state, Brazil

Metabolite class	Reactions	<i>Arrabidaea chica</i>	
		Leaves	Stem
Anthraquinones	NaOH	-	-
Flavonoids	AlCl ₃	+	+
Triterpenes/steroids	Anhydrous acetic/ sulphuric acid	-	-
Saponin	Foaming index	-	-
Phenolic compound	FeCl ₃	+	-
Tannin	Gelatin	+	+
Anthocyanidins/chalcones	HCl/NaOH	+	+

(+) presence; (-) absence

IC₅₀ determination

In screening tests to evaluate the potential leishmanicidal effect on *L. amazonensis* promastigotes, *A. chica* stem extract showed no activity against the protozoan, even at the highest tested concentration (500 mg/mL), and so was not used in continuity tests.

In the screening tests of the crude ethanolic extract of *A. chica* leaves, there was a reduction of 50 % of *L. amazonensis* promastigote viability with a concentration of 125 µg/mL (Fig. 1) at the intervals of 24, 48 and 72 h after incubation. However, the concentration of 250 µg/mL displayed antileishmanial activity in the first 24 h of treatment and a total absence of viable parasites after 72 h of incubation. The concentration of 500 µg/mL of the leaf ethanol extract had the best leishmanicidal efficacy at the three tested intervals.

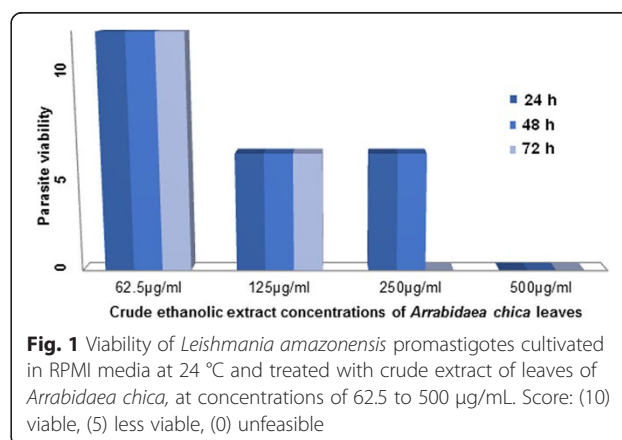
After screening, the 50 % inhibitory concentration (IC₅₀) of *L. amazonensis* promastigote forms was determined as 155.9 µg/mL (Fig. 2).

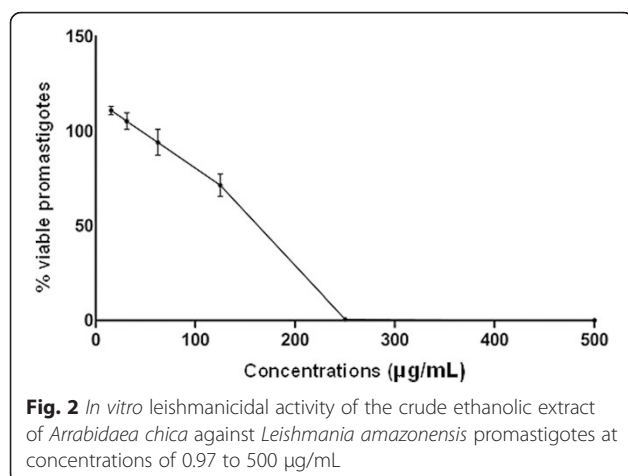
When evaluating the leishmanicidal activity of the chloroform, methanol and ethyl acetate fractions, there was a 50 % reduction of *L. amazonensis* promastigotes at concentrations of 120 µg/mL, 120 µg/mL and 60 µg/mL, respectively (Table 2). This result demonstrated that the fractions acted against the parasite at lower concentrations than the crude extract, and that the ethyl acetate fraction had the most effective result.

CC₅₀ determination

Cytotoxicity assay in the peritoneal macrophages after 24 h showed that a 189.9 µg/mL concentration of *A. chica* leaf extract reduced the number of viable cells by 50 %, representing CC₅₀ (Fig. 3).

Regarding CC₅₀ and IC₅₀ values of the crude extract, it was observed that *A. chica*'s bioactive compounds have a higher Selectivity Index (1.218) to the parasite than to the host cell, proving to be a potential treatment for leishmaniasis.





Healing effect

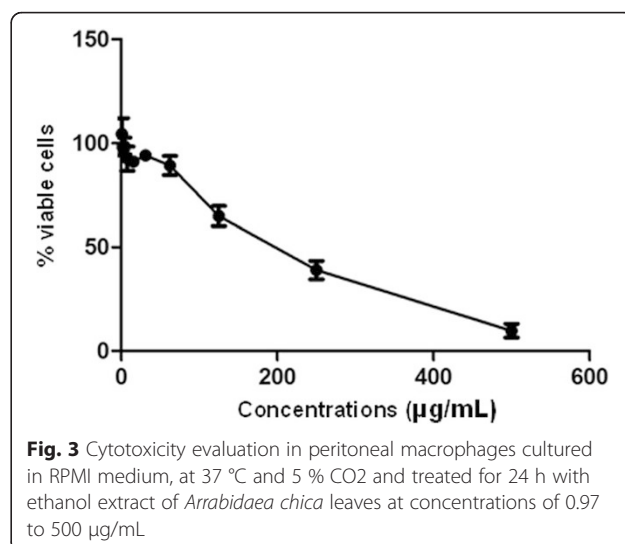
Regarding the evaluation of dermal irritation potential of the crude extract of *A. chica* leaves, a concentration of 20 mg/g caused skin irritation, with the presence of moderate and persistent erythema during the treatment period. Thus, the healing test was performed with a concentration of 10 mg/g.

In macroscopic analysis of the wound healing process, the beginning of the tissue repair process, with the presence of crusts on the third day, was noted. On the seventh day, a reduction in the size of the wound and the formation of granulation tissue in both the treated and control groups was observed (Fig. 4).

In the 0–3 day interval an increase in the lesion area was observed in the group treated with *A. chica*, as compared to the control group (Fig. 5). In the third to the 10th day, there was a marked reduction in the wound area, with a gradual approximation of the edges in both the control and treated groups.

Between the 14th and 21th post-injury days, the retraction of the wound was almost complete, with marked healing and tissue repair.

On the third day of treatment cellular debris and moderate to intense inflammatory process was observed in both groups. Discrete angiogenesis, collagen deposition



and epithelialization were observed in the treated group (Fig. 6a–b), as well as an increased amount of mast cells.

On the seventh day of treatment, re-epithelialization and the reduction of cell debris and the inflammatory process was quite evident. While there was no difference between the two groups, the patterns of re-epithelialization, angiogenesis and collagen deposition were higher in the control group.

Healing process evaluation between the 14th and 21st days showed epithelialization and intense collagen deposition in the control group (Fig. 6c–d), while the treated group (Fig. 6e–f) revealed patterns ranging from mild to intense. In both groups, there was a low number of inflammatory cells and mast cells. Angiogenesis was discreet in the treated group and varied from moderate to mild between the 14th and the 21st day.

The topical preparation with the crude extract of *A. chica* at a concentration of 10 mg/g displayed a more effective healing activity in the initial phase of the experiment, but was not able to induce a stronger response throughout the 21 day observation period.

Discussion

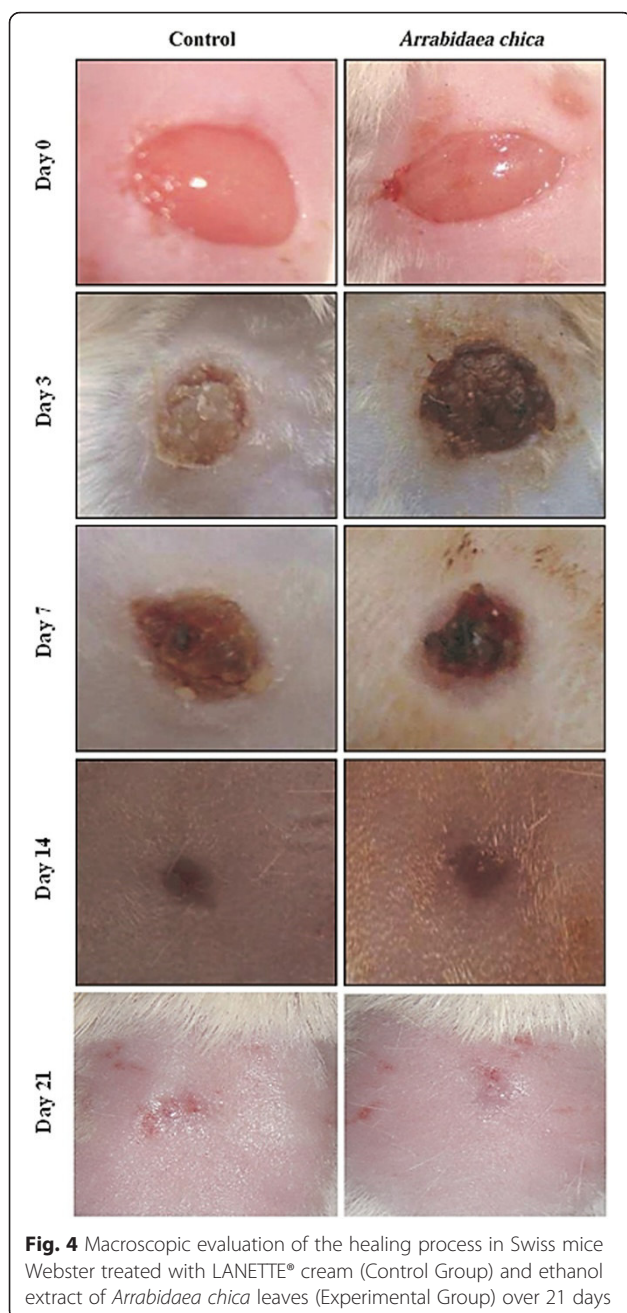
Plants are important sources of bioactive compounds, and are a major potential source of new therapeutic agents against several diseases, including those of interest to public health, in particular neglected diseases such as leishmaniasis.

The bioactive compounds produced by plant cells may vary depending on soil, climate and the season that the plant, fruit or leaves were collected. Soil and climate conditions, along with the plant's need for adaptation and defense, bring about this change in chemical constituents [25]. Phytochemical analysis of *A. chica* revealed the presence of flavonoids, tannins, anthocyanidins,

Table 2 50 % inhibitory concentration (IC₅₀) of *Leishmania amazonensis* promastigote forms after 72 h treatment with crude ethanolic extract and chloroform, methanolic and ethyl acetate fractions of *Arrabidaea chica*

Treatments	IC ₅₀ (µg/mL)
Crude ethanolic fraction	155.9 ± 0.1185
Chloroform fraction	>120
Methanolic fraction	>120
Ethyl acetate fraction	>60
Amphotericin B (reference drug)	0.76 ± 0.9218

Values represent mean ± standard deviation



chalcones and phenolic compounds, as previously described for this species [26, 27].

The first chemical study with the leaves of *A. chica* was performed by Chapman et al. and reported the isolation of the substance 3-deoxyanthocyanidin, more specifically 1,6,7-dihydroxy-5,4'-dimethoxy-flavylium (carajurin) and 6,7,4'-trihydroxy-5', methoxy-flavylium (carajurone), responsible for the reddish color of the extract [28]. In other studies, other compounds, such as flavone acacetin, oleonic triterpene acid and two deoxyanthocyanidins (6, 7, 3'-4'-tetrahydroxy-5-methoxy-flavylium and 6, 7, 3'-trihydroxy-5, 4' dimethoxy-flavylium) were isolated [29-31].

Anthocyanins are plant pigments belonging to the family of flavonoids, included in the group of phenolic compounds [32, 33]. More than 50 new anthocyanins have been isolated from the flowers, fruits, seeds and leaves of plants. They play a pivotal role in flower pollination, seed dispersal by insects and defense against predatory insects [34].

Pharmacological studies with anthocyanins reveal their high antioxidant effect [15] on metabolic diseases [35]. The antioxidant, cytotoxic, antimicrobial and diuretic potential of the ethanol extract and fractions of *A. chica* was evaluated by Amaral et al. that confirmed this antioxidant and diuretic action, and the absence of an antimicrobial and cytotoxic response [14]. The therapeutic activity of the phenolic compounds is mainly attributed to their antioxidant capacity, the chelation of metal ions, modulation of gene expression and interaction with cell signaling pathways [36].

Phenolic composition and antioxidant activity of the leaves of *A. chica* was evaluated by Siraichi et al. by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), and verified the presence of two important flavonoids, apigenin and scutellarein, which are most responsible for the significant antioxidant action [15].

In pharmacological tests, Alves et al. determined the chemical potential of *A. chica* dye, which is widely used by indigenous tribes, and verified the presence of alkaloids, anthocyanidins, anthocyanins, anthraquinone, steroids, triterpenoids, phenols, flavanones, flavanols, flavanones, saponins and catechin tannin [37]. This study described some compounds undetected in the present study, differing in the presence of anthraquinones, steroids, triterpenoids and saponins. This fact is probably due to the climate differences between the two places where the plant was collected, the states of Maranhão and Pará, as well as the method by which the extract was obtained. Both were obtained using ethanol as a solvent; however, in the present study this was submitted to 72 h rotoevaporation at 40 °C, while in the aforementioned study the extract was macerated for 10 days in a capped percolator.

In vitro pharmacological studies have demonstrated the antineoplastic and antioxidant potential of *A. chica*, and this action was directly related to the high content of flavonoids, especially anthocyanins [38]. These and other benefits made the search for new sources of anthocyanins essential [35].

The secondary metabolites in *A. chica*, such as flavonoids, the antimicrobial, antiviral, anti-inflammatory, antineoplastic, antifungal and anti-protozoan action of which has already been reported in literature [17, 38-40], encourages many researchers to study this plant.

In the present study, the leishmanicidal potential of the crude extract and fractions of *A. chica*, which displayed satisfactory results at concentrations between 60

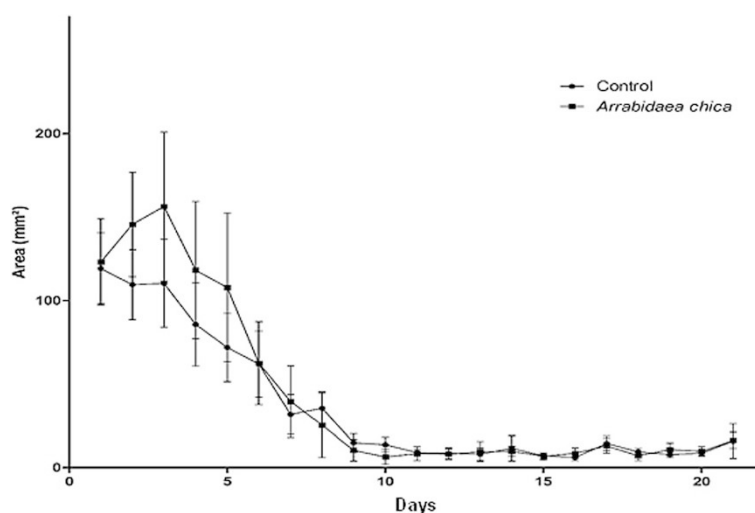


Fig. 5 Wound healing kinetics of surgical lesions in Swiss Webster mice treated with Lanette® cream (Control Group) and ethanol extract of *Arrabidaea chica* leaves (Experimental Group) over 21 days

and 155.9 µg/ml, depending on the type of fraction. The chemical constituents of the plant were more selective for the protozoan than the host cell, with a cytotoxicity effect at concentrations higher than those with leishmanicidal action.

Several studies have aimed to identify and characterize crude extracts, fractions and essential oils [41–48], as well as the isolated compounds of the plant [49, 50], with leishmanicidal activity. The screening of these natural compounds is performed with cultures of *Leishmania* spp., as it is easy to maintain and produce [51].

According to the World Health Organization, a candidate drug must undergo *in vitro* cytotoxicity and antiparasitic activity testing. After this initial screening, the results are evaluated to define the continuity of studies and the possibility of *in vivo* assays [52].

Studies performed by Rodrigues et al. [53] investigated the leishmanicidal effect of five fractions obtained from the crude hexane extract of *A. chica*, on *L. amazonensis* and *L. infantum* promastigote forms, by determining the minimum inhibitory concentration (MIC), which was found to be 37.2 and 18.6 µg/ml, respectively. This result is related to one of the fractions used, the main chemical components of which were linoleic acid methyl ester (25.38 %), n-hexadecanoic acid (19.61 %), octadecanoic acid (14.10 %) and gamma-sitosterol (12.85 %). The study also evaluated ultrastructural alterations of the parasite under the influence of this fraction, and observed mitochondrial swelling, disruption of mitochondrial membrane, presence of vesicles in the cytoplasm, mitochondria and flagellar pocket and modifications in the Golgi complex.

The significant increase in studies of plant compounds with leishmanicidal potential is due to the major

importance of this disease, of which there are large numbers of new cases annually. According to the World Health Organization 1.3 million new cases are recorded per year, being 300,000 cases of visceral leishmaniasis and 1 million cutaneous leishmaniasis [5].

Leishmaniasis are characterized as neglected diseases, as they are associated with low social indicators and are more prevalent in developing countries, whose resources in the areas of control, treatment and research are insufficient [4].

Additionally, plant extracts or plant compounds can represent a valuable source of new medicinal agents and alternative, effective and less toxic treatments, compared to conventional drugs (pentavalent antimonials, amphotericin B, pentamidine), leading to a search for natural products with a potential leishmanicidal action. Furthermore, the benefits obtained from the search for natural products have encouraged interest in valuable synthetic compounds [42].

The crude extract from the leaves of *A. chica* had a cytotoxic effect at a concentration of 189.9 µg/mL. Maliofete et al. [54], also testing the ethanol extract from the leaves of *A. chica*, observed low oral toxicity, absence of cytotoxicity and antibacterial activity against *Helicobacter pylori*. *In vivo* experimental studies did not show any clinical or histopathological signs of extract toxicity in the pleura [13], open or sutured wounds or burns [55]. Studies by Barbosa et al., assessing the antifungal, antimicrobial and antiprotozoal potential of the ethanol extract and fractions of *A. chica*, observed growth inhibition of *Trichophyton mentagrophytes* and a significant trypanocidal effect against *Trypanosoma cruzi*, as well as the absence of relevant acute toxicity, even at the highest dose tested (1000 mg/kg) [17].

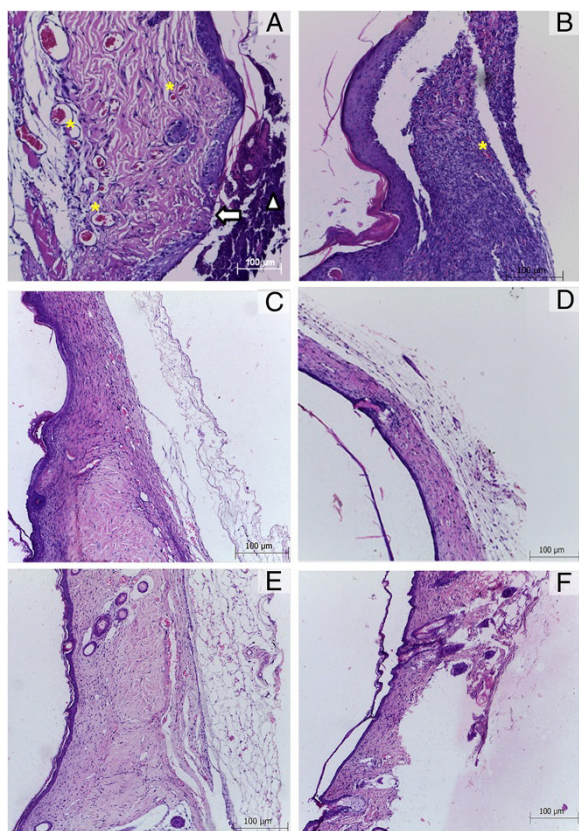


Fig. 6 Swiss Webster. Skin. Healing process through topical treatment with a cream containing the crude ethanolic extract of *A. chica* (experimental group) or only Lanette® cream (control group). **a** (3 days) - Experimental group: cellular debris (Δ), reepithelization (arrow), angiogenesis (*). **b** (7 days) - Experimental group: Absence of cellular debris and angiogenesis (*). **c** (14 days) and **d** (21 days) - Control group: Absence of cellular debris and reepithelization. **e** (14 days) and **f** (21 days) - Experimental group: Absence of cellular debris and reepithelization. HE

Several factors must be taken into consideration when evaluating the toxic effects of a plant extract, such as seasonal and environmental factors, the genotypic variations of the species, the part of the plant used to produce the extract, the time of collection and the age of the plant [25].

In traditional medicine in the Amazon region, *A. chica* is widely used as an anti-inflammatory, healing and anti-anemic medicine, and is prepared from the leaves as a tea, for oral or vaginal administration [16, 17, 56]. However, despite its wide popular use, several studies are still necessary to evaluate the medical potential of this plant.

In the present study, the healing potential of the crude extract of the leaves of *A. chica* in skin lesions was evaluated. A satisfactory response was initially observed, with angiogenesis and collagen deposition, but over the 21-day observation period it was found that the extract did not accelerate healing, presenting a similar profile to the control group.

Several local and systemic factors can cause variations in the healing process, leading to a delay or even prolongation of healing. Among these are the local of the lesion, existence of infection, surgical technique, tissue ischemia and bandages, as well as malnutrition and/or deficiency of trace minerals and vitamins [57, 58].

As cited above the importance of bandages in the healing process should be noted. Several studies have demonstrated that there the healing process is more successful when bandages are placed on the location of the wound after the application of the test product [16, 59] to protect the injury area. This step, which was not performed in the present study, may have contributed to the retardation of healing. The application of a suitable compress enables an increase of the natural debridement and simplifies healing, as it keeps the exudates rich on mediators on the wound surface [60].

Wound healing is a complex and dynamic process of the replacement of a devitalized tissue by a new tissue, which occurs through a series of biochemical cascade events; usually divided into three phases: the inflammatory, proliferative, and remodeling phases [61]. Wound healing treatment has improved considerably over time, notably in order to achieve the best scarring results in the shortest possible time.

In the present study, a strong inflammatory response was observed at the beginning of the healing process, with an increased lesion area until the 3rd day after surgical incision and the presence of inflammatory cells in histological evaluation. The inflammatory phase is characterized by increased vascular permeability and immune cells chemotaxis to the site of injury, due to the release of mediators (histamine, serotonin, bradykinin, prostaglandins, thromboxanes) [62].

The most pronounced reduction period in the wound area was between the third and the 10th day, with retraction of the wound area, representing the proliferative phase. During this phase fibroplasia, granulation tissue formation, and removal of cellular debris were observed, with the formation of a temporary repair tissue during inflammation, and development of tissue substitutes [63].

In the healing process, fibroplasia occurs together with neovascularization [61]. As the wound healing process advances, fibroblasts undergo phenotypic changes, with an abundance of rough endoplasmic reticulum, due to intense protein synthesis; and subsequent transformation into myofibroblasts, which help in the retraction of the lesion [64, 65].

Studies performed by Jorge et al. [16], when evaluating the healing, anti-inflammatory, antiulcerogenic and antioxidant effect of crude methanolic extract of *A. chica*, found that there was no reduction of paw edema induced by carrageenan or ear edema induced by croton oil in rats. However, *in vitro* fibroblast proliferation induction, stimulation

of collagen synthesis *in vivo* and *in vitro*, anti-ulcer activity and moderate antioxidant capacity were observed.

After 10 days, it was verified that 96 % of wounds in the experimental group had healed, with a prominent collagen deposition, while in the control group, treated with saline solution, only 36 % of the wounds had healed, with a mild collagen deposition [16].

The topic healing effect of *A. chica* extract in the tendons of Wistar rats was investigated by Aro et al., that found better collagen organization in the treated groups than in the control group, as well as an increase in sulfate, on the 14th day treatment [20].

Compounds exhibiting antioxidant activity can indicate good therapeutic agents in the healing process, according to Houghton et al. [57]. Non-enzymatic antioxidant activity is promoted by flavonoids, carotenoids, ascorbic acid, glutathione, and vitamin E, among others [58, 66, 67]. Therefore, the healing activity of *A. chica* is attributed to the antioxidant activity of anthocyanins and to fibroblast proliferation stimuli and consequent collagen synthesis [16, 68].

Fibroblasts are connective tissue cells, essential for dermis formation, due to collagen production, and responsible for structural firmness. After tissue injury, fibroblasts near the lesion site proliferate, migrate to the wound and produce a large amount of matrix material rich in collagen (type I and III), which helps to isolate and repair the injured tissue [63].

The proliferation of fibroblasts is directly related to the presence of macrophages in the healing process, as these release growth factors and cytokines essential for the maturation of the inflammatory phase and the beginning of the healing. Among the growth factors responsible for the proliferation, insulin-like growth factor 1 (IGF-I) and transforming growth factor beta (TGF- β) play an important role. Studies have demonstrated that IGF-I increases the procollagen chain expression in cultured dermal fibroblasts [63].

In the present study, on day 14 the control and experimental groups presented a smaller healing area than on day 07. At the end of the experiment, after 21 days, both groups were in the final stages of the healing process, in which the remodeling phase predominates, characterized by the reduction of inflammatory cells [69] and blood vessels at the lesion site [61], being the stage in which the scar acquires maximum resistance [70].

Several plants are used in popular medicine for the treatment of skin disorders. A number of these, such as *Aloe vera* ("babosa"), *Schinus terebenthifolius* ("aroeira"), *Stryphnodendrom barbatiman* ("barbatimão"), *Calendula officinalis* ("calendula") and *Triticum vulgare* ("trigo") are especially worthy of note due to the number of Brazilian studies involving the phytopharmaceutical use of these species [71–74].

Conclusions

The present study demonstrates that the compounds extracted from *A. chica* leaves are potential leishmanicidal agents in non-cytotoxic concentrations, with further research required to continue the evaluation of this potential, in order to develop new drugs based on these compounds, or associate them with drugs already used in therapeutic protocols for the disease. The wound healing potential of the plant requires additional studies, in order to eliminate the interference of external factors and optimize the action of *A. chica* extract.

Abbreviations

°C: Celsius degree; $\mu\text{g/mL}$: microgram per milliliter; μL : microliter; μm : micrometer; μM : micromolar; AlCl_3 : Aluminum chloride; ANOVA: Analysis of variance; CC_{50} : 50 % cellular cytotoxicity; CO_2 : Carbon dioxide; DMSO: Dimethyl sulfoxide; FeCl_3 : Iron chloride; FGF2: Fibroblast growth factor 2; G: giros; HCl: Hydrochloric acid; HE: Hematoxylin-eosin; IC_{50} : 50 % inhibitory concentration; IGF-I: Insulin-like growth factor 1; mg/kg : milligrams per kilogram; mg/mL : milligrams per milliliter; mM: millimolar; MTT: (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide); NaOH: Sodium hydroxide; PBS: Phosphate buffered saline; RPMI: Roswell Park Memorial Institute medium; TGF- β : Transforming growth factor beta.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JCS and FAS performed phytochemical analysis, leishmanicidal and cytotoxic assays, and made substantial contributions to the conception of the study and the acquisition and interpretation of data. RMO, ISSO, IFBM and LL contributed to the interpretation of data and the review and editing of the manuscript. AFAL and HSF collected the plant material, prepared the extracts and contributed to the interpretation of data. ALAS conceived the study, and participated in its design and coordination and helped to draft the manuscript. All the authors read and approved the final manuscript.

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