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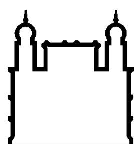
Doutorado em Programa de Pós-Graduação em Medicina Tropical

AVALIAÇÃO DA ATIVIDADE *IN VITRO* E *IN VIVO* DE FLAVONOIDES DE *Arrabidaea chica* (HUMB. & BONPL.) B. VERLOT CONTRA *Leishmania amazonensis* EM MODELO MURINO

JOÃO VICTOR DA SILVA E SILVA

Rio de Janeiro
Novembro de 2021



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Programa de Pós-Graduação em Medicina Tropical

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Avaliação da atividade *in vitro* e *in vivo* de flavonoides de *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot contra *Leishmania amazonensis* em modelo murino

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Medicina Tropical

Orientador (es): Profa. Dra. Kátia da Silva Calabrese
Prof. Dr. Fernando Almeida de Souza

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Novembro de 2021

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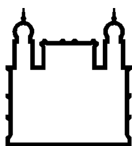

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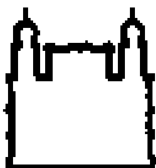

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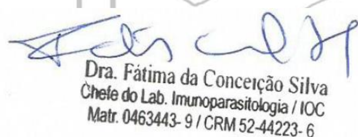
Ata da defesa de tese de doutorado acadêmico em Medicina Tropical de **João Victor da Silva e Silva**, sob orientação da Drª. Kátia da Silva Calabrese e co-orientação do Dr. Fernando Almeida de Souza. Ao vigésimo nono dia do mês de novembro de dois mil vinte e um, realizou-se às nove horas, de forma síncrona remota, o exame da tese de doutorado acadêmico intitulada: **"Avaliação da atividade *in vitro* e *in vivo* de flavonoides de *Arrabidaea chica* (humb. & bonpl.) B. Verlot contra *Leishmania amazonensis* em modelo murino"**, no Programa de Pós-graduação em Medicina Tropical do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutor em Ciências - área de concentração: Diagnóstico, Epidemiologia e Controle, na linha de pesquisa: Relação Parasito-Hospedeiro. A banca examinadora foi constituída pelos Professores: Drª. Fátima da Conceição Silva – IOC/FIOCRUZ (Presidente), Drª. Silvia Amaral Gonçalves da Silva – UERJ/RJ, Dr. Adriano Defini Andricopulo – USP/SP e como suplentes: Drª. Marcia Pereira de Oliveira Duarte – IOC/FIOCRUZ e Dr. Eduardo Caio Torres dos Santos – IOC/FIOCRUZ. Após arguir o candidato e considerando que o mesmo demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela **APROVAÇÃO** da defesa da tese de doutorado acadêmico. De acordo com o regulamento do Programa de Pós-Graduação em Medicina Tropical do Instituto Oswaldo Cruz, a outorga do título de Doutor em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, a Presidente da Banca atesta a decisão e a participação do aluno e de todos o membros da banca de forma síncrona remota. O Coordenador Adjunto do Programa Dr. Marco Aurélio Pereira Horta, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 29 de novembro de 2021.



Drª. Fátima da Conceição Silva (Presidente da Banca):

Dr. Marco Aurélio Pereira Horta (Coordenador Adjunto do Programa):

Dedico esta tese aos meus pais Terezinha Soares e Sebastião Siqueira e aos meus irmãos Dayana, João Paulo, Danila e Idalina da Silva.

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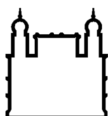
“Todas as vitórias ocultam uma abdicação”

Simone de Beauvoir



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AVALIAÇÃO DA ATIVIDADE *IN VITRO* E *IN VIVO* DE FLAVONOIDES DE *Arrabidaea chica* (HUMB. & BONPL.) B. VERLOT CONTRA *Leishmania amazonensis* EM MODELO MURINO

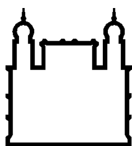
RESUMO

TESE DE DOUTORADO EM MEDICINA TROPICAL

João Victor da Silva e Silva

As leishmanioses estão na lista da OMS como doenças tropicais negligenciadas, considerada um grave problema de saúde pública. Devido às limitações relacionadas ao tratamento, torna-se necessária a busca por novas alternativas terapêuticas. O objetivo do presente estudo foi avaliar a atividade leishmanicida *in vitro* dos flavonoides e os efeitos imunomoduladores da fração rica em carajurina obtida de *Arrabidaea chica* no modelo murino. *A. chica* é uma planta com atividade antiparasitária que utilizamos para avaliar *in vitro* e *in vivo* a atividade leishmanicida do extrato bruto (ACCE), da fração rica em antocianidinas e flavonas, e dos compostos isolados. O extrato obtido por maceração em solução hidroalcoólica de folhas de *A. Chica* foi fracionado, resultando em uma mistura de flavonoides (ACFF) e uma fração rica em antocianidinas (ACAF), seguida de uma fração rica em carajurina (ACCF). A partir daí, antocianidinas carajurina, carajurona e 3-hidroxicarajurona e os flavonoides luteolina e apigenina foram isolados. Nos ensaios *in vitro*, ACCE, ACAF, ACFF e seus compostos isolados mostraram atividade contra promastigotas de *Leishmania amazonensis*. Alterações ultraestrutural com inchaço pronunciado do cinetoplasto, diminuição do potencial de membrana mitocondrial, aumento na produção de espécies reativas de oxigênio e morte celular por apoptose tardia em promastigotas foram observadas após tratamento com carajurina. Além disso, a atividade contra amastigotas intracelulares da carajurina foi comparada com a anfotericina B, com redução de todos os parâmetros de infecção e indução do aumento da quantidade de nitrito em macrófagos, cujos estudos de acoplamento mostraram uma possível interação da carajurina no local de ativação da enzima NOS**.** As propriedades físico-químicas da carajurina não violaram nenhuma das regras estabelecidas por Lipinski, Ghose, Veber, Egan e Mueggue. Nos ensaios *in vivo*, camundongos BALB/c infectados com *L. amazonensis* por via intragástrica, receberam por 30 dias doses diárias de 200 $mg.kg^{-1}$ de ACCF por gavagem, após 30 dias do início da infecção. As avaliações de toxicidade foram negativas e o qPCR mostrou diminuição da carga parasitária no sítio da infecção dos animais tratados, com aumento da expressão de citocinas iNOS e IFN-γ no sítio de infecção, assim como aumento de TNF-α e IFN-γ. Redução de IL-4 e IL-10 nos níveis séricos de citocinas também foram observados. Os resultados sugerem que a carajurina atua como marcador biológico de *A. Chica* e a fração rica deste componente constituem um passo importante na procura de uma nova alternativa terapêutica no tratamento das leishmanioses.

Palavras-chave: *Leishmania amazonensis*, flavonides, *Arrabidaea chica*, macrófagos, camundongos, citocinas.



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EVALUATION OF *IN VITRO* AND *IN VIVO* ACTIVITY OF FLAVONOIDS FROM *Arrabidaea chica* (HUMB. & BONPL.) B. VERLOT AGAINST *Leishmania amazonensis* IN MURINE MODEL

ABSTRACT

PHD THESIS IN MEDICINA TROPICAL

João Victor da Silva e Silva
Leishmaniasis is on the WHO list as neglected tropical diseases, considered a serious public health problem. Due to limitations related to the treatment, the search for new therapeutic alternatives becomes necessary. The aim of the present study was to evaluate the in vitro leishmanicidal activity of flavonoids and the immunomodulatory effects of the carajurin-rich fraction obtained from *Arrabidaea chica* in the murine model. *A. chica* is a plant with antiparasitic activity that we used to evaluate *in vitro* and *in vivo* the leishmanicidal activity of the crude extract (ACCE), the fraction rich in anthocyanidins and flavones, and the isolated compounds. The extract obtained by steeping *A. Chica* leaves in hydroalcoholic solution was fractionated, resulting in a mixture of flavonoids (ACFF) and an anthocyanidin-rich fraction (ACAF), followed by a carajurin-rich fraction (ACCF). From there, anthocyanidins carajurin, carajurone and 3-hydroxycarajurone and the flavonoids luteolin and apigenin were isolated. In in vitro assays, ACCE, ACAF, ACFF and their isolated compounds showed activity against *Leishmania amazonensis* promastigotes. Ultrastructural changes with pronounced swelling of the kinetoplast, decreased mitochondrial membrane potential, increased production of reactive oxygen species and cell death by late apoptosis in promastigotes were observed after treatment with carajurin. Furthermore, the activity against intracellular amastigotes of carajurin was compared with amphotericin B, with a reduction in all infection parameters and induction of an increase in the amount of nitrite in macrophages, whose coupling studies showed a possible interaction of carajurin at the site of activation. of the NOS enzyme. The physicochemical properties of carajurin did not violate any of the rules established by Lipinski, Ghose, Veber, Egan and Mueggue. In *in vivo* assays, BALB/c mice infected with *L. amazonensis* intragastrically received daily doses of 200 mg.kg-1 of ACCF by gavage for 30 days, 30 days after the onset of infection. Toxicity evaluations were negative and qPCR showed a decrease in the parasite load at the site of infection of the treated animals, with increased expression of cytokines iNOS and IFN-γ at the site of infection, as well as an increase in TNF-α and IFN-γ. Reduction of IL-4 and IL-10 in serum cytokine levels was also observed. The results suggest that carajurin acts as a biological marker of *A. Chica* and the rich fraction of this component constitutes an important step in the search for a new therapeutic alternative in the treatment of leishmaniasis.

Key words: *Leishmania amazonensis*, flavonoids, *Arrabidaea chica*, macrophages, mice, cytokines.

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LISTA DE SIGLAS E ABREVIATURAS

OMS	Organização Mundial de Saúde
FDA	Administração de Alimentos e Medicamentos dos Estados Unidos (do inglês *Food and Drug Administration*)
RENISUS	Lista Nacional de Plantas Medicinais de Interesse do Sistema Único de Saúde
Sb^V	Antimônio pentavalente
ATP	Adenosina trifostato
v/v	Volume/Volume
w/v	Peso/Volume
IC_{50}	Concentração Inibitória 50%
IC_{90}	Concentração Inibitória 90%
CC_{50}	Concentração Citotóxica 50%
EC_{50}	Concentração de lise 50%
µg	Micrograma
µM	Micromolar
µL	Microlitro
mL	Mililitro
CIM	Concentração Inibitória Minima
mg	Miligrama
kg	Quilograma
PCR	Reação de Cadeia de Polimerase (do inglês *polymerase chain reaction*)
IFN-γ	Interferon gama
TNF-α	Fator de necrose tumoral
TGF-β	Fator de transformação de crescimento beta
iNOS	Óxido nítrico sintase induzida
IL	Interleucina
NO	Óxido nítrico
ROS	Espécie reativa de oxigênio
NAC	N-acetilcisteína
VC	voltametria cíclica
HOMO	Orbital molecular ocupado mais alto (do inglês *Highest Occupied Molecular Orbital*)

LUMO	Orbital molecular não ocupado mais baixo (do inglês *Lowest Unoccupied Molecular Orbital*)
PGE2	Prostaglandina E2
COX-2	Ciclooxigenase-2
ACCE	Extrato bruto obtido de *Arrabidaea chica*
ACAF	Fração rica em antocianidinas de *Arrabidaea chica*
ACFF	Fração rica em flavonas de *Arrabidaea chica*
ACCF	Fração rica em carajurina de *Arrabidaea chica*
IS	Índice de Seletividade
kDNA	Ácido desoxirribonucleico do cinetoplasto
mt-DNA	Ácido desoxirribonucleico mitocondrial
TMRE	Tetrametilrodamina, éster etílico

1 INTRODUÇÃO

As leishmanioses constituem um complexo de doenças causadas por protozoários intracelulares do gênero *Leishmania*. Na lista das doenças negligenciadas da Organização Mundial de Saúde (OMS) são consideradas um grave problema de saúde pública e representam um desafio à saúde global, devido sua ampla distribuição mundial associada a investimentos limitados em diagnóstico, tratamento e controle, bem como às condições de pobreza da população[1-3]. Estima-se que mais de um bilhão de pessoas residem em cerca de 92 países ou territórios considerados endêmicos e sob risco de infecção por *Leishmania*, com 1 milhão de casos de leishmaniose cutânea relatados nos últimos 5 anos, 300 mil casos de leishmaniose visceral estimados anualmente e mais de 20 mil mortes por ano[3]. O Brasil é listado entre os nove países onde ocorre a maioria dos casos cutâneos de leishmaniose, e entre os sete países responsáveis por mais de 90% dos novos casos de leishmaniose visceral notificados à OMS em 2017[3].

Somado a esse cenário, as opções quimioterápicas disponíveis pouco mudaram nos últimos 80 anos e o tratamento ainda depende do uso de antimoniais pentavalentes como o estibogluconato de sódio (Pentostam) e o antimonato de meglumina (Glucantime), ou de anfotericina B lipossomal e convencional, pentamidina, paromomicina e miltefosina[4,5]. Esses medicamentos apresentam sérias limitações como alto custo, eficácia limitada, toxicidade, e administração prolongada, o que pode explicar a baixa adesão ao tratamento, impactando no aparecimento de cepas resistentes[4,6,7]. Além disso, as vacinas candidatas para utilização em humanos ainda precisam ser avaliadas em mais estudos clínicos[8].

Diante das limitações da quimioterapia anti-*Leishmania* somada a falta de novas alternativas terapêuticas, diversos grupos de pesquisas têm direcionado os olhares para produtos naturais derivados de plantas, por representarem um repositório de compostos bioativos potencialmente úteis para uma abordagem alternativa e segura contra a leishmaniose[9-11]. Nesse contexto, para ilustrar esse cenário, é sabido que dos 20 antiparasitários aprovados pela agência reguladora dos Estados Unidos (FDA, EUA), entre janeiro de 1981 e dezembro de 2019, 45,0% são produtos naturais ou derivados[12].

O Brasil é detentor da maior floresta equatorial e tropical úmida do planeta, com uma vasta biodiversidade ainda pouquíssimo estudada. Em 2009 foi emitida a Lista Nacional de Plantas Medicinais de Interesse do Sistema Único de Saúde (RENISUS), com destaque para a espécie *Arrabidaea chica*, listada pelas autoridades sanitárias brasileiras entre as 71 plantas medicinais mais importantes utilizadas na medicina popular[13].

Arrabidaea chica (Humb. & Bonpl.) B. Verlot, syn. *Bignonia chica*, pertence à família Bignoniaceae, é uma planta trepadeira, nativa da América tropical[14] encontrada no Cerrado e na Mata Atlântica[15], além de ser muito comum na região amazônica[16-18]. É considerada a espécie mais conhecida do gênero *Arrabidaea* na Colômbia[19], e é popularmente conhecida como crajiru ou pariri[20].

A espécie é tradicionalmente utilizada para fins medicinais na região amazônica[16] no tratamento de inflamações cutâneas[21,22], limpeza de feridas e úlceras para auxiliar na cicatrização[23], e cura de feridas[24]. Estudos *in vivo* descrevem seu efeito na cicatrização de feridas[25,26]. Outros estudos também relatam atividade antiparasitária *in vitro* contra *Trypanosoma cruzi*[27,28] e *Leishmania* spp.[14,26,29]. Essas atividades possivelmente estão relacionadas à presença de flavonoides[30], pois na triagem fitoquímica de extratos de *A. chica*, as folhas demonstram presença de dois diferentes tipos de flavonoides; as antocianidinas, como carajurina e carajurona[29]; além das flavonas[31], como a luteolina e a apigenina[32].

Resultados recentes do nosso grupo de pesquisa mostram que extrato hidroalcoólico de *A. chica* rico em antocianidinas afeta a viabilidade das formas promastigotas de *Leishmania amazonensis*[29], e relaciona a atividade leishmanicida com a presença de carajurina e que esta seria, portanto, um potencial marcador biológico da espécie. Entretanto, até a realização desta tese, não foram encontrados dados na literatura sobre os possíveis mecanismos de ação leishmanicida para extratos ou substâncias isoladas de *A. chica*.

1.1 Leishmanioses

As leishmanioses são um grupo de doenças causadas por protozoários parasitos de mais de 20 espécies do gênero *Leishmania*[3]: o subgênero Viannia Lainson & Shaw, 1987, com ocorrência apenas no Novo Mundo, com as espécies

Leishmania (Viannia) braziliensis Vianna, 1911 emend Matta, 1916, *Leishmania (Viannia). guyanensis* Floch, 1954, *Leishmania (Viannia) panamensis* Lainson & Shaw, 1972, e *Leishmania (Viannia) peruviana* Velez, 1913; e o subgênero Leishmania Ross, 1903, incluindo as espécies *Leishmania (Leishmania) chagasi* Cunha & Chagas, 1937. Alguns autores consideram que nome *chagasi* seria sinônimo de *infantum,* sendo descrita como *Leishmania (Leishmania) infantum (syn. chagasi*)[33,34,35]; *Leishmania (Leishmania) enriettii* Muniz & Medina, 1948; *Leishmania (Leishmania) mexicana* Biagi, 1953 emend. *Garnham*, 1962; *Leishmania (Leishmania) amazonensis* Lainson & Shaw, 1972; *Leishmania (Leishmania) aristidesi* Lainson & Shaw, 1979; *Leishmania (Leishmania) venezuelensis* Bonfante-Garrido, 1980; *Leishmania (Leishmania) garnhami* Scorza et al. 1979; *Leishmania (Leishmania) pifanoi* (Medina & Romero, 1959) Medina & Romero, 1962; *Leishmania (Leishmania) hertigi* Herrer, 1971; *Leishmania (Leishmania) deanei* Lainson & Shaw, 1977[36].

Segundo a OMS, mais de 1 bilhão de pessoas vivem em áreas endêmicas e estão expostas ao risco de contrair leishmaniose com mais de 1 milhão de novos casos ocorrendo anualmente[3]. A leishmaniose é classificada pela OMS em três formas principais da doença: leishmaniose cutânea, a forma da doença mais disseminada em todo o mundo; a leishmaniose visceral, também conhecida como calazar, listada como a forma mais grave; e a leishmaniose mucocutânea, que em casos mais severos, podem ser consideradas a forma mais incapacitante da doença, com lesões que destroem parcial ou totalmente a mucosa do nariz, boca e garganta[3]. No Brasil a leishmaniose cutânea é clinicamente dividida em leishmaniose cutânea (forma localizada, disseminada, recidiva cútis e cutânea difusa) e leishmaniose mucosa (forma tardia, sem lesão cutânea prévia, concomitante, contígua e primária)[37].

A leishmaniose cutânea constitui um problema de saúde pública, com dados em 2018, de 92 países ou territórios considerados endêmicos ou com relatos de casos de leishmaniose cutânea[3]. Sua distribuição ocorre nas Américas, na bacia do Mediterrâneo, no Oriente Médio e na Ásia Central e são responsáveis por cerca de 95% dos casos. Mais de 87% dos novos casos de leishmaniose cutânea ocorreram em 10 países: Afeganistão, Argélia, Brasil, Colômbia, Irã, Iraque, Líbia, Paquistão, República Árabe Síria e Tunísia, segundo dados de 2019, com estimativa de 600.000 e 1 milhão de novos casos em todo o mundo anualmente[3].

Casos de leishmaniose cutânea são notificados desde o extremo sul dos Estados Unidos até o norte da Argentina, com exceção do Chile e do Uruguai[37]. Dados coletados em 2020 identificaram 16.432 casos de leishmaniose cutânea no Brasil, destes 7.312 (44,4%) casos ocorreram na região Norte, sendo que no Estado do Pará registrou-se o maior número de casos, 2.997 (40,9%), seguido pelos Estados do Amazonas, 1.563 (21,3%) e Acre com 948 (12,9%)[38].

O ciclo de vida da *Leishmania* é digenético (heteroxênico), vivendo alternadamente alterna entre hospedeiros vertebrados e insetos vetores (Figura 1). Todas as espécies do gênero *Leishmania* são transmitidas pelo repasto sanguíneo, popularmente conhecido como "picada", de insetos fêmeas infectadas, conhecidos como flebótomos ou flebotomíneos, pertencentes aos gêneros Lutzomyia – no Novo Mundo, e Phlebotomus – no Velho Mundo. O parasito *Leishmania* alterna sua morfologia em cada tipo de hospedeiro (vertebrado e invertebrado), se diferenciando entre amastigotas e promastigotas[39]. O flebotomíneo inicialmente é infectado após a "picada" de um hospedeiro infectado. Uma vez dentro do flebotomíneo fêmea, no aparelho digestivo[40-42], ocorre a primeira diferenciação, e o parasito se transforma em promastigotas procíclicos (formas flageladas e móveis)[43,44]. O flagelo auxilia o parasito a se fixar no intestino do inseto onde irão passar por mudanças morfológicas, fisiológicas e bioquímicas, diferenciando-se em promastigotas nectomonas, leptomonas para finalmente se diferenciam nas promastigotas metacíclicas e se movem para a probóscide do inseto, de onde estão prontas para transmissão a um hospedeiro mamífero[39,45]. Esta parte do ciclo de vida chamada metaciclogênese ocorre em um período de 7-10 dias, e com a "picada" de um flebotomíneo, os promastigotas são transmitidos para o hospedeiro mamífero[46,47]. A "picada" transfere as promastigotas metacíclicas juntamente com sua saliva para o hospedeiro, e essas promastigotas desencadeiam um processo fagocitário ao aderirem à membrana plasmática[48,49]. Os promastigotas, desta forma, penetram nos macrófagos e infectam os vacúolos parasitóforos. É aqui que os promastigotas se diferenciam em amastigotas ovóides[50,51]. À medida que as formas amastigotas vão se multiplicando dentro do vacúolo parasitóforo, os macrófagos se rompem liberando parasitas que são fagocitados por outros macrófagos[52], dando início a uma cadeia de novas infecções[53,54], levando a uma das manifestações clínicas discutidas anteriormente.

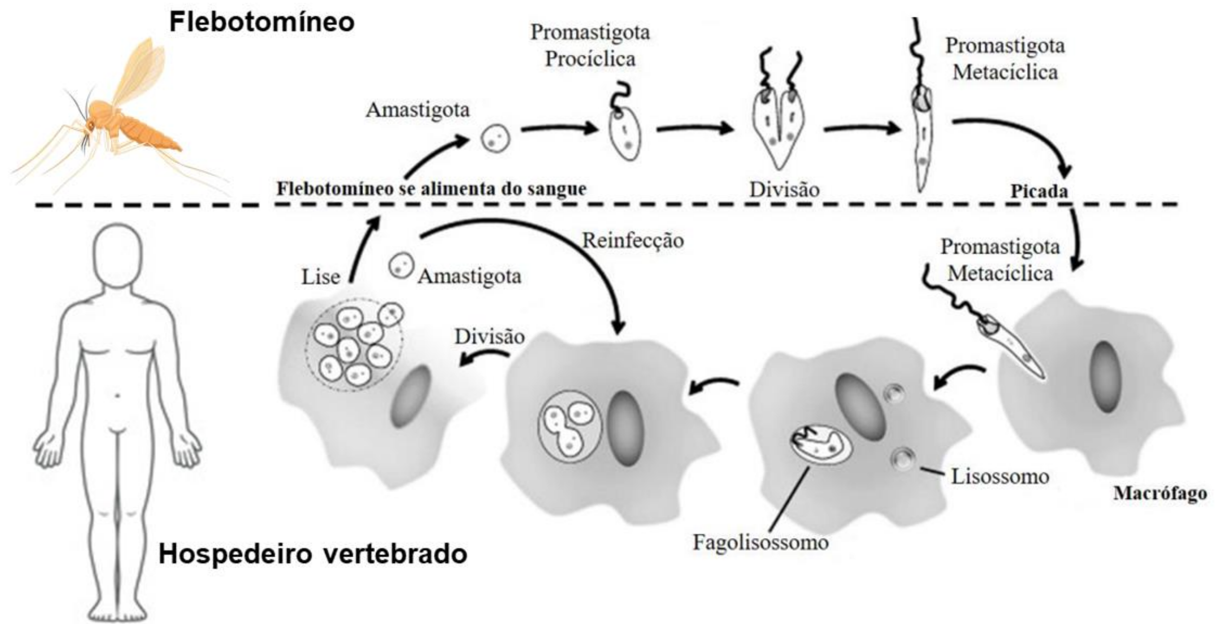

Figura 1 Ciclo biológico da *Leishmania* spp.

O tratamento convencional para leishmaniose visceral e cutânea é utilizado desde 1945[56], através do uso dos sais de antimônio pentavalentes (Sb^{v})[37,57], estibogluconato de sódio, não comercializado no Brasil, e antimoniato de meglumina. Além destes fármacos, o isetionato de pentamidina, desoxicolato de anfotericina B e a anfotericina B lipossomal são consideradas alternativas terapêuticas preconizadas pelo ministério da saúde[37]. Em 2018, a Portaria n° 56, de 30 de outubro, incorpora a miltefosina na primeira linha de tratamento da Leishmaniose cutânea, no âmbito do Sistema Único de Saúde (SUS)[58].

A paromomicina, um antibiótico aminoglicosídeo, foi aprovado pelo governo indiano para o tratamento da leishmaniose visceral em 2006, e em combinação com estibogluconato de sódio apresentou efeito sinérgico em casos de leishmaniose visceral[59].

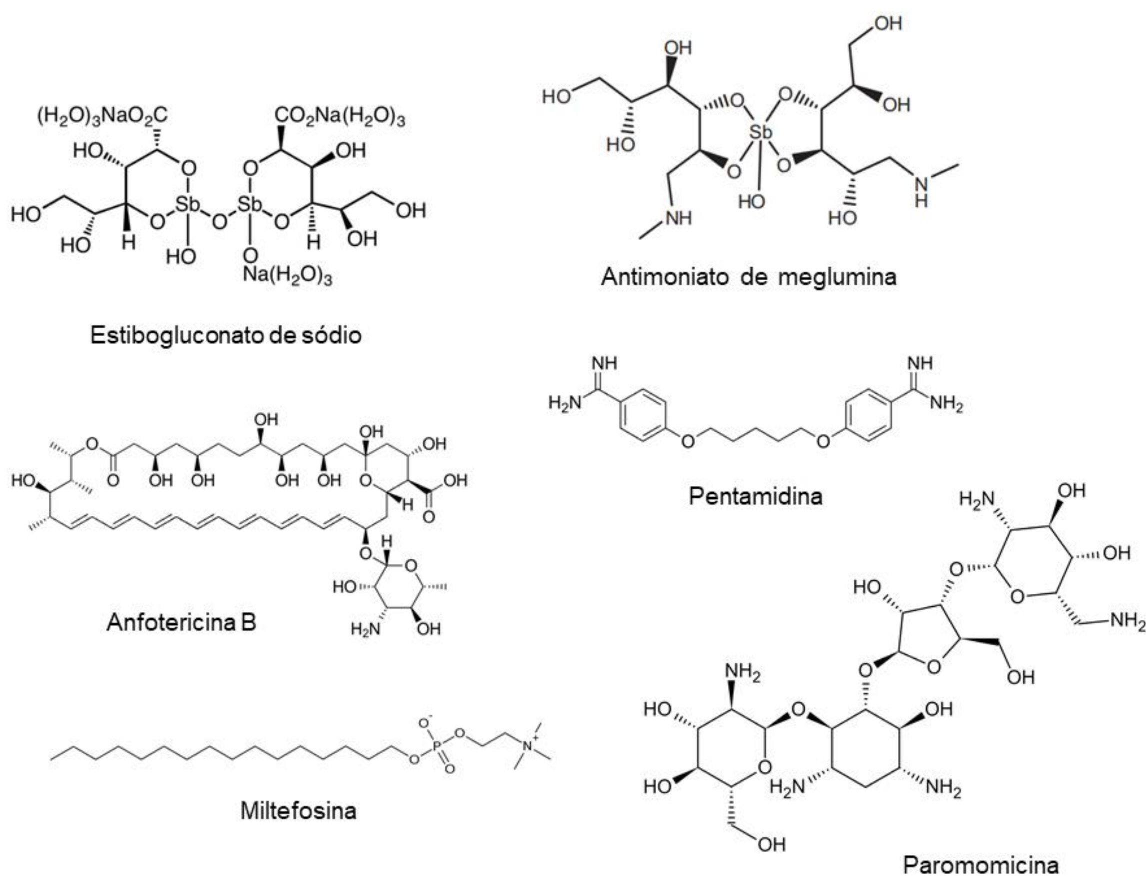

Figura 2 Estruturas químicas dos fármacos anti-*Leishmania.*

O longo período de hospitalização, cardiotoxicidade[60], cirrose, toxicidade pancreática[61] e o surgimento de altas proporções de casos resistentes aos antimoniais[62-64] levaram ao uso da pentamidina no início dos anos 1980 como alternativa de segunda escolha em casos refratários aos antimoniais. Entretanto, o tratamento é de alto custo, com toxicidades graves, como pancreatite levando a diabetes insulino-dependente, hipoglicemia, hipotensão e hipercalemia[65,66]. Essa limitação de alternativa terapêutica levou ao conceito de reaproveitamento de medicamentos, onde os medicamentos clinicamente aprovados usados para o tratamento de outras doenças podem ser usados para a leishmaniose[66]. Os medicamentos reaproveitados incluíram os medicamentos convencionais – anfotericina B, miltefosina e paromomicina. A anfotericina B desoxicolato é um agente antifúngico, utilizada para o tratamento da leishmaniose Leishmaniose. As reações adversas incluem reações à infusão, nefrotoxicidade, hipocalemia e miocardite, tornam obrigatório o monitoramento e hospitalização do paciente, aumentando assim o custo da terapia[59]. Para minimizar essas reações adversas, foi introduzida a formulação lipossomal da anfotericina B, eficaz para várias infecções fúngicas e benéfica em pacientes com insuficiência renal, bem como neutropenia[67]; mais tarde, no ano de 1997, foi aprovado para o tratamento da leishmaniose[68], e o único medicamento aprovado pelo FDA dos EUA[59]. A miltefosina, o primeiro e único

medicamento oral aprovado para leishmaniose[69], foi originalmente descoberta por suas propriedades anticancerígenas[70]. No entanto, seu uso é limitado devido ao alto custo, potencial teratogênico, alta incidência de toxicidade gastrointestinal e ocasional toxicidade hepática e nefrotoxicidade que requer monitoramento[59,71]. A paromomicina, um antibiótico aminoglicosídeo de amplo espectro, se mostrou eficaz contra infecções por protozoários, como giardíase, amebíase[72] e, posteriormente, contra leishmaniose na década de 1960[73-75], sendo aprovado na Índia para o tratamento da leishmaniose visceral No entanto, por ser aminoglicosídeo, apresenta risco de desenvolvimento de resistência aos medicamentos, se usado em monoterapia[59,66], e sua administração parenteral dificulta a adesão ao tratamento[76].

Em síntese, as quimioterapias atuais disponíveis para o tratamento da leishmaniose apresentam uma série de limitações e muitas reações adversas indesejáveis[77], somado a falta de novas alternativas terapêuticas e o surgimento de cepas resistentes às atuais quimioterapias[7].

1.2 *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot

Arrabidaea chica (Humb. & Bonpl.) B. Verlot (Figuira 3), syn. *Bignonia chica*, pertence à família Bignoniaceae. Compreende cerca de 100 gêneros e 860 espécies[78]. Segundo a classificação taxonômica de Cronquist[79], a espécie *A. chica* pertence à divisão Magnoliophyta, classe Magnoliopsida, subclasse Asteridae, ordem Scrophulariales, família Bignoniaceae, gênero *Arrabidaea*.

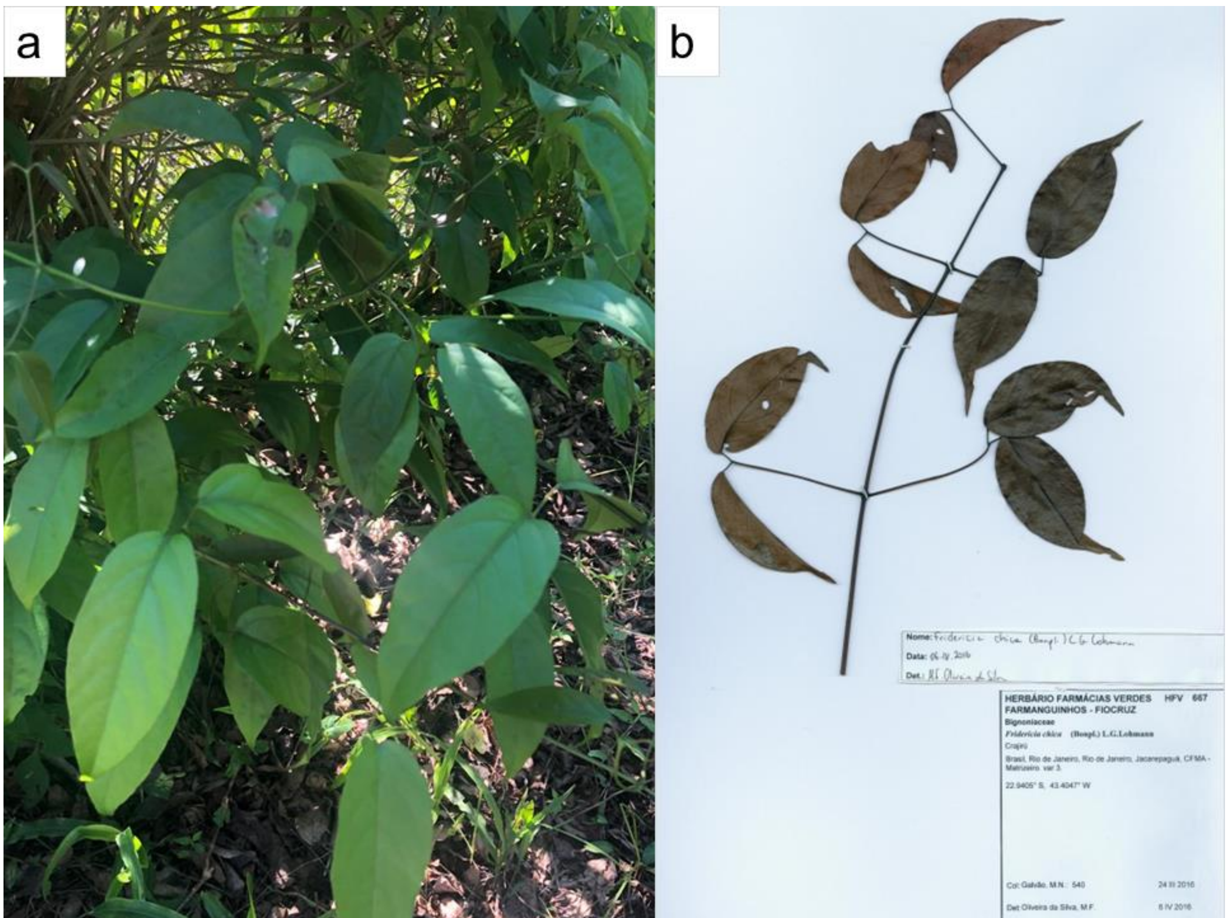

Figura 3 *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot. **a** Fotografia de Carla Moragas-Tellis, Laboratório de Produtos Naturais para Saúde Pública (Farmanguinhos - FIOCRUZ). **b** Exsicata depositada no Herbário de Farmanguinhos - FIOCRUZ.

Essa espécie é amplamente distribuída nas florestas tropicais da América do Sul[80]. Seu gênero, *Arrabidaea,* é generalizado no continente Americano, indo do Sul do México para o Brasil central, com ocorrência limitada no sul do Brasil, Paraguai e nordeste da Argentina[16]. *A. chica* não têm um habitat único e pode ser encontrada no Cerrado, Mata Atlântica e na Amazônia[15], porém outros autores acreditam ser uma espécie muito comum na região amazônica[16], sendo considerada nativa dessa região[17].

É conhecida popularmente com várias denominações, tais como, crajiru (Amazonas), pariri (Pará)[81], e em outras regiões como carajiru[82], puca-panga[83], cipó-pau, cipó-cruz, carajuru, carapiranga, carajeru, crejer[21], crejeru, cajuru, carajunu, chica, china, cuica, coá-piranga, guajuru, guajuru-piranga, guarajuru, oajuru, oajuru-piranga, paripari[20] e cajuru-do-índio[84].

A. chica apresenta-se como uma liana (trepadeiras lenhosas, comumente conhecida como cipós), arbustiva ou arbórea, com caule apresentando estruturação reticulada de parênquima e esclerênquima junto aos tecidos condutores e aos cristais prismáticos na medula. Suas com folhas compostas, bi ou trifolioladas, penaticompostas do tipo imparipenadas, de folíolos glabros, oblongo-lanceolados, com glândulas esparsas e com fitotaxia tipo oposta dística. Sua cutícula é estriada e os estômatos são anisocíticos. As flores possuem entre 18 e 20 cm de comprimento e são campanuladas róseo-lilacinas dispostas em panículas terminais. O fruto possui sementes ovóides, porém os frutos têm aspecto de uma cápsula linear, alongada, aguda em ambos os lados, glabra e castanho-ferrugínea, com uma nervura média saliente nas valvas. Além disso, as folhas modificadas, conhecidas como gavinhas, consideradas órgãos de suporte do vegetal, são responsáveis pela fixação ao substrato[20].

Essa espécie é tradicionalmente usada como uma planta medicinal na região amazônica[16], com uso de folhas para aplicação como tônico[85]. O chá feito das folhas foi listado para alterações patológicas orais, como anti-inflamatória[22], para tratar inflamação da pele, distúrbios gastrointestinais, leucemia, icterícia, albuminúria, impingens (nome popular para as dermatofitoses), micoses[21], leucorreia, cólica intestinal, diarreia sanguínea[86,87] e propriedades adstringentes[15]. Para as comunidades, indígenas *A. chica* é uma planta medicinal importante e amplamente utilizada. Existem inúmeros relatos de sua utilização por índios amazônicos através da decocção das folhas para a limpeza de feridas e úlceras para ajudar na cicatrização, além do uso para tratamento de infecções fúngicas e herpes[23]. Outras aplicações incluem problemas renais[84], lavagem ocular, especialmente para infecções oculares[88], tratamento de sintomas relacionados à tuberculose[89], disfunção sanguínea (anemia, hemorragia), inflamação uterina, hemorroidas e afecções cutâneas[27]. Além do mais, a infusão (uso oral) é usada para cura de feridas e “limpeza” do sangue[24].

As folhas de *A. chica,* maceradas ou em forma de chá, são usadas para tratamento de fraqueza, de anemia e de malária. O chá, ou infusão, é utilizado para cistos nos ovários, amenorreia, secreção vaginal, cistite, hepatite, problema hepático e diarreia. Na forma de xarope é utilizado para gripe, congestionamento e tosse. Na forma de garrafada é utilizado para engravidar e tratar úlcera; e banho para prurido vaginal[90]. Um método alternativo para preparar um chá para tratar diarreia infantil

envolve a adição das folhas de *A. chica*, dando ao medicamento uma cor vermelha atraente[90].

Algumas tribos preparam uma infusão das folhas, utilizada no tratamento de conjuntivite aguda, contra “ataque” de insetos, utilizam uma pasta na forma de cataplasma, há ainda, relatos de grande efeito contra câncer de boca, de útero e como anti-inflamatório[87]. Também há relatos de uso para problemas no estômago, intestino, prevenção de cárie; usado como cosmético para manter a pele macia e úmida[86]; e, além de tudo, é usado como corante natural[18].

As folhas desta planta foram tradicionalmente utilizadas pelas populações indígenas do Rio Meta e do Orinoco para obtenção de tintura no uso de pintura corporal[91]. Para além disso, o corante é utilizado pelos indígenas da região Amazônica para tingir enfeites, utensílios e vestuários; nas artes, rituais e até como método profilático contra picada de mosquitos[92].

Em triagem fitoquímica de extratos de *A. chica*, poucos foram os estudos que utilizaram caule, sendo identificados flavonoides, taninos, antocianidinas, chalconas[26] e polifenois totais[93]. Entretanto, as folhas são maioria entre os estudos, com fitoquímica rica em antocianinas e uma fonte de flavonoides[31] e taninos[18,31] (Tabela 1).

Nas folhas também foi possível identificar outras classes de metabólitos secundários (Tabela 1): antocianidinas[26,27,92], antraquinonas[27,92], catequinas[15,27,92], ácidos orgânicos[15,27,94], açúcares redutores[15,27,92,94], esteroides[15,27,92], xantonas[27], flavanois[92,94], flavanonas[27,92], saponinas[15,17,92], fenóis[15,92,94], depsídeos, depsidonas, naftoquinonas e fenantraquinonas[15], triterpenoides[15,92], cumarina[15,95], derivados de benzoquinona[15], fitoesteróis[16], taninos[15,26,27,92,94], chalconas e com compostos fenólicos encontrados apenas nas folhas[26], polifenois totais[31,93,96], antocianinas[27,92,94], taninos catéquicos[92], compostos fenólicos[17,26,97] e flavonoides[15,17,26,27,31,93,95,97].

A análise da quantidade de compostos fenólicos e flavonoides mostrou maior concentração desses metabólitos nas tinturas quando comparada com infusões (Tabela 1), como observado por Torres et al.[97].

Extratos com diferentes polaridades obtidos das folhas, apresentaram maiores conteúdo para flavonoides totais e compostos fenólicos totais[31]. Outros compostos fenólicos já foram identificados em extrato etanol-água (9:1 v/v) obtido das folhas de *A. chica*, incluindo isoscutellareína, 6-hidroxiluteolina, hispidulina,

scutellareína, apigenina e luteolina[98]. A luteolina também foi identificada em outros extratos, como metanólico a 95%[16], etanólico[32] e aquoso[99], o que se confirma nos estudos de Paula et al.[96,100], com análise em diferentes tipos de extratos (Tabela 1).

A tabela 1 mostra autores que identificaram a presença de alcaloides nas folhas de *A. chica*, em análise de prospecção fitoquímica[15,17,85,92]. Entretanto, o resultado positivo observado com esse metabólito, pode ser consequência de reação cruzada do reativo de Dragendorff com quinonas, pois a fração alcaloídica, obtida por processo de partição ácido-base, não forneceu resultados positivos para tal classe de metabólito[92].

Também foram caracterizados nos extratos das folhas (Tabela 1) a carajurina[18,30,31,96,100-102], carajurona[18,30,31,96,100-102], 6,7,3',4'-tetraidroxi-5-metoxiflavilio[18,31,96,100-102], kaempferol[18], 7,4'-dihidroxi-5-metoxiflavona[16], ácido gálico, rutina, ácido elágico[99], ácido n-hexadecanóico, ácido linoleico, ácido linolênico, éster metílico, ácido octadecanóico, ácido eicosanóico, vitamina E, campesterol, estigmasterol e gama-sitosterol[14].

Tabela 1 Metabólitos identificados e isolados de *Arrabidaea chica*.

Órgão vegetal	Local da coleta	Tipo de extrato	Metabólicos identificados	Referência
Folha	Curitiba – PR (abril de 1989)	Metanol 95%	Fitoesteróis e 7,4'-dihidroxi-5-metoxiflavona	Takemura et al.[16]
Folha	Belém – PA	Etanólico	Antocianidinas, antocianinas, antraquinonas, catequinas, ácidos orgânicos, açúcares redutores, esteroides, xantonas, taninos, flavonoides e flavonas	Barbosa et al.[27]
Folha	Paulinia – SP	Metanol/0,3% ácido cítrico	Carajurina e carajurona	Jorge et al.[30]
Folha	Região metropolitana de Belém – PA (janeiro de 2007)	Hidroalcoólico 70% (v/v)	Açúcar redutor, alcaloides, antocianidinas, antocianinas, antraquinonas, esteroides, triterpenoides, fenóis, flavanonóis, flavanóis, flavanonas, saponinas e taninos catéquicos	Alves et al.[92]
Folha	Leste da Nicarágua	Aquoso	Alcaloide	Coe et al.[85]
Folha	Região metropolitana de Belém – PA (maio de 2009)	Hidroalcoólico bruto (etanol 70%, v/v)	Saponinas, açúcares redutores, ácido orgânicos, fenóis, taninos, alcaloides, depsídeos, depsidonas, esteroides, triterpenoides, flavonoides, catequinas, cumaria, derivados de benzoquinonas, naftoquinona e fenatraquinonas	Lima de Medeiros et al.[15]
Folha	Maringá – PR	Etanolico	Fenóis, taninos, flavanóis, antocianinas, ácidos orgânicos e açúcares redutores	Ribeiro et al.[94]
Folha	Tabaí - RS	Aquoso	Saponinas, alcaloides, flavonoides e	dos Santos et al.[17]

			compostos fenólicos	
Folha	Campinas – SP	CO_2/Etanol/Àguda (80/20/0) CO_2/Etanol/Àguda (80/14/6) Etanol-água (70:30) Aquoso	Polifenois totais, flavonoides totais, carajurina, carajurona e 6,7,3’,4’-tetra-hidroxi-5-metoxiflavilio	Paula et al.[31]
Folha	Juina – Mato Grosso	70% hidroetanólico (1:10 w/v)	Carajurina, carajurona, 6,7,3’,4’-tetra-hidroxi-5-metoxiflavilio e kaempferol	Mafioleti et al.[18]
Folha	Obtida do Oeste do Colorado (RO), cultivada e coletada Curitiba – PR	Etanol-água (9:1, v/v)	Isoscutellareína, 6-hidroxiluteolina, hispidulina (6-metilscutellareína), luteolina e apigenina	Siraichi et al.[98]
Folha	Paulínia – SP	Metanol/ácido cítrico 0,3% (1:5, v/v)	Carajurina, carajurona e 6,7,3’,4’-tetra-hidoxi-5-metoxiflavilio	Taffarello et al.[101]
Folha	Norte da Argentina	Tintura Infusão	Compostos fenólicos e flavonoides	Torres et al.[97]
Folha	Campinas – SP	CO_2 Etanol (0,3% ac. Cítrico) Água (0,3% ac. Cítrico)	Polifenois totais, carajurina, carajurona e 6,7,3’,4’-tetra-hidroxi-5-metoxiflavilio e luteolina	Paula et al.[96]
Folha	Manaus - AM	Hexânico	Ácido n-hexadecanóico. Ácido linoleico, Ácido linolênico, éster metílico, ácido octadecanóico, ácido eicosanóico, vitamina E, campesterol, estigmasterol e gama-sitosterol	Rodrigues et al.[14]
Folha	Tabaí - RS	Aquoso	Ácido gálico, rutina, ácido elágico, luteolina	Gemelli et al.[99]
Folha	Paulinia – SP	Hidroalcoólico	Carajurina, carajurona e 6,7,3’,4’-tetra-hidroxi-5-metoxiflavilio	Servat-Medina et al.[102]

Caule	São Luís – MA	Etanólico	Flavonoides, taninos, antocianidinas e chalconas	Cortez de Sá et al.[26]
Folha			Flavonoides, compostos fenólicos, taninos, antocianidinas e chalconas	
Folha	Belém, PA	Etanol (70%, v/v)	Flavonoides e cumarinas	Martins et al.[95]
Folha	Pará	Metanol, etanol, água destilada e ácido clorídrico (69:20:10:1, v:v:v:v)	Polifenois totais e flavonoides totais	Silva et al.[93]
Caule			polifenois totais	
Folha	Campinas – SP	Supercrítico	Carajurina, carajurona, 6,7,3’,4’-tetra-hidroxi-5-metoxiflavilio e luteolina	Paula et al.[100]
		Etanólico		
		Hicroalcoólico		

A caracterização fitoquímica da *A. chica* possibilitou o isolamento das seguintes substâncias: carajurina (6,7-diidroxi-5,4'-dimetoxiflavilio)[80,91,103]; carajurona (6,7,4'-triidroxi-5-metoxiflavilio)[80,91,103]; 6,7,3',4'-tetraidroxi-5-metoxiflavilio[80,103]; luteolina[16,32]; carajuflavona (6,7,3',4'-tetraidroxi-5-metoxiflavona)[16]; 6,7,3'-triidroxi-5,4'-dimetoxiflavilio[103]; Kaempferol; vicenina-2; 4'-hidroxi-3,7-dimetoxiflavona[27]; e feoforbídeo A[28] (Figura 4).

Entre as substâncias isoladas, vale ressaltar o trabalho de Chapman et al.[91], por ser o primeiro a caracterizar o corante vermelho obtido a partir das folhas frescas de *A. chica*, identificando no extrato metanólico uma 3-desoxiantocianidina (6,7-diidroxi-5,4'-dimetoxiflavilio), denominada carajurina, como uma das substâncias responsáveis pela cor vermelha. Isso foi relatado há mais de 90 anos, no entanto Scogin[104] acreditava que a carajurina é única para o gênero *Arrabidaea* e talvez para a espécie *A. chica*, hipótese que perdura até o momento, devido não ter sido encontrado relato na literatura de identificação ou caracterização química da carajurina em outras espécies vegetais.

A carajurina é considerada o principal pigmento da *A. chica* e foi identificada e isolada das folhas dessa espécie em outros trabalhos, junto com outros pigmentos do tipo 3-desoxiantocianidinas: 6,7,3'-tridroxi-5,4'-dimetoxiflavilio; 6,7,4'-triidroxi-5-metoxiflavilio, conhecida como carajurona; e 6,7,3',4'-tetraidroxi-5-metoxiflavilio[103]. No estudo de Paula et al.[31], foi considerada componente majoritário no extrato etanólico das folhas. Além disso, a presença de carajurina também foi observada em flores de *A. chica*[103].

É conhecido que carajurona difere da carajurina pela ocorrência de apenas um grupo metoxi[91]; e que a 6,7,3',4'-tetraidroxi-5-metoxiflavilio, difere da carajurona por apresentar um grupo hidroxi adicional. Já a 6,7,3'-triidroxi-5,4'-dimetoxiflavilio mostrou diferença nos deslocamentos químicos para os prótons do anel B em comparação com a carajurina, revelando uma substituição de 3'-OH, 4'-OCH3 do anel B[105].

Curiosamente, as 3-desoxiantocianidinas raramente são encontradas nas plantas, e nas Bignoniáceas, elas só foram encontradas em *A. chica*[104]. Além disso, as antocianidinas são mais frequentemente encontradas na forma 3-O-glicosilada[106] e todas as antocianidinas isoladas possuem uma 6-hidroxilação. Um estudo anterior relatou a presença de uma flavona diferente, 6-hidroxilada em *A. chica*[16]. Essas descobertas sustentam a visão de Harborne[106] de que a 6-hidroxilação é uma

característica comum dos flavonóides de Bignoniaceae e contradiz a de Blatt et al.[107], que propuseram que a 6-hidroxilação seria apenas característica para a tribo Tecomeae, mas não para a tribo Bignonieae às quais pertence o gênero *Arrabidaea*.

Além disso Takemura[16] relatou a ocorrência de uma flavona, 7,4'-diidroxi-5-metoxiflavona, e mais tarde, o isolamento da luteolina e de uma nova flavona, 6,7,3',4'-tetraidroxi-5-metoxiflavona, chamada carajuflavona[16]. Em estudos posteriores foram isolados três flavonoides (4'-hidroxi-3,7-dimetoxiflavona, vicenina-2, kaempferol), sendo a 4'-hidroxi-3,7-dimetoxiflavona a primeira vez descrita para o gênero *Arrabidaea*[27]; e o achado mais recente, o feoforbídeo A[28].

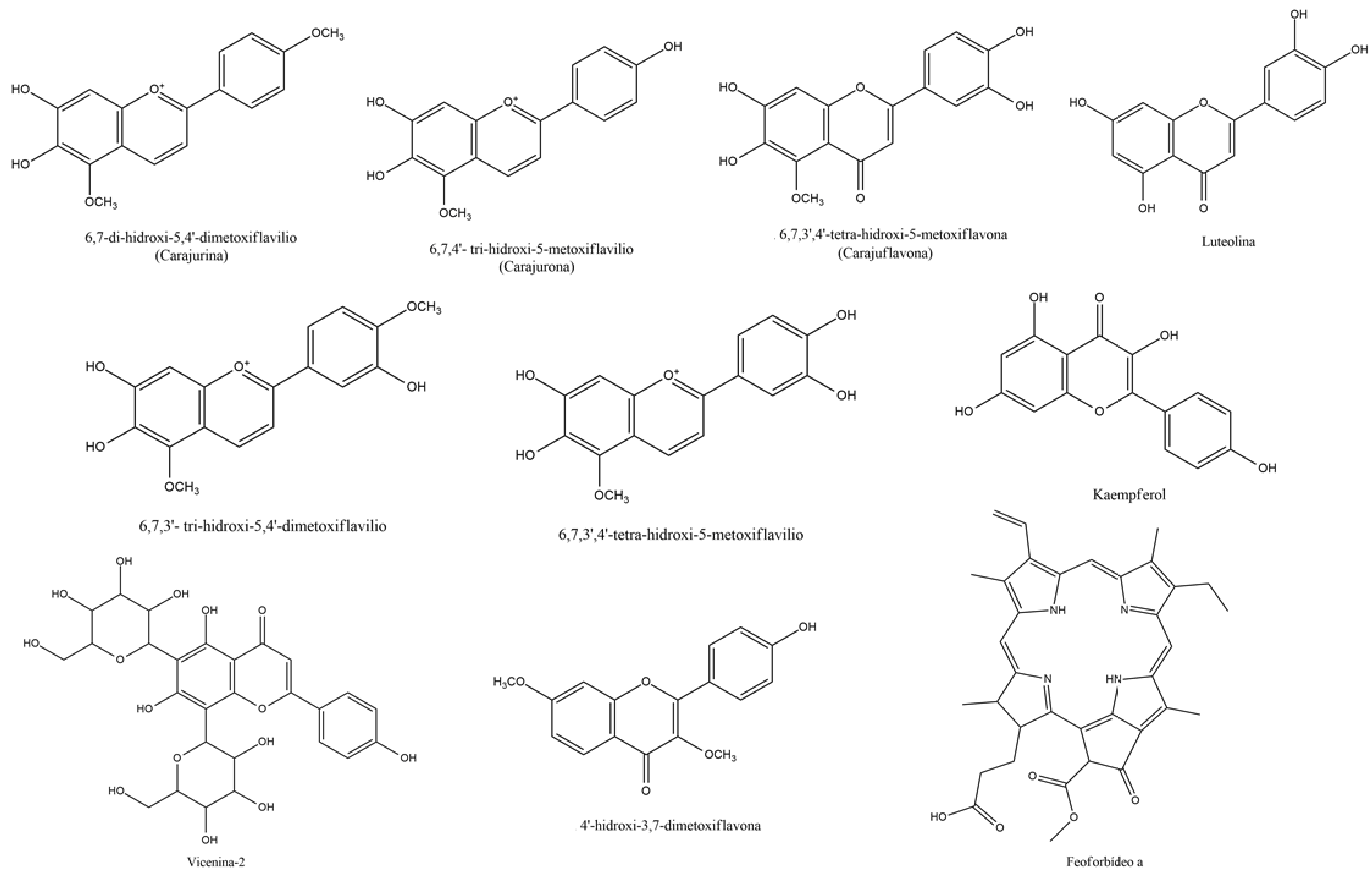

Figura 4 Substâncias isoladas da espécie *Arrabidaea chica*.

1.2.1 Atividade tripanossomicida

Dados da literatura relatam a busca por compostos bioativos das folhas de *A. chica* e avaliação de sua atividade contra diferentes formas de *T. cruzi*[27,28]. Barbosa et al.[27] obteve extrato etanólico por maceração, seguido de fracionamento por cromatografia em coluna sobre gel de sílica, obtendo-se diferentes frações (éter de petróleo 100%; éter de petróleo-hexano 50/50; hexano 100%; hexano-diclorometano 50/50; diclorometano 100%; diclorometano-metanol 50/50; metanol 100%). A avaliação fitoquímica do extrato etanólico possibilitou detectar os seguintes metabólitos: antocianidinas, antocianinas, antraquinona, catecinas, ácidos orgânicos, açúcares redutores, esteróides, xantonas, taninos, flavanonóis, flavanona e indicação de glicosídeos cardíacos. Além disso, foram isolados os compostos 4'-hidroxi-3,7-dimetoxiflavona a partir da extração sob refluxo das folhas utilizando n-hexano, e os compostos vicenina-2 e kaempferol, a partir da fração de acetato de etila[27]. Outro estudo obteve extrato hidroetanólico (etanol: água; 9: 1, v/v) por maceração, seguido de partição com éter de petróleo, hexano, clorofórmio, diclorometano e acetato de etila[28].

O extrato etanólico foi testado a uma concentração de 4 mg/mL mostrando uma atividade contra as formas tripomastigotas de *T. cruzi*, induzindo 41% de lise celular, e seu fracionamento contribuiu para melhora da atividade, quando testadas na concentração de 2 mg/mL: a fração CH_2Cl_2/MeOH (50:50), produziu lise em 71% de lise, enquanto a fração MeOH a 100%, produziu 54%. Duas outras frações, ambas eluídas por CH_2Cl_2 a 100%, ainda apresentaram atividade com 20% e ~ 28% de lise, respectivamente[27]. Entretanto, no estudo de Miranda et al. (28), foi possível determinar a concentração inibitória (IC_{50}), em que o extrato hidroetanólico e frações das folhas de *A. chica* apresentaram atividade contra as formas epimastigotas, porém o melhor resultado foi observado frente as formas tripomastigotas. Assim, na tentativa de elucidar os compostos ativos destes extratos, foi realizado fracionamento, no qual a partição das folhas de *A. chica* não contribuiu de forma significativa para resposta tanto para as formas epimastigotas quanto para tripomastigotas. Miranda et al.[28] relata inibição contra amastigotas intracelulares, ao utilizar a fração de clorofórmio, de 96,4% e 40,2% para concentrações de 50,0 e 10,0 μg/mL, respectivamente, além de apresentar baixo nível de hemólise (3,7%).

Diante desses resultados, somado ao fato de não ser observada citotoxicidade no extrato e nas frações (CC_{50} > 1000.0 μg/mL), a fração de clorofórmio foi selecionada para novo fracionamento, obtendo-se seis subfrações e a subfração F2a foi a mais efetiva, com valores de IC_{50} (ou seja, a concentração que inibiu 50% do crescimento do parasito) de 33,5 e 8,2 μg/mL contra epimastigotas e amastigotas, respectivamente, e um valor EC_{50} (ou seja, a concentração que lisou 50% dos parasitos) de 8,6 μg/mL contra tripomastigotas. Após refracionamento da subfração F2a, obteve-se a subfração F4b com boa atividade contra formas de tripomastigota (CE_{50} 5,2 μg/mL). E desta foi possível obter três subfrações, do qual a subfração F3c, sendo identificada como feoforbídeo A[28].

Para avaliar a atividade do composto feoforbídeo A isolado contra as três formas *de T. cruzi*, os parasitos foram tratados com diferentes concentrações e depois submetido a irradiação durante 5 minutos (sistema laser de díodo, com um comprimento de onda de 658 nm), com 1 cm de distância entre a luz e a amostra. Todas as amostras também foram testadas na ausência de luz. Além disso, também foram realizadas análise das alterações morfológicas das formas de tripomastigota e amastigota tratadas com feoforbídeo A. Na ausência de luz não foi observada atividade, mesmo a uma concentração de 20,0 μg/mL, bem como não foram observadas alterações morfológicas e ultraestruturais por microscopia eletrônica de transmissão nas formas de tripomastigota expostas a uma concentração de 100,0 μg/mL. No entanto, na presença de luz o composto feoforbídeo A exibiu maior atividade contra as formas de tripomastigota (EC_{50} de 2,3 μg/mL) e amastigota intracelulares (IC_{50} de 2,3 μg/mL), com 92,9% de inibição quando tratadas com a concentração de 10,0 μg/mL. Além disso, houve alterações morfológicas com arredondamento e encurtamento do corpo das tripomastigotas e feoforbídeo A quando quando tratadas com 2,3 μg/mL (IC_{50}) e 10,0 μg/mL (IC_{90}) na presença de luz. Alterações na morfologia também foram observadas nas amastigotas intracelulares, com arredondamento, tamanho menor e alterações na membrana plasmática. Nesse estudo, foi realizado apenas avaliação ultraestrutural em tripomastigota, sendo possível observar inchaço mitocondrial, a presença de vacúolos grandes (por exemplo, autofagosomas), a formação de estruturas de membrana concêntricas no citosol, envolvendo organelas e pequenas vesículas e alterações nucleares, como a distribuição anormal da cromatina. Em síntese, a substância isolada quando comparada ao extrato bruto hidroetanólico melhorou os

valores inibitórios aproximadamente 10 vezes, logo, vale ressaltar que o fracionamento bioguiado do extrato contribuiu para melhora da atividade tripanossomicida[28].

Outros estudos reportam a atividade tripanossomicida para outros gêneros de Bignoniaceae e atribui o efeito inibitório à presença de quinonas[108,109]. Alterações ultraestruturais observadas no estudo de Miranda et al. (28) podem estar envolvidas na autofagia e no processo apoptótico[110-113], o que pode auxiliar na compreensão do mecanismo de ação do feoforbídeo A.

1.2.2 Atividade leishmanicida

Até a realização da presente tese existiam poucas informações sobre a atividade anti-*Leishmania* de *A. chica*. No levantamento bibliográfico realizado foram encontrados estudos voltados para a avaliação leishmanicida contra cepas de *Leishmania infantum e L. amazonensis*[14,26,29,114].

Sobre o estudo do uso popular das plantas como alternativa terapêutica, o trabalho de Odonne et al.[115] avaliou o conhecimento sobre leishmaniose nas diferentes populações da bacia do médio e alto Oiapoque, com foco nas estratégias terapêuticas adotadas por pessoas afetadas pela leishmaniose e o uso de remédios fitoterápicos para a leishmaniose. Das 132 pessoas, 70 foram capazes de citar pelo menos uma espécie de vegetação antileishmanial. Havia 85 receitas medicinais, correspondendo a 38 espécies vegetais. Destas, 14 foram realmente utilizadas pelas pessoas que foram questionadas, incluindo espécie do gênero *Arrabidaea, Arrabidaea nigrescens* Sandwith (Bignoniaceae), dentre as nove espécies mais citadas para fitoterapia. Esses resultados evidenciam o uso de remédios fitoterapêuticos pelas populações da bacia do superior e médio Oiapoque contra a leishmaniose[115].

Outro estudo partindo do conhecimento popular citou o uso das folhas de *Arrabidaea chica* para o tratamento de leishmaniose[114]. Nesse trabalho a seleção das plantas foi baseada em uso local: as espécies explicitamente indicadas para "uta" (um termo local para leishmaniose cutânea) foram coletadas sistematicamente, juntamente com espécies indicadas como úteis no tratamento de problemas dermatológicos (cicatrização de feridas, inchaço ou dermatite). Espécies relatadas pelo grupo Chayahuitas (Amazônia peruana) foram selecionadas para avaliação do

potencial leishmanicida: usos tradicionais e atividade leishmanicida *in vitro* contra promastigotas e amastigotas axênicos de *L. amazonensis*. Entre estas espécies, foram selecionadas as folhas de *A. chica* que apresentou relato do uso de sua tintura (cor vermelha). Entretanto, no estudo *in vitro* com extrato etanólico (95%), esta espécie não apresentou atividade inibitória para formas amastigotas (IC_{50}> 100 µg/mL) e nenhum valor foi determinado para formas promastigotas. Muitas razões podem ser defendidas para explicar a falta de atividade de plantas leishmanicidas presumidas. Segundo Estevez et al.[114], em primeiro lugar, a cicatrização de uma úlcera da pele devido à leishmaniose cutânea pode ocorrer espontaneamente, daí a reputação usurpada da planta utilizada. Mesmo que as pessoas tendam a diferenciar entre "uta de água" (leishmaniose) e "uta seca" (dermatose devido a micoses ou outros agentes patogênicos bacterianos), a sobre-infecção é comum na leishmaniose cutânea e é possível que uma planta com boa atividade antimicrobiana possa ser percebida como ativa. Em segundo lugar, as culturas de amastigotas axênicas não refletem estritamente o que acontece nos macrófagos infectados com amastigota e alguns extratos podem não atuar diretamente nas amastigotas intracelulares, mas aumentam as capacidades do macrófago para destruir o parasito[116]. Portanto, os autores sugerem que novos estudos devem ser realizados com macrófagos infectados e administração oral em modelo murino para melhor entendimento da resposta inibitória da espécie *A. chica*[114].

Outro estudo avaliou o perfil fitoquímico, a citotoxicidade da espécie *Arrabidaea chica*, bem como a avaliação da ação do extrato bruto e as frações em promastigotas de *L. amazonensis*[26]. Os extratos etanólicos das folhas e caule de *A. chica* apresentaram rendimento de 59,2 e 3,33%, respectivamente, o que mostra que as folhas têm melhor eficiência de extração, pois o mesmo método de extração foi utilizado tanto para o caule quanto para as folhas. A triagem fitoquímica mostrou que as folhas e os extratos de caule contêm flavonoides, taninos, antocianidinas e chalconas. Entretanto, os compostos fenólicos foram encontrados apenas nas folhas. Na triagem sobre os promastigotas de *L. amazonensis*, o extrato etanólico do caule não apresentou atividade contra o protozoário, mesmo na maior concentração testada (500 µg/mL). Porém, a concentração de 125 µg/mL do extrato etanólico das folhas apresentou uma redução de 50% da viabilidade das formas promastigotas nos intervalos de 24, 48 e 72 h após a incubação. No entanto, a concentração de 250 µg/mL mostrou atividade antileishmanial nas primeiras 24 h de tratamento e

uma ausência total de parasitos viáveis após 72 h de incubação. A concentração de 500 µg/mL do extrato etanólico das folhas teve a melhor eficácia leishmanicida nos três intervalos testados. Após a triagem, a concentração inibitória 50% (IC_{50}) das formas promastigotas foi determinada como 155,9 µg/mL. Ao avaliar a atividade leishmanicida das frações foi possível determinar que o fracionamento contribuiu para melhora da atividade com resultado mais efetivo para a fração de acetato de etila. Macrófagos peritoneais após 24h de tratamento com extrato de folha de *A. chica* apresentaram a concentração citotóxica 50% (CC_{50}) de 189,9 µg/mL. Isso demonstra que o extrato foi discretamente mais seletivo para o protozoário do que a célula hospedeira (Índice de seletividade, IS 1,218). Isso talvez seja explicado ao analisar a composição fitoquímica de *A. chica*, em que revelou a presença de flavonoides, taninos, antocianidinas e chalconas[26].

Na tentativa de melhor compreender a atividade leishmanicida observada na espécie *A. chica*, foram investigados os efeitos de 5 frações obtidas a partir do extrato hexânico contra cepas de *L. amazonensis* e *L. infantum*[14]. O extrato foi obtido por extração de 1 semana em hexano, seguido de fracionamento em coluna cromatográfica de gel de sílica com um gradiente crescente de polaridade, começando com 100% de n-hexano e 100% de acetato de etila até 100% de etanol, obtendo cinco frações (B1, B2, B3, B4 e B5). B2 (1:1 n-hexano/acetato de etila) foi a fração mais ativa com valores de concentração inibitória mínima (CIM) de 37,2 e 18,6 µg/mL para promastigotas *L. amazonensis* e *L. infantum*, respectivamente. As formas promastigotas de *Leishmania* mostraram-se mais sensíveis a fração B2 e, portanto, a análise química desta fração foi realizada e os principais componentes identificados foram ácido linolênico, éster metílico (25,38%) de ácido n-hexadecanóico (19,61%), ácido octadecanóico (14,10%) e gama-sitosterol (12,85%). A fração B2 também demonstrou inibição significativa da peptidase quando testada em lisados celulares de *Leishmania*. Após a incubação dos lisados celulares tratados com CIM (37,2 e 18,6 µg/mL) e duas vezes CIM (74,4 e 37,2 µg/mL) de B2, a atividade da peptidase foi completamente inibida para *L. amazonensis* e *L. infantum*, respectivamente. Além disso, nesse estudo foram observadas alterações importantes na ultraestrutura de promastigotas de *L. infantum* em parasitos tratados com B2 a 18,6 µg/mL (CIM), após 24h. Foram observadas perda de conteúdo da matriz e alterações no complexo de Golgi seguidas de um processo de vacuolização do citoplasma, um processo exocítico intenso de conteúdo citoplasmático na bolsa

flagelar. A mitocôndria drasticamente danificada, sendo possível observar um inchaço intenso com presença de vesículas e em alguns casos, a membrana das mitocôndrias parece ser interrompida[14].

A atividade leishmanicida observada na espécie *A. chica* pode ser explicada, segundo os trabalhos de Cortez de Sá et al.[26] e Rodrigues et al.[14], pela sua composição metabólica. Cortez de Sá et al.[26] aborda que as antocianidinas são pigmentos vegetais pertencentes à família de flavonoides, estes incluídos no grupo de compostos fenólicos, e atividade terapêutica dos compostos fenólicos são atribuídas principalmente à sua capacidade antioxidante. Porém, Rodrigues et al.[14] aborda que os ácidos graxos são relatados como ativos contra *Leishmania*; no entanto, a atividade de tais compostos parece estar relacionada principalmente com ácidos graxos insaturados em vez de seus análogos saturados. Na tentativa de contribuir para essa análise, nosso grupo de pesquisa realizou estudo do perfil fitoquímico voltado para as antocianidinas avaliando os morfotipos (herbáceo e arbóreo) de *A. chica* coletados em duas estações (verão e inverno) para avaliar a influência da época de coleta e do morfotipo no seu conteúdo, bem como sua atividade contra formas promastigotas de *L. amazonensis*[29]. Nesse estudo, foi possível identificar a presença das antocianidinas 6,7,3',4'-tetraidroxi-5-metoxiflavilio (3'-hidroxicarajurona), carajurona, 6,7,3'-triidroxi-5,4'-dimetoxiflavílio (3'-hidroxi-carajurina) e carajurina. Apenas carajurona foi identificada em todos os extratos analisados, o que pode inferir como possível marcador químico desses morfotipos. Também foi observado que o extrato que apresentou menores valores de concentração inibitória (IC_{50}), logo maior atividade leishmanicida, foi coletado no verão. Da mesma forma, realizando a análise multivariante da composição das antocianidinas, verifica-se que o grupo que continha carajurina, tem mais extratos coletados no verão, enquanto o grupo sem carajurina, tem mais extratos coletados no inverno. Esse achado sugere uma possível influência da sazonalidade no aumento da probabilidade de os morfotipos de *A. chica* conterem carajurina. Dentre as antocianidinas identificadas nos extratos, apenas a carajurina apresentou correlação estatística significativa ($p = 0,030$) com atividade contra *L. amazonensis*, o que indica seu potencial como um possível marcador de atividade leishmanicida de *A. chica* a ser explorado[29]. Assim, ciente dos efeitos sinérgicos, antagônicos e aditivos entre os compostos presentes em um extrato vegetal, torna-se interessante

a avaliação bioguiada do extrato de *A. chica* para melhor entendimento de sua atividade anti-*Leishmania*.

A Tabela 2 resume as atividades antiparasitárias de *Arrabidaea chica* descrita na literatura.

Tabela 2 Atividades antiparasitárias de *Arrabidaea chica*.

Atividade	Parte da planta	Preparação	Concentração testada	Parasito	Forma de uso	Principais resultados	Referência
Tripanossomicida	Folha	Extrato etanólico, frações éter de petróleo 100%, éter de petróleo hexano 50/50, hexano a 100%, hexano-diclorometano 50/50, diclorometano 50/50, diclorometano 100%, diclorometano-metanol 50/50 e metanol 100%)	Extrato (4 mg/mL) e frações (2 mg/mL)	Tripomastigotas de *T. cruzi* (cepa Y) foram obtidas de camundongos infectados.	*In vitro*	Extrato ativo contra tripomastigotas do *T. cruzi* (41% da lise celular). Na concentração de 2 mg/mL: a fração F7, eluída com CH_2Cl_2/MeOH (50:50), produziu lise em 71% dos parasitos, enquanto a fração F9, eluída com MeOH 100%, produziu 54%. Frações, F4 e F5, ambas eluídas por H_2Cl_2 100%, exibiram 20% e ~ 28% de lise, respectivamente.	Barbosa et al.[27]
	Folha	Extrato hidroetanólico (etanol:água; 9: 1, v/v)	Não declarado	Tripomastigotas de *T. cruzi* (cepa Y)	*In vitro*	Fracionamento contribui para o	Miranda et al.[28]

		por maceração, seguido de partição com éter de petróleo, hexano, clorofórmio, diclorometano e acetato de etila; e feoforbídeo A (isolado da fração clorofórmica).				aumento progressivo da atividade contra epimastigota, tripomastigota e amastigota. Isolamento do composto feoforbide-a, ativo contra o protozoário na presença de luz, com alterações morfológicas e ultraestruturais, demonstrando seu potencial na terapia fotodinâmica.	
Leishmanicida	Folha	Extrato etanólico: Maceração, etanol a 95%.	Extrato etanólico (10-100 μg/mL)	Os experimentos foram conduzidos em promastigotas e amastigotas axênicas de *L. amazonensis* (cepa MHOM / BR / 76 / LTB-012)	*In vitro*	Promastigotas: IC_{50}; não determinado; Amastigotas axênicas: IC_{50} > 100 μg/mL.	Estevez et al.[114]
	Folha	Extrato hexânico; frações começando 100% de n-	Concentrações	Formas promastigotas de duas espécies de	*In vitro*	O extrato hexânico, especialmente a	Rodrigues et al.[14]

		hexano e 100% de acetato de etilo a 100% de etanol, obtendo cinco frações (B1, B2, B3, B4 e B5), como B2 (1:1 n-hexano/acetato de etila).	(1-500 μg/mL)	*Leishmania*, *L. amazonensis* (IFLA / BR / 1967 / PH8) e *L. infantum* (MHOM / BR / 1974 / PP75) da Coleção de Culturas de Tipos de Leishmania do Instituto Oswaldo Cruz / Fiocruz (Rio de Janeiro / RJ / Brasil) foram utilizados em todos os experimentos.		fração B2, possui atividade contra ambas espécies. Ácidos graxos e esteróis provavelmente são os principais componentes envolvidos na atividade antileishmanial.	
	Caule	Extrato etanólico	Concentrações (0,97-500 µg/mL)	Cepa de *L. amazonensis* (MHOM/BR/76/MA-76-IOC/FIOCRUZ-RJ), isolada de paciente com leishmaniose cutânea difusa.	*In vitro*	Promastigotas: IC_{50}> 500 µg/mL.	Cortez de Sá et al.[26]
	Folha					Fracionamento contribuiu para melhora da atividade com resultado mais efetivo para a fração de acetato de etila (IC_{50} > 60 µg/mL).	
	Folha	Extrato etanólico: maceração, álcool etílico-água (70/30, v/v)	Concentrações (1,95-500 µg/mL)	Formas promastigotas de *L. amazonensis* (MHOM/BR/76/MA-76),	*In vitro*	Carajurona é possível marcador químico dos diferentes morfotipos	Moragas-Tellis et al.[29]

				obtidas de um caso humano de leishmaniose difusa.		de *A. chica*; porém é a o teor de carajurina que possivelmente estar influenciando a atividade anti-*Leishmania* e neste caso poderia ser relacionado como um marcador biológico da espécie.	

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar a atividade leishmanicida *in vitro* dos flavonoides e os efeitos imunomoduladores da fração enriquecida de carajurina obtida de *Arrabidaea chica* no modelo murino.

2.2 Objetivos Específicos

– Identificar/isolar as antocianidinas e flavonas de frações obtidas do extrato hidroalcoólico de *A. chica*;

– Determinar o índice de seletividade da fração de antocianidinas e flavonas, bem como da carajurina por meio de ensaios *in vitro* de atividade leishmanicida contra formas promastigotas de *L. amazonensis* e ensaios de citotoxicidade em macrófagos;

– Avaliar as alterações ultraestruturais provocadas pelo tratamento com fração de antocianidinas e flavonas e seus respectivos componentes majoritários, luteolina e carajurina, em formas promastigotas de *L. amazonensis*;

– Avaliar a atividade da fração de antocianidinas e flavonas e seus respectivos componentes majoritários, luteolina e carajurina, contra formas amastigotas intracelulares de *L. amazonensis*;

– Avaliar o efeito modulador da fração de antocianidinas e flavonas e seus respectivos componentes majoritários, luteolina e carajurina, em macrófagos infectados ou não por *L. amazonensis*;

– Avaliar a toxicidade *in vivo* da fração rica em carajurina e seu perfil farmacocinético;

– Determinar a carga parasitária do sítio de inoculação e linfonodo drenante por meio de PCR em tempo real em camundongos infectados por *L. amazonensis* e tratados com fração rica em carajurina;

– Determinar a expressão de RNAm das citocinas IFN-γ, TNF-α, IL-4, IL-10, IL-12 e iNOS na lesão de camundongos infectados por *L. amazonensis* e tratados com fração rica em carajurina e seus respectivos controles;

– Determinar os níveis de IFN-γ, TNF-α, IL-4, IL-10 e IL-12 no soro de camundongos infectados por *L. amazonensis* e tratados com fração rica em carajurina e seus respectivos controles;

– Avaliar as alterações histopatológicas no sítio de infecção, linfonodo, baço e fígado de camundongos infectados por *L. amazonensis* e tratados com fração rica em carajurina e seus respectivos controles.

3 ARTIGOS

3.1 Antileishmanial activity of flavones-rich fraction from *Arrabidaea chica* Verlot (Bignoniaceae)

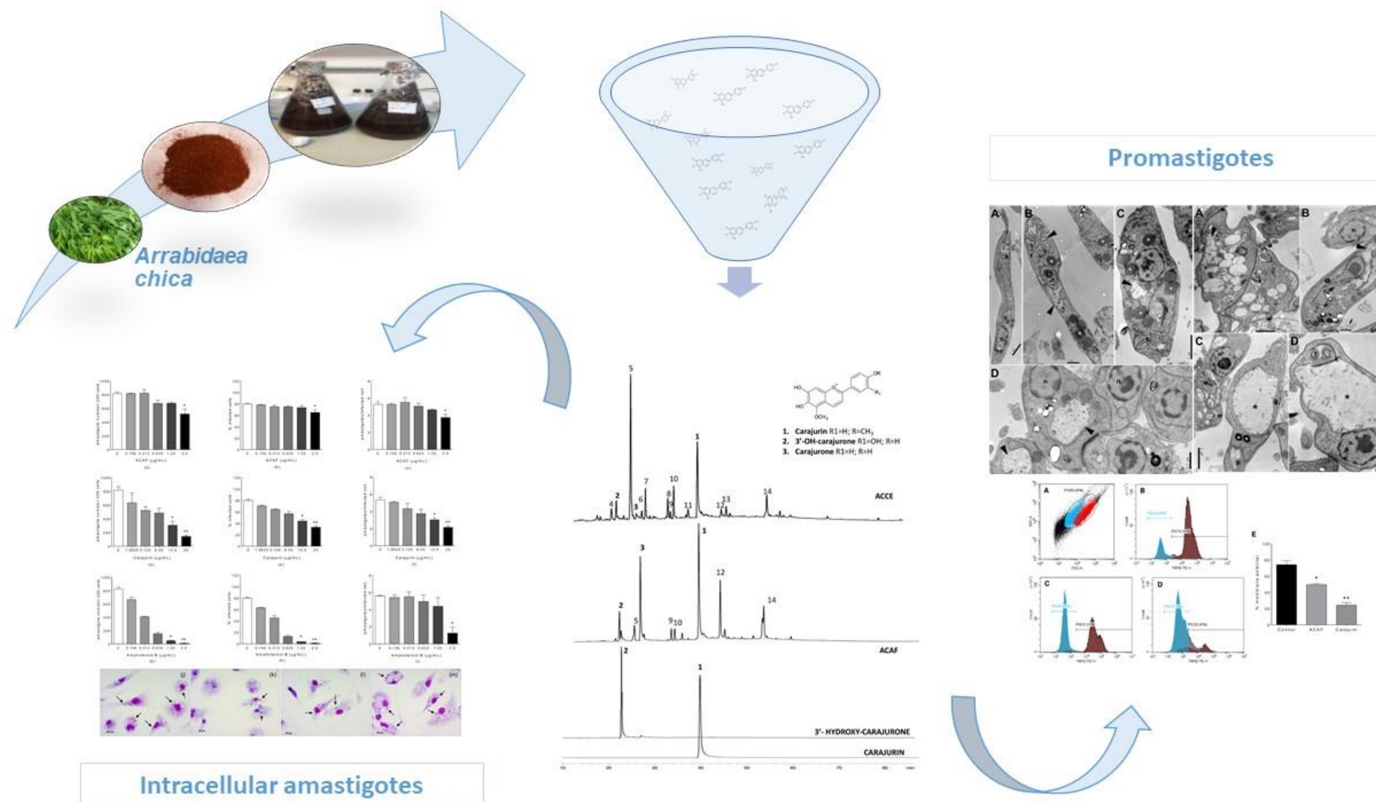

Figura 5 Resumo gráfico dos principais resultados da fração rica em flavonas obtidas da *Arrabidaea chica* Verlot (Bignoniaceae).

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Antileishmanial Activity of Flavones-Rich Fraction From *Arrabidaea chica* Verlot (Bignoniaceae)

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Acknowledging the need of identifying new compounds for the treatment of leishmaniasis, this study aimed to evaluate, from *in vitro* trials, the activity of flavones from *Arrabidaea chica* against *L. amazonensis*. The chromatographic profiles of the hydroethanolic extract and a flavone-rich fraction (ACFF) from *A. chica* were determined by high-performance liquid chromatography coupled with a diode-array UV-Vis detector (HPLC-DAD-UV) and electrospray ionization mass spectrometry in tandem (LC-ESI-MS-MS). The flavones luteolin (**1**) and apigenin (**2**), isolated from chromatographic techniques and identified by Nuclear Magnetic Resonance of ^{1}H and ^{13}C, were also quantified in ACFF, showing 190.7 mg/g and apigenin 12.4 mg/g, respectively. The other flavones were identified by comparing their spectroscopic data with those of the literature. The *in vitro* activity was assayed against promastigotes and intramacrophagic amastigote forms of *L. amazonensis*. Cytotoxicity tests were performed with peritoneal macrophages of BALB/c mice. Nitrite quantification was performed with Griess reagent. Ultrastructural investigations were obtained by transmission electron microscopy. Anti-*Leishmania* assays indicated that the IC_{50} values for ACFF, apigenin, and luteolin were obtained at 40.42 ± 0.10 and 31.51 ± 1.13 µg/mL against promastigotes, respectively. ACFF and luteolin have concentration-dependent cytotoxicity. ACFF and luteolin also inhibited the intra-macrophagic parasite (IC_{50} 3.575 ± 1.13 and 11.78 ± 1.24 µg/mL, respectively), with a selectivity index of 11.44 for ACFF. Promastigotes exposed to ACFF and luteolin exhibited ultrastructural changes, such as intense cytoplasm vacuolization and mitochondrial swelling. These findings data evidence the antileishmanial action of flavone-rich fractions of *A. chica* against *L. amazonensis*, encouraging further studies.

Keywords: *L. amazonensis*, flavonoids, *Arrabidaea chica*, macrophages, nitric oxide, transmission electron microscopy

INTRODUCTION

Flavonoids are an important class of secondary metabolites with a low molecular weight polyphenolic structure, widely distributed in the plant kingdom among subgroups that include chalcones, flavones, flavonols, and isoflavones (Panche et al., 2016). This metabolic class has its biological and therapeutic activity experimentally determined (Nijveldt et al., 2001), being able to affect enzymes and various cellular systems, having beneficial effects on the body (Silva et al., 2010). Furthermore, it is largely known that flavonoids have a wide spectrum of antileishmanial activity (Fotie, 2008; Wong et al., 2012; Rocha et al., 2018).

Leishmaniasis is a neglected tropical disease that seriously affects humans and can lead to death if left untreated (Reithinger and Dujardin, 2007). This protozoosis represents a global health challenge, since it has a worldwide distribution, with an estimate of more than one billion people living in endemic areas and at risk of *Leishmania* infection (World Health Organization, 2020). In addition to these circumstances, the anti-leishmanial drugs currently in use exhibit drug resistance, toxicity, and high cost, which may explain the low adherence to treatment (Sundar et al., 2019). The lack of new therapeutic alternatives to leishmaniasis highlights the need to seek new compounds with leishmanicidal activities. In this context, the use of natural products in traditional medicine has contributed to the identification of candidate compounds for the development of new drugs. Therefore, medicinal plants represent a repository of bioactive compounds potentially useful for the development of new therapeutic alternatives for leishmaniasis (da Silva et al., 2018).

Arrabidaea chica (Humb. & Bonpl.) B. Verlot, syn. *Bignonia chica*, belongs to the family Bignoniaceae. It comprises about 120 genera and 860 species (Fischer et al., 2004; Sampaio et al., 2016). *A. chica* occurs in tropical America, being a very common species in the Amazon region (Takemura et al., 1995; dos Santos et al., 2013) and is popularly known as crajiru or pariri (Behrens et al., 2012). It is traditionally used as a medicinal plant in the Amazon region (Takemura et al., 1995), with the use of tea made from leaves as an anti-inflammatory (Evangelista et al., 2013), to treat skin inflammation and mycoses (Corrêa, 1984), and has astringent properties (Lima de Medeiros et al., 2011). Amazonian Indians use the decoction of leaves to clean wounds and ulcers to aid in healing, in addition to the use to treat fungal infections and herpes (Lorenzi and Matos, 2002), as well as for other skin conditions (Barbosa et al., 2008). Furthermore, the infusion (oral use) is used to heal wounds and cleanse the blood (Bieski et al., 2012). Previous studies have demonstrated its antioxidant (do Amaral et al., 2012; dos Santos et al., 2013), wound healing (Aro et al., 2013; Cortez de Sá et al., 2015), trypanocidal (Barbosa et al., 2008; Miranda et al., 2017) and leishmanicidal activities (Rodrigues et al., 2014; Cortez de Sá et al., 2015; Moragas-Tellis et al., 2020). In the phytochemical screening of extracts of *A. chica*, the leaves are rich in anthocyanidins, such as carajurin and carajurone (Moragas-Tellis et al., 2020); in addition to some flavones (Paula et al., 2013), such as luteolin and apigenin (do Amaral et al., 2012).

Thus, the present study aimed to evaluate the antileishmanial activity *in vitro* of the hydroethanolic extract derived from *A. chica*, as well as of its flavone-rich fraction and the isolated flavones, luteolin and apigenin, against promastigotes, and intracellular amastigotes of *Leishmania amazonensis*.

MATERIALS AND METHODS

Plant Material

Leaves of *A. chica* Verlot (Bignoniaceae) (morphotype IV) were cultivated and collected in March 2016 at Fiocruz Atlantic Forest Campus, municipality of Rio de Janeiro, Rio de Janeiro State, Brazil (S22.9406 W43.4046). Plant material was identified by Dr. Marcus Felipe Oliveira da Silva at the Botanical Collection of Medicinal Plants of Farmanguinhos/FIOCRUZ, where a voucher specimen was deposited and registered under the number CBPM666.

Extraction and Isolation

Arrabidaea chica leaves were dried in a forced circulation oven at a temperature of 45°C for 4 days. After drying, the plant material was powered using a knife-mill affording 0.85 mm particles that were stored in an amber flask. The hydroethanolic extract was prepared by exhaustive maceration of dried and powdered leaves (1.5 kg) in 70% ethanol solution (v/v) with three changes of solvent, once every 48 h for 7 days at room temperature. After filtration, the solvent was evaporated under reduced pressure to yield 226.19 g of a red extract (ACCE) corresponding to yielding of 15.07%. The crude hydroethanolic extract (60 g) was then submitted to liquid-liquid partition with *n*-hexane (3 × 200 mL), dichloromethane (3 × 200 mL), ethyl acetate (3 × 200 mL) and *n*-butanol (3 × 200 mL). The final aqueous residue was discarded. Dichloromethane fraction was successively chromatographed by column chromatography on Sephadex LH-20 (Sigma, St Louis, MO, United States), using methanol as eluent to produce a purified flavone-rich fraction determined by thin layer chromatoghapy (TLC) analysis, and named ACFF. Successive chromatographic fractionation steps of ACFF on Sephadex LH-20 led to the isolation of two flavonoids (F1 = 24 mg; F2 = 4 mg). Isolated compounds and a flavone-rich fraction were analyzed by TLC (silica gel F_{254}, Merck, Darmstadt, Germany) using acetone: chloroform: formic acid (75:16:0.8 v/v/v) as eluent and, subsequently, sprayed with 1% NP/PEG reagent (diphenylboriloxyethilamine/polyetileneglicol, Sigma, St Louis, MO, United States), as well as ^{1}H and ^{13}C NMR spectrometry. Comparison with literature records allowed the identification of **(1)** luteolin and **(2)** apigenin (Ersöz et al., 2002; Özgen et al., 2011; Siraichi et al., 2013; Grabsk et al., 2017).

(1) Luteolin 1H NMR (400 MHz-methanol-d6) d: 6.47 (s, 1H, H-3); 6.13 (d, 1H, H-6, J = 2.0 Hz); 6.35 (d, 1H, H-8, J = 2.0 Hz); 7.35 (d, 1H, H-2′, J = 2.2 Hz); 6.88 (d, 1H, H-5′, J = 8.2 Hz); 7.35 (dd, 1H, H-6′, J = 8.2 Hz, J = 2.2 Hz). 13C NMR (400 MHz-methanol-d6) d: 166.05 (C-2); 103.41(C-3); 183.49 (C-4); 163.05 (C-5); 95.91(C-6); 166.05 (C-7); 101.31 (C-8); 159.72 (C-9); 104.27 (C-10); 123.43 (C-1′); 113.89 (C-2′); 147.31 (C-3′); 151.65 (C-4′); 116.86 (C-5′); 120.23 (C-6′).

(**2**) Apigenin 1H NMR (400 MHz, Methanol-d6) d: 6.55 (s, 1H, H-3); 6.16 (d, 1H, H-6, J = 2.0 Hz); 6.39 (d, 1H, H-8, J = 2.0 Hz); 7.35 (d, 1H, H-2′ and H-6′, J = 2.2 Hz); 6.88 (d, 1H, H-3′ and H-5′, J = 8.2 Hz); 13C NMR (400 MHz-Methanol-d6) d: 170.32 (C-2); 103.60 (C-3); 183.69 (C-4); 163.15 (C-5); 100.93 (C-6); 166.08 (C-7); 95.65 (C-8); 163.93 (C-9); 104.65 (C-10); 123.25 (C-1′); 129.42 (C-2′ and C-6′); 117.14 (C-3′ and C-5′); 159.65 (C-4′).

High-Performance Liquid Chromatograph Coupled With a Diode-array UV-Vis Detector

Chromatographic analyses were performed on HPLC-DAD-UV using a Shimadzu Nexera XR® liquid chromatographer coupled to a Shimadzu UV detector with diode array SPDM20A, equipped with a CBM20A controller, DGU20A degasser, LC20AD binary pump, CTO20A oven, and SILA20A auto-injector. A Shimadzu LabSolutions Software Version 5.3 (Shimadzu, Kyoto, Japan) was used to analyze chromatograms. Combinations of acidified ultrapure water (pH 3.0, with anhydrous acetic acid, Merck, Darmstadt, Germany) (A) and acetonitrile (HPLC grade, Tedia, Rio de Janeiro, Brazil) (B) were used as the mobile phase (initially 5% A rising to 95% in 80 min). HPLC column was silica-based C18 (250 mm × 4.6 mm i.d. × 5 μm particle size, ODS Hypersil, Thermo, Waltham, MA, United States). The oven was set at 50°C and the injection volume was 10 μL for all analyses.

Preparation of *A. chica* Hydroethanolic Extract (ACCE) and Flavone-Rich Fraction (ACFF) Samples

A total of 1,000 μL of acetonitrile: methanol (both HPLC grade, Tedia, Rio de Janeiro, Brazil) mixture (75:25; v/v) was added to 5 mg of ACCE and ACFF, previously weighed in a 4 mL vial. The vial was sealed and the sample was sonicated for 10 min with occasional swirling. Posteriorly, the sample was vortexed to mix thoroughly, followed by filtering through a 0.45 μm PTFE filter (Merck Millipore, Darmstadt, Germany) before further analyses into an HPLC vial.

Preparation of Standard Solutions and Quantification of Luteolin and Apigenin

Stock solutions of analytical standards luteolin and apigenin (Lot. 2,578 and 2,968, Phytolab, Vestenbergsgreuth, Germany) were prepared at 1,000 mg/mL in MeOH (Tedia, Rio de Janeiro, Brazil) in volumetric flasks. Six concentration of work solutions (1; 4; 8; 12; 16, and 20 μg/mL) were done on the day for calibration curves of each compound. The solutions were filtered in a 0.45 μm PTFE filter (Merck Millipore, Darmstadt, Germany) before analyses by HPLC-DAD-UV. Injections of 20 μL were performed in triplicate to obtain the calibration curves from the areas corresponding to the peaks of luteolin and apigenin. The analytical curve (1–20 μg/mL) of the standards was plotted based on the UV-Vis signal at 254 nm: luteolin content (μg/mL) = (Abs (mAu) + 21,030)/83,557; R2 = 0.9995 and apigenin content (μg/mL) = (Abs (mAu) + 27,059)/77,296; R2 = 0.996. Flavones amounts were calculated in mg/g of dry extract. The following dilution factors were used for luteolin and apigenin quantitative analysis: 147.06 and 11.47, respectively.

Liquid Chromatography Coupled to Electrospray ionization Mass spectrometry in Tandem Analysis

Liquid chromatography coupled to electrospray ionization mass spectrometry in tandem (LC-ESI-MS-MS) was performed with an LC Shimadzu Nexera Ultra-Fast Liquid Chromatography (UFLC) coupled to an ion trap Bruker amaZon MS. Analyses were performed at ambient temperature in a silica-based C18 column (150 mm × 4.6 mm i.d. x 2.6 μm particle size, Kinetex C18 gravity column, Phenomenex, CA, United States). The mobile phase consisted of ultrapure water obtained from the Milli-Q Millipore purification system, acidified at pH 3.0 with anhydrous acetic acid (Merck, Darmstadt, Germany) (A) and acetonitrile (HPLC grade, Tedia, Rio de Janeiro, Brazil) (B). The gradient of B was as follows: in 54.86 min from 5 to 95%; from 54.86 to 55.54 min returns to 5% B, remaining like this until 62.0 min to column re-equilibration. The flow rate was set at 0.5 mL/min and the injection volume was 1 μL. ESI-MS/MS were recorded in a Bruker Ion trap amazon SL mass spectrometer in the positive ionization mode (ESI+). The operating conditions were 1 μL/min infusion, 3.0–4.0 kV capillary voltage, 100°C temperature source, and cone voltage of 20–40 V. Mass spectra were recorded and interpreted by Bruker Compass Data Analysis 4.2 (Bruker Daltonics, Boston, MA, United States).

Animals and Ethical Statements

All procedures performed with 4–6-weeks old female BALB/c mice were in accordance with the National Council for Control of Animal Experimentation (CONCEA). These animals were obtained from the Institute of Science and Technology in Biomodels of Oswaldo Cruz Institute and the experiments were approved by the local Ethics Committee on Animal Care and Utilization (CEUA-IOC L53/2016).

Parasites

Leishmania amazonensis H21 (MHOM/BR/76/MA-76) was maintained in the laboratory by successive passages in BALB/c mice. Parasites were isolated from a non-ulcerated nodular lesion in the footpad and amastigote viability was checked by light microscopy. 10^6 amastigote forms were transferred to the NNN medium (Novy-MacNeal-Nicolle) and maintained for seven days to differentiate into promastigote forms. Then, these forms were cultured at 26°C in Schneider's Insect medium (Sigma, St Louis, MO, United States), supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, United States), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin in a maximum of ten *in vitro* passages (Almeida-Souza et al., 2016).

Peritoneal Macrophage Obtaining and Cell Culture

The animals were previously inoculated intraperitoneally, with 3.0 mL of 3% sodium thioglycolate. After 72 h of stimulation, the

animals were euthanized with 10% ketamine and 2% xylazine according to the weight of each animal and, after death, the abdomen skin was retracted for peritoneum exposure. 10.0 mL of sterile pH 7.2 phosphate-buffered saline was inoculated and a light manual massage was performed. The cells were harvested from the peritoneum with the same syringe and dispensed in a sterile conical tube to prepare the cell suspension. The cells were centrifuged at 2,000 rpm for 5 min and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and (100 μg/mL) streptomycin, at 37°C and 5% CO_2 and grown overnight (Almeida-Souza et al., 2018).

Activity Against *L. amazonensis* Promastigote Forms

To evaluate the anti-promastigote effects of the ACCE, ACFF, and isolated flavonoids from *A. chica* on the promastigote forms of *L. amazonensis*, viable promastigotes were counted in a Neubauer chamber according to the method described by Rottini et al. (2019). In brief, 50 μL of the promastigotes (2×10^6 cells/mL) harvested from the logarithmic growth phase were added to 96-well flat-bottomed microtiter plates. Then, 50 μL of the ACCE (62.5–1,000 μg/mL), ACFF (12.5–200 μg/mL) or isolated flavonoids (3.125–100 μg/mL) were added to each well and incubated at 26 ± 1°C for 72 h. Wells with parasites and DMSO 1% only were used as untreated control and amphotericin B (0.03125–1.0 μg/mL) was used as a reference drug. After the incubation, viable promastigotes were counted in a Neubauer chamber. The experiments were conducted in triplicate. Percentage of growth inhibition was calculated from the count of viable parasites relative to the untreated control, and 50% inhibitory concentration (IC_{50}) values were determined.

Cytotoxicity Assay

Peritoneal macrophages collected as previously described (*Peritoneal Macrophage Obtaining and Cell Culture*) were seeded at 5×10^5 cells per milliliter in a 96-well plate and allowed to adhere overnight at 37°C and 5% CO_2. Subsequently, the cells were treated with different concentrations of the ACCE, ACFF (both with concentrations of 7.81–1,000 μg/mL), flavonoids (1.95–500 μg/mL) or amphotericin B (0.19–25 μg/mL), in a final volume of 100 μL/well, incubated for 72 h at the same conditions. Wells without cells were used as blank and wells with cells and 1% DMSO were used as controls. The cytotoxicity was determined with the MTT (Sigma, St Louis, MO, United States) assay. Half-maximal cytotoxic concentration (CC_{50}) was calculated according to Oliveira et al. (2018).

Activity Against *L. amazonensis* Intracellular Amastigotes and Selectivity Index

Peritoneal macrophages of BALB/c mice were cultured in 24-well plates (5×10^5 cells/well), containing round coverslips and incubated at 37°C in 5% CO_2 overnight. The cells were then infected with promastigote forms of *L. amazonensis*, in the parasite/cell ratio of 10:1, for 6 h followed by washing with PBS to remove free parasites. Infected cells were treated with different concentrations of ACFF (0.15–2.5 μg/mL) or luteolin (1.56–25 μg/mL), and the plates were incubated in similar conditions for 24 h. The macrophages containing amastigotes without compounds treatment and those treated with amphotericin B (2.5–0.15 μg/mL) were considered the negative and positive controls, respectively. Finally, the coverslips of the infected and treated cells were fixed in Bouin's solution, stained by Giemsa and observed under a light microscope. The activity anti-intramacrophage amastigotes of the compounds was evaluated by counting the number of amastigotes in each macrophage by examining 200 macrophages in comparison with the untreated control. The percentage of infected cells was obtained from the number of infected cells divided by two. The mean number of amastigotes per cell was obtained from the number of intracellular amastigotes in 200 cells divided by the number of infected cells (Almeida-Souza et al., 2020). The percentage inhibition was calculated and IC_{50} was obtained by GraphPad Prism® version 7 (GraphPad Software Inc., San Diego, CA, United States). Selectivity index (SI), which was calculated using the equation of CC_{50} for murine macrophage/IC_{50} for the intracellular amastigote forms of *L. amazonensis*, was used to compare the toxicity and activity of the compounds.

Nitrite Quantification

Nitric oxide (NO) release was indirectly measured in the supernatants of macrophage culture (5×10^6 cells/mL) by the Griess reaction for nitrite according to the method described by Almeida-Souza et al. (2016). About 50 μL of the supernatants were collected 48 h after treatment with ACFF (2.5 μg/mL) or luteolin (25 μg/mL) and/or stimulation twith *L. amazonensis* (3×10^7 parasites/mL), and added in 96-well plates. Then, were added to supernatants 50 μL of Griess reagent (25 μL of sulfanilamide 1% in 2.5% H_3PO_4 solution and 25 μL of N-(1-naphthyl ethylenediamine 0.1% solution). After 10 min, the plates were read at 570 nm on the spectrophotometer and the nitrite values were obtained from the standard sodium nitrite curve (1.5–100 μM).

Transmission Electron Microscopy

Promastigote forms of *L. amazonensis* were treated with IC_{50} for ACFF or luteolin, for 24 h. Non-treated parasites were used as a negative control. After 24 h-incubation at 26°C promastigotes were collected by centrifugation at 5,000 rpm for 5 min. The parasites were fixed with 2.5% glutaraldehyde (Sigma, St Louis, MO, United States) in 0.1 M sodium cacodylate buffer, pH 7.2, overnight. Then, parasites were washed three times with 0.1 M sodium cacodylate buffer and post-fixed in a solution containing 1% osmium tetroxide, 0.8% potassium ferrocyanide, and 5 mM calcium chloride, washed in 0.1 M sodium cacodylate buffer, dehydrated in graded acetone, and embedded in EPON 812 resin (Sigma, St Louis, MO, United States). Ultrathin sections were obtained from 100 nm cuts in Sorvall MT 2-B (Porter Blum) ultramicrotome (Sorvall, Newtown, CT, United States) stained with 5% uranyl acetate aqueous solution and lead citrate (1.33% lead nitrate and 1.76% sodium citrate), and examined in a

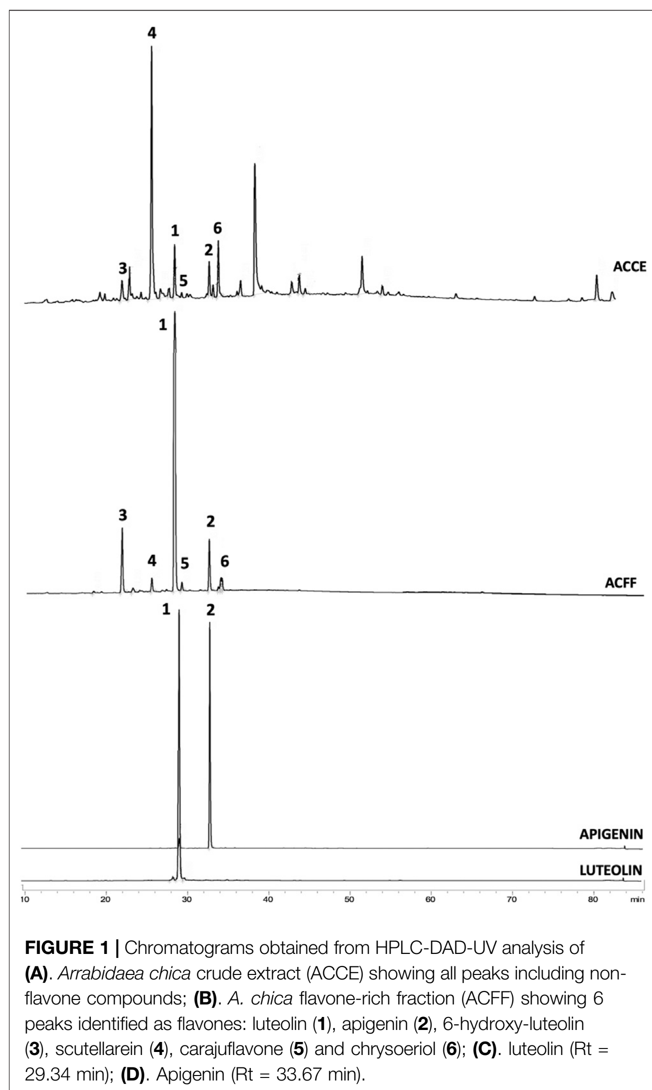

FIGURE 1 | Chromatograms obtained from HPLC-DAD-UV analysis of **(A)**. *Arrabidaea chica* crude extract (ACCE) showing all peaks including non-flavone compounds; **(B)**. *A. chica* flavone-rich fraction (ACFF) showing 6 peaks identified as flavones: luteolin (**1**), apigenin (**2**), 6-hydroxy-luteolin (**3**), scutellarein (**4**), carajuflavone (**5**) and chrysoeriol (**6**); **(C)**. luteolin (Rt = 29.34 min); **(D)**. Apigenin (Rt = 33.67 min).

transmission electron microscope JEM-1011 (JEOL, Tokyo, Japan) operating at 80 kV (Mondêgo-Oliveira et al., 2021).

Statistical Analysis

The numerical results were expressed as mean ± standard deviation and the statistical analyses were conducted through the statistical software GraphPad Prism® version 7. The differences were considered significant when $p < 0.05$ by one-way analysis of variance (ANOVA) and Mann-Whitney test.

RESULTS

High-Performance Liquid Chromatograph Coupled With a Diode-Array UV-Vis Detector and Liquid Chromatography Coupled to Electrospray Ionization Mass Spectrometry in Tandem

The comparison between the profiles of ACCF and ACFF of *A. chica* (**Figure 1**) by HPLC-DAD-UV showed that the chromatographic fractionation steps were useful both to obtain a fraction rich in flavones, and isolating two of them, luteolin **(1)** and apigenin **(2)**, which had already been reported in the literature, and had their structures confirmed by ^{1}H and ^{13}C NMR (Özgen et al., 2011; Siraichi et al., 2013).

LC-ESI-MS-MS analysis in positive mode of the flavone-rich fraction (ACFF) also resulted in a chromatogram with six peaks (**Figure 2**). Mass spectrometry in tandem (MS-MS) and pseudomolecular ions $[M + H]^+$ were useful to identify and confirm the structures of the six flavones in flavone-rich fraction.

The results of HPLC-DAD-UV and LC-ESI-MS-MS allowed the identification of the six component flavones of the rich fraction in flavonoids of *A. chica*, as shown in **Table 1**.

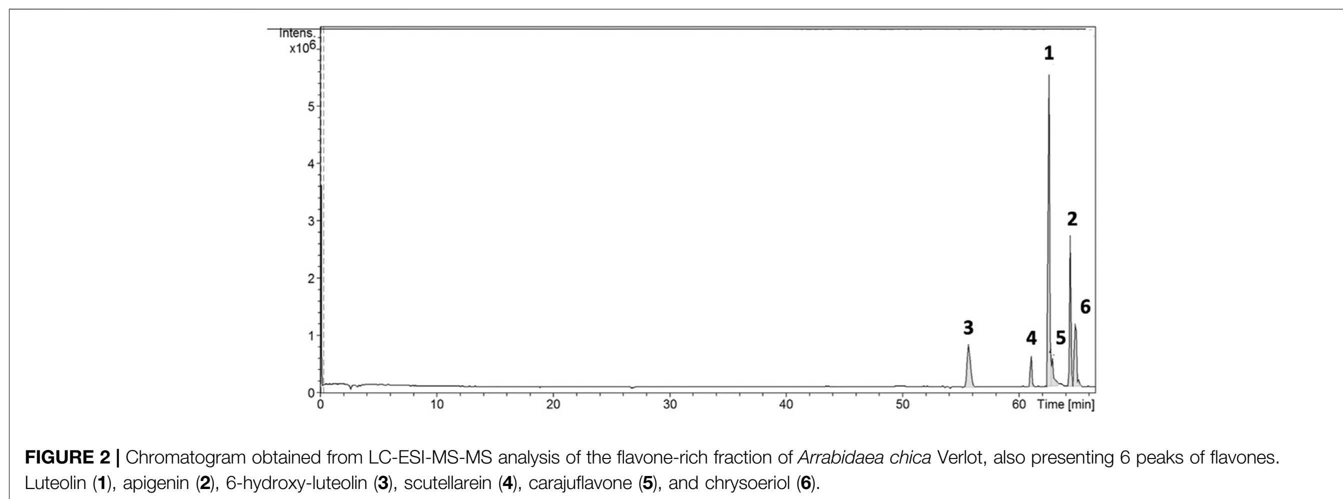

FIGURE 2 | Chromatogram obtained from LC-ESI-MS-MS analysis of the flavone-rich fraction of *Arrabidaea chica* Verlot, also presenting 6 peaks of flavones. Luteolin (**1**), apigenin (**2**), 6-hydroxy-luteolin (**3**), scutellarein (**4**), carajuflavone (**5**), and chrysoeriol (**6**).

TABLE 1 | LC-ESI-MS-MS and HPLC-DAD-UV data of flavone-rich fraction obtained from *Arrabidaea chica* Verlot.

Peak	LC-ESI-MS-MS		MS-MS	HPLC-DAD-UV		Identification
	Rt (min)	[M + H]		Rt (min)	UV data (nm)	
3	55.7	303.0451	303, 257, 169	22.9	346, 282	6-OH-luteolin
4	61.0	287.0496	287, 169	26.6	338, 283	Scutellarein
1	62.6	287.0493	287, 241, 153	29.8	348, 256	Luteolin
5	62.9	317.0594	317, 302, 168	30.3	346, 271	Carajuflavone
2	64.4	271.0548	271, 153	33.6	339, 267	Apigenin
6	64.8	301.0464	301, 286, 258,153	35.0	343, 267	Chrysoeriol

LC-ESI-MS-MS: Liquid chromatography coupled to electrospray ionization mass spectrometry. HPLC-DAD-UV: High-performance liquid chromatography coupled to a diode-array detector. [M + H]: pseudomolecular ions. MS-MS: Mass spectrometry. Rt: retention time.

TABLE 2 | Quantification of luteolin and apigenin (mg/g dry extract) content in the flavone-rich fraction of *Arrabidea chica* Verlot.

Flavone	Area (mAU)	Concentration (mg/g)	RSD (%)
Luteolin (**1**)	1,062,597 ± 1,326.92	190.717 ± 0.015	0.12
Apigenin (**2**)	811051.3 ± 4,500.54	12.4367 ± 0.058	0.54

*Values are expressed as the mean ± SD (n = 3, see experimental). RSD: relative standard deviation; Content (mg/g) for luteolin = (area +21,030)/83,557*147.06 (dilution factor); content (mg/g) for apigenin = (area +27,059)/77,296*11.47 (dilution factor).*

Quantification of Luteolin and Apigenin in the Flavone-Rich Fraction

Chromatographic investigation of the analytes was realized by comparing the retention time (Rt) and UV spectra of the corresponding peaks in the flavone-rich fraction with authentic standards apigenin and luteolin. Flavones quantification was carried out through calibration curves obtained by triplicate injections. Calibration curves showed to be linear in the ranges of 1–20 µg/mL for luteolin and apigenin. Besides that, good regression coefficients (r^2) for linear regression equations of both standards were obtained: 0.9995 for luteolin and 0.9960 for apigenin. Luteolin and apigenin contents were calculated using the equations Absorbance (mAu) = 83,557 (concentration)—21,030 and Absorbance (mAu) = 77,296 (concentration)—27,059, respectively. The content of luteolin and apigenin (**Table 2**) were obtained in mg/g of dry weight, after correction by dilution factor, when necessary.

Leishmanicidal Activity of Flavonoids Obtained From *A. chica*

Growth inhibitory activity by the selected compounds was performed on *L. amazonensis* promastigotes forms. In the test, all evaluated compounds from *A. chica* showed antipromastigote effects (**Table 3**). The results also revealed that ACFF caused leishmanicidal effects on the promastigotes of *L. amazonensis* 3-fold more potent in comparison with the ACCE. Moreover, the IC_{50} value for the flavonoids apigenin and luteolin against promastigotes of *L. amazonensis* were similar to their original fraction, ACFF.

Evaluation of cytotoxicity showed that ACFF was the less cytotoxic compound, followed by ACCE, and the flavonoids apigenin and luteolin were more cytotoxic having CC_{50} similar to amphotericin B (**Table 3**). The cytotoxicity against peritoneal macrophage and *L. amazonensis* were compared using the selectivity index (SI) (**Table 3**).

From the results described in **Table 3**, ACFF and luteolin were selected for evaluation against intra-macrophage forms. We found that luteolin inhibited the intracellular amastigote number. However, results demonstrated that the ACFF was 3.3-fold more effective for the amastigotes than luteolin. Therefore, considering this increased activity against intracellular amastigote, we observed a promising selectivity for the fraction rich in flavonoids (SI/24 h > 10.0). Amphotericin B showed leishmanicidal activity and cytotoxicity as expected.

TABLE 3 | Cytotoxicity and antileishmanial activity of extracts and isolated flavonoids from *Arrabidaea chica*.

Compounds	Cytotoxicity	*L. amazonensis*		
	CC_{50} (µg/mL)	Promastigote	Intracellular amastigote	SI
		IC_{50} (µg/mL)	IC_{50} (µg/mL)	
ACCE	38.64 ± 1.23	121.8 ± 1.41	—	—
ACFF	40.93 ± 1.18	40.42 ± 0.10	3.575 ± 1.13	11.44
Apigenin	11.87 ± 1.32 (43.92 µM)	45.60 ± 1.08 (168.7 µM)	—	—
Luteolin	8.005 ± 1.23 (27.97 µM)	31.51 ± 1.13 (110.08 µM)	11.78 ± 1.24 (41.15 µM)	0.679
Amphotericin B	9.352 ± 1.11 (10.1 µM)	0.07198 ± 1.15 (0.078 µM)	0.2752 ± 1.28 (0.298 µM)	33.98

ACCE: A. chica *crude extract; ACFF: fraction rich in flavonoids of* A. chica*. Data represent mean ± SD of at least two experiments realized in triplicate. CC_{50}: half-maximal cytotoxic concentration for 50% of cells; IC_{50}: 50% inhibitory concentration of parasites; SI: selectivity index, SI = CC_{50}/IC_{50} intracellular amastigote.*

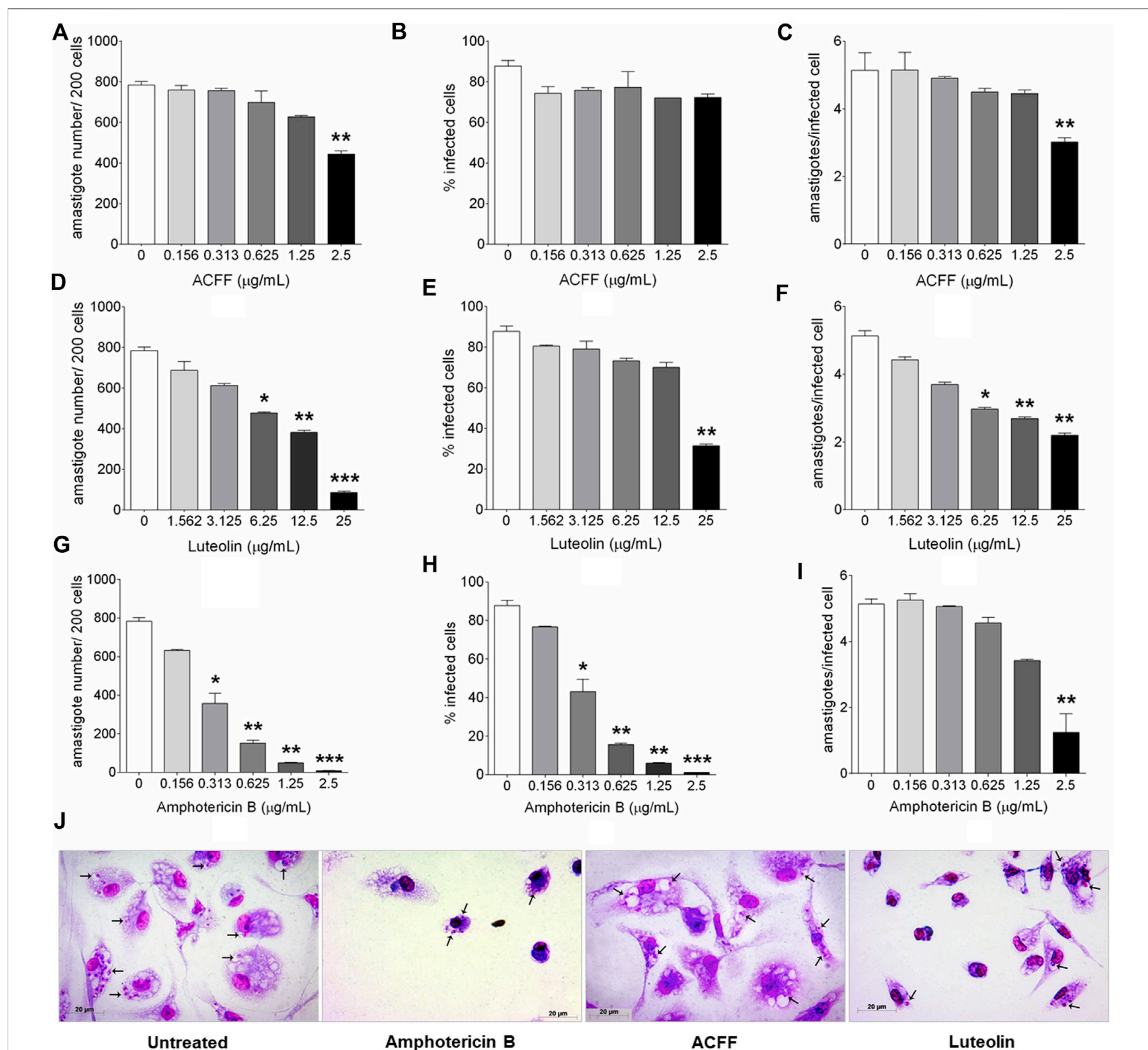

FIGURE 3 | BALB/c peritoneal macrophages infected with *Leishmania amazonensis* and treated for 24 h with amphotericin B, *Arrabidaea chica* flavone-rich fraction (ACFF) or luteolin. **(A–I)** Parameters of infection and **(J)** light microscopy of untreated, and treated with amphotericin B (2.5 μg/mL), ACFF (2.5 μg/mL) or luteolin (25 μg/mL) infected cells. (black arrows) Intracellular amastigotes inside macrophages. The images and data (mean ± standard deviation) represent two independent experiments performed in triplicate. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 when compared with untreated infected cells by Kruskal–Wallis and Dunn's multiple comparison test. Giemsa, 40× objective.

The parameters of infection of untreated cells were used as comparative control for the treatment with the different compounds at different concentrations. Infected and untreated macrophages presented 783.1 ± 80.43 amastigotes per 200 cells, 87.74 ± 7.95% of infected cells, and mean of amastigotes per infected cell of 5.135 ± 0.53 (**Figure 3**). The treatment with ACFF significantly reduced the number of amastigotes per 200 cells (443.50 ± 30.60, p = 0.0010, **Figure 3A**) and the mean of amastigotes per infected cell (3.19 ± 0.21, p = 0.0043, **Figure 3C**) at 2.5 μg/mL. In infected cells treated with luteolin, a statistically significant reduction was observed at the highest concentration evaluated (25 μg/mL) in all parameters of infection according to the intracellular amastigote number (84.50 ± 13.28, p = 0.0002, **Figure 3D**), percentage of infected cells (31.50 ± 1.73, p = 0.0011, **Figure 3E**), and the mean of amastigotes per infected cell (2.20 ± 0.10, p = 0.0049, **Figure 3F**). Amphotericin B showed a statistically significant reduction in all infection parameters at 2.5 μg/mL (9.00 ± 1.73, p = 0.0005, **Figure 3G**; 1.09 ± 0.21, p = 0.0002, **Figure 3H**; 1.25 ± 1.37, p = 0.0002, **Figure 3I**). As shown in **Figure 3J**, images of BALB/c peritoneal macrophages infected

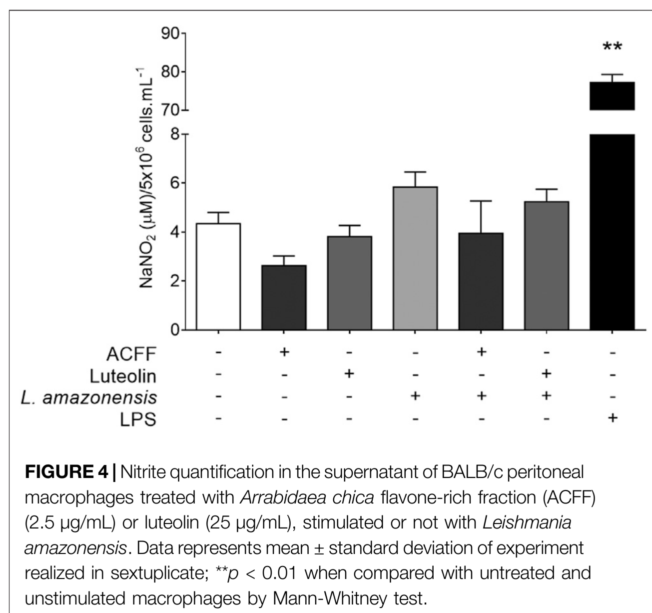

FIGURE 4 | Nitrite quantification in the supernatant of BALB/c peritoneal macrophages treated with *Arrabidaea chica* flavone-rich fraction (ACFF) (2.5 μg/mL) or luteolin (25 μg/mL), stimulated or not with *Leishmania amazonensis*. Data represents mean ± standard deviation of experiment realized in sextuplicate; $^{**}p < 0.01$ when compared with untreated and unstimulated macrophages by Mann-Whitney test.

with *L. amazonensis* and treated with amphotericin B, ACFF and luteolin corroborate with the results of **Figures 3A–I**.

Nitrite Quantification in *L. amazonensis*-Infected Peritoneal Macrophages Treated With ACFF and Luteolin

The effect of *A. chica* compounds on nitrite production in the supernatant of BALB/c peritoneal macrophages is shown in **Figure 4**. The macrophages showed low nitrite levels in cells treated with ACFF (2.63 ± 0.79 μM $NaNO_2$, p = 0.0857) and luteolin (3.55 ± 0.91 μM $NaNO_2$, p = 0.4000), when compared to untreated cells (4.34 ± 1.12 μM $NaNO_2$). Low nitrite levels were also observed in cells stimulated with *L. amazonensis* and treated with ACFF (3.34 ± 2.65 μM $NaNO_2$, p = 0.4476) and luteolin (4.43 ± 0.30 μM $NaNO_2$, p = 0.4048), when compared to stimulated and untreated cells (5.83 ± 1.69 μM $NaNO_2$), although the difference was not statistically significant. In the test, macrophages when stimulated with lipopolysaccharide (LPS) produced high levels of nitrite compared to cultures not stimulated with LPS.

Flavones From *A. chica* Promotes Ultrastructural Changes in *L. amazonensis* Promastigotes

To assess whether the treatment with compounds from *A. chica* promoted morphological and structural changes, analysis by transmission electron microscopy (TEM) was performed. Parasites were treated or not with ACFF and luteolin IC_{50} for 24 h. Promastigote forms showed the cellular morphology with an elongated body and all its intact organelles (**Figure 5A**). It was possible to observe that the treatment with ACFF promoted several vacuoles containing granular, circular and electron-

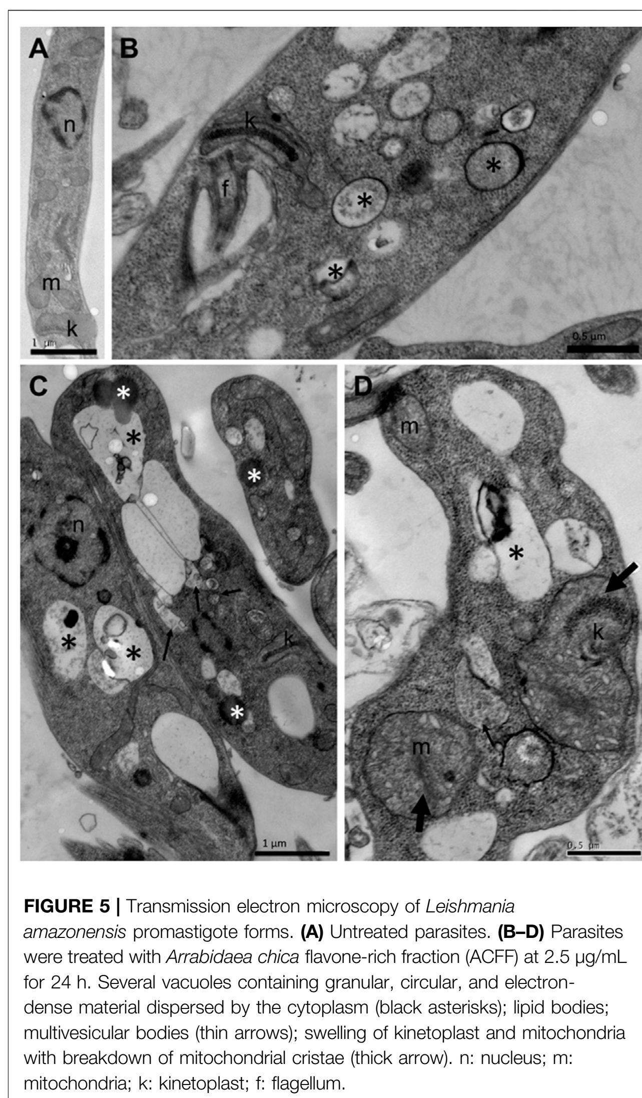

FIGURE 5 | Transmission electron microscopy of *Leishmania amazonensis* promastigote forms. **(A)** Untreated parasites. **(B–D)** Parasites were treated with *Arrabidaea chica* flavone-rich fraction (ACFF) at 2.5 μg/mL for 24 h. Several vacuoles containing granular, circular, and electron-dense material dispersed by the cytoplasm (black asterisks); lipid bodies; multivesicular bodies (thin arrows); swelling of kinetoplast and mitochondria with breakdown of mitochondrial cristae (thick arrow). n: nucleus; m: mitochondria; k: kinetoplast; f: flagellum.

dense material dispersed by the cytoplasm (**Figure 5B**), lipid bodies, multivesicular bodies (**Figure 5C**), swelling of kinetoplast and mitochondria with the breakdown of mitochondrial cristae (**Figure 5D**). Luteolin promoted small vacuoles containing electron-dense microvesicles dispersed in the cytoplasm (**Figures 6A,B**), change in the nuclear chromatin (**Figure 6A**), lipid bodies (**Figures 6A,B**), kinetoplast and mitochondria fully degenerated (**Figures 6A,B**). Also, it was possible to observe that luteolin promoted vacuoles containing material of different shapes and density, lipid bodies (**Figures 7A–D**), several layers of circular membranes involving multivesicular bodies (**Figure 7B**), and autophagosome-like vacuoles (**Figure 7D**).

DISCUSSION

Dichloromethane fraction obtained from hydroalcoholic extract of *A. chica* is admittedly rich in flavonoids (Takemura et al., 1995;

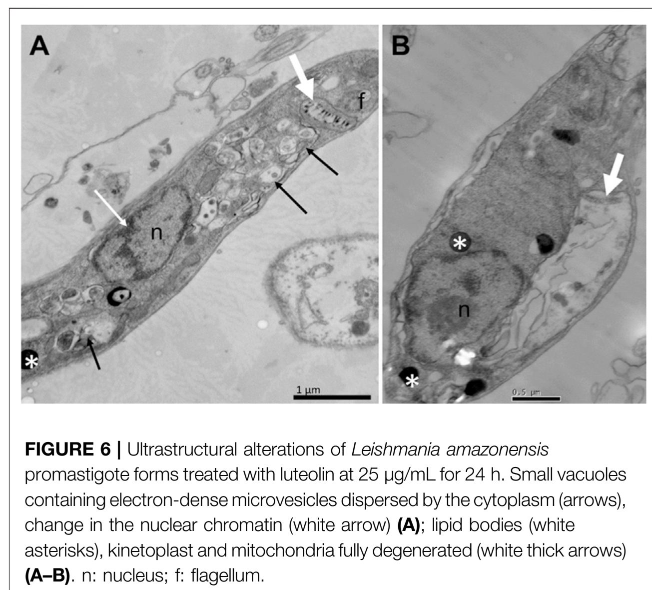

FIGURE 6 | Ultrastructural alterations of *Leishmania amazonensis* promastigote forms treated with luteolin at 25 μg/mL for 24 h. Small vacuoles containing electron-dense microvesicles dispersed by the cytoplasm (arrows), change in the nuclear chromatin (white arrow) **(A)**; lipid bodies (white asterisks), kinetoplast and mitochondria fully degenerated (white thick arrows) **(A–B)**. n: nucleus; f: flagellum.

Zorn et al., 2001; Devia et al., 2002; Barbosa et al., 2008; Siraichi et al., 2013), among which stand out anthocyanidins, the chemical markers of the species, in addition to flavones and flavonols. The fractionation carried out in this study had as the main objective obtaining a flavone-rich fraction, without anthocyanidins, and the isolation of some major compounds.

HPLC-DAD-UV analysis showed a chromatogram with six peaks. UV/Vis spectrum showed that the six compounds had broad bands of absorption with maximum peaks in the range of 338 and 348 nm (band I) and 256 and 283 nm (band II). Band I is associated with absorption due to the B ring of cinnamoyl system and when registered in the range of 304–350 nm it is characteristic of flavones (Mabry et al., 1970). Band II is related to absorption in the A ring benzoyl system. This ring when trisubstituted shows higher absorption at band II (**Supplementary material—S1**).

The flavone-rich fraction (ACFF) was also analyzed by LC-ESI-MS-MS, whose results compared with literature data (Siraichi et al., 2013; Grabsk et al., 2017) allowed to identify four flavonoids: 6-hydroxy-luteolin **(3)**, scutellarein **(4)**, carajuflavone **(5)** and chrysoeriol **(6)**.

Mass spectrometry has been an extremely useful method to identify and characterize polyphenolic secondary plant metabolites, and flavonoids are of particular importance (Cuyckens and Claeys, 2004). The collision of the precursor ions produced in the positive ion mode from the detected flavones yielded mass spectra for product ions. These spectra were used for their structural identification. The most useful fragmentations in terms of flavonoid aglycone identification are those that require cleavage of two carbon-carbon bonds of the flavonoid C ring (dihydropyran ring), which can be rationalized in terms of retro-Diels-Alder reactions (Pinheiro and Justino, 2012; Vaca et al., 2017). Such fragmentation in general allows a quick identification of the flavonoid type, as well as the number of substituents in each ring (Pinheiro and Justino, 2012). Therefore, in positive mode, fragments at *m/z* 153 and 169 are characteristic

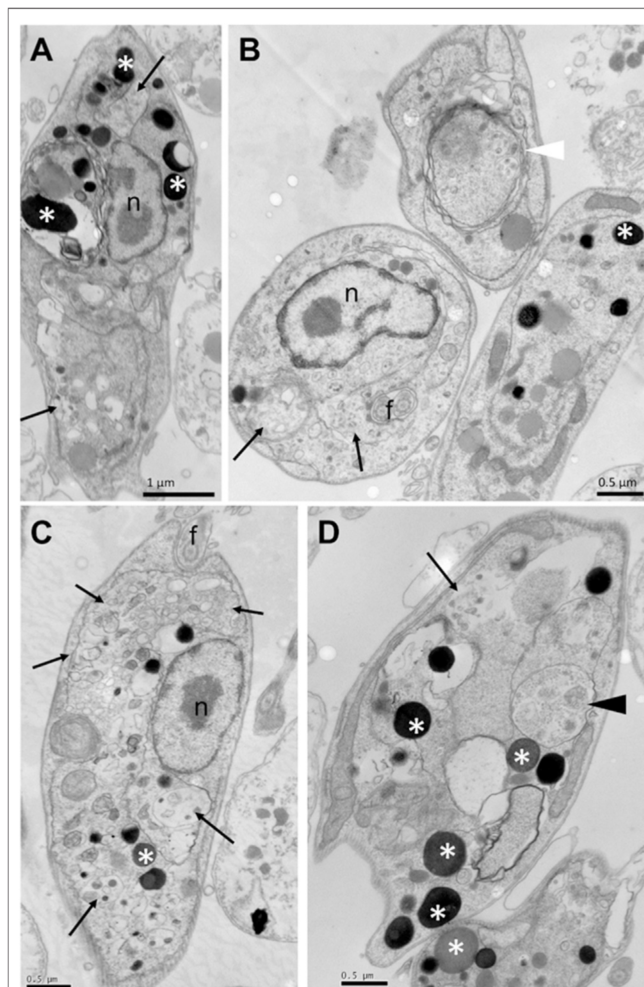

FIGURE 7 | Ultrastructural alterations of *Leishmania amazonensis* promastigote forms treated with luteolin at 25 μg/mL for 24 h. Vacuoles containing material of different shapes and density (arrows), lipid bodies (white asterisks) **(A–D)**, several layers of circular membranes involving multivesicular bodies (white arrowhead) **(B)**, autophagosome-like vacuoles (black arrowhead) **(D)**. n: nucleus.

of compounds containing di-hydroxylated and tri-hydroxylated A ring, respectively. Retro-Diels-Alder fragmentation also produces information about B ring. Fragments at *m/z* 119 and 135 are related to mono- and di-hydroxylated B ring, respectively. Positive LC-ESI-MS-MS data analyses were performed to confirm structures in addition to data already obtained in HPLC-DAD-UV and Rt of flavones from ACFF. Both luteolin **(1)** and apigenin **(2)** have been identified by ^{1}H NMR and ^{13}C NMR spectrometric techniques, UV, elution order, and Rt, and were also evaluated by fragmentation pattern. These compounds showed the Retro-Diels-Alder fragmentation pattern confirming the di-hydroxylation of A ring (ions at *m/z* 153.0151 and 153.0148, respectively). Luteolin **(1)** showed experimental pseudo-molecular ion $(M + [H]^+)$ at *m/z* 287.0496, which is compatible to the molecular formula $C_{15}H_{10}O_6$ as well as the mass fragment at *m/z* 241.0447 $[(M\text{-}H_2O\text{-}CO)]^+$. Apigenin **(2)**

was confirmed by the pseudo-molecular ion at *m/z* 271.0548, corresponding to the molecular formula $C_{15}H_{10}O_5$, as well as the fragments at *m/z* 243 characterized by C=O loss. Retro-Diels-Alder fragments correspondent to a tri-hydroxylated A ring at *m/z* 169.0103 and 169.0105 were observed to 6-hydroxy-luteolin **(3)** and scutellarein **(4)**, respectively. The compound 6-hydroxy-luteolin or 5,6,7,3′,4′-penta-hydroxy flavone **(3)** also showed the experimental pseudo-molecular ion $(M + [H]^+)$ at *m/z* 303.0449, compatible to the molecular formula $C_{15}H_{11}O_7$, as well as the mass fragment at *m/z* 257.0389 $[(M\text{-}H_2O\text{-}CO)]^+$, confirming their identification. The structure of scutellarein **(4)** was also confirmed by the experimental pseudo-molecular ion at *m/z* 287.0496, which is compatible with the molecular formula $C_{15}H_{10}O_6$. Carajuflavone or 6,7,3′,4′-tetrahydroxy-5-methoxy flavone **(5)** showed a pseudo-molecular ion at *m/z* 317.0595 corresponding to the molecular formula $C_{16}H_{13}O_7$. The fragment at *m/z* 168.0017 indicated that in Retro-Diels-Alder fragmentation, one of the three hydroxyl groups of A ring was substituted. It was confirmed by their fragmentation pattern that produced ions at *m/z* 302.0360 confirming the loss of a methyl group $(M—[CH_3]^+)$. Chrysoeriol **(6)** showed experimental pseudo-molecular ion at *m/z* 301.0646 compatible with the molecular formula $C_{16}H_{13}O_6$. Chrysoeriol fragmentation pattern produced ions at *m/z* 286.0414 $[(M)\text{-}CH_3]^+$ and at *m/z* 258.0467 $[(M)\text{-}CH_3\text{-}CO]^+$. These pieces of evidence confirm compound **(6)** as chrysoeriol (**Supplementary Material S2**).

All identified flavones in ACFF have been previously described for *A. chica*. Compound 6-hydroxy-luteolin, besides of luteolin, apigenin, and chrysoeriol had been described for *A. chica* leaves hydroethanolic extract (Vasconcelos et al., 2019). In another study, also with an *A. chica* extract, Siraichi et al. (2013) identified six flavones, from which, four—6-hydroxyluteolin **(3)**, luteolin **(1)**, apigenin **(2)**, and scutellarein **(4)**—were identified in ACFF. Carajuflavone **(5)**, another 6-hydroxylated compound from *A. chica* had already been described by Takemura et al. (1995). Compound 6-hydroxy-luteolin **(3)** has been reported in the Bignoniaceae family, and 6-hydroxylation is a common structural feature of the Bignoniaceae flavonoids having particular chemotaxonomy relevance for this reason. Besides, 6-hydroxylation is also characteristic of the structures of the red pigments anthocyanidins carajurin and carajurone found in *A. chica* (Harborne, 1967).

Quantitative analysis showed luteolin **(1)** as the most representative flavone of ACFF (**Figure 1**), whose concentration was calculated as 190.7 mg/g dry extract. Apigenin **(2)** represented only 12.43 mg/g dry extract. The results obtained in the present study are already better than other publications on concentrations and contents of luteolin and apigenin in extracts obtained from *A. chica* leaves (Paula et al., 2013). It demonstrated that the developed method for obtaining the flavone-rich fraction showed great results.

Publications by our research network previously reported on the leishmanicidal activity of hydroalcoholic extracts from four morphotypes of *A. chica* (Moragas-Tellis et al., 2020). However, as the species *A. chica* exhibit a great set of polyphenolic compounds—mainly flavonoids—we decided to expand this study, previously done only with anthocyanidins, to evaluate whether flavones may also be involved in the leishmanicidal activity.

The increase in antipromastigote activity from bioguided fractionation facilitated the identification of the best fractionation stage that contributes to the best results for leishmanicidal activity. As a result, the ACFF was more effective than ACCE, and the isolated flavone luteolin showed better activity than ACFF. Apigenin, however, showed activity similar to ACFF.

Studies conducted by Cortez de Sá et al. (2015) presented a screening test for the crude ethanolic extract of the leaves of *A. chica* with the inhibitory concentration of 50% (IC_{50}) of the promastigote forms of *L. amazonensis* determined at 155.9 μg/mL. Phytochemical screening of that study showed an extract containing flavonoids, phenolic compounds, tannins, anthocyanidins and chalcones. In addition, the crude extract of the leaves of *A. chica* had a cytotoxic effect at a concentration of 189.9 μg/mL. Factors that can explain the difference in phytochemical and biological results are probably due to the climatic differences between the two locations where the plant was collected, as well as the way of obtaining the extract (Cortez de Sá et al., 2015). However, it should be noted that the difference in cytotoxicity found with our study is probably related to the exposure time used, since we used 72 h, while in the study by Cortez de Sá et al. (2015) the time was reduced to 24 h. Besides, and even more important, the different morphotypes of *A. chica* vary in chemical composition and, consequently, in biological activity (Moragas-Tellis et al., 2020).

Aware that the extraction and fractionation methods can help in biological activity, we opted to optimize the bioguided fractionation process favoring the obtaining of a flavone-rich fraction in an attempt to better explore the phytochemical profile of *A. chica* species. Flavones have demonstrated a wide range of biological activities that include antioxidant, antimicrobial, anti-inflammatory and other activities. In addition, structure-activity relationships have generated interest among medicinal chemists, making the flavones an important class of natural products of new therapeutic agents (Singh et al., 2014).

Literature data show flavones with leishmancidal activity. Apigenin was tested for its anti-*Leishmania* activity against the promastigote forms of *L. amazonensis*, inhibiting the growth of the parasites at IC_{50} values of 23.7 μM (Fonseca-Silva et al., 2015) and 22.77 μM against *Leishmania donovani* strains (Antwi et al., 2019). This inhibition was also observed by luteolin at IC_{50} value of 12.5 μM against *L. donovani* (Mittra et al., 2000). Another study shows apigenin and luteolin having inhibitory activity against *L. donovani* axenic amastigotes (IC_{50} 1.9 and 0.8 μg/mL, respectively) (Tasdemir et al., 2006). The antipromastigote activity of the ACFF may be related to the high concentration of these flavones in their composition. The high cytotoxicity of these flavones against L6 cells (derived from rat skeletal myoblasts) has also been reported, at IC_{50} values of 18.1 μg/mL for apigenin and 9.44 μg/mL for luteolin—data that corroborate the toxicity observed in our study. Besides, the structure-activity relationship of these flavones was investigated, and it was observed that the presence of the 5,7-dihydroxybenzochromone structure greatly increases

leishmanicidal activity, occurring the same with the presence of the double bond between positions 2 and 3 (C-2,3). Such characteristics are observed in apigenin and luteolin. Thus, it is possible to infer that the different leishmanicidal activity of these two flavones might be due to the replacement pattern in the B-ring, since luteolin has a catechol portion (3′,4′-dihydroxyphenyl) while apigenin has only one hydroxyl in 4’ (Tasdemir et al., 2006).

Our findings against the promastigote form recommend selecting the ACFF and luteolin, the main component of the fraction—to verify the activity against the forms of *L. amazonesis* intracelular amastigote. In the search for new drugs against *Leishmania* spp., intracellular amastigote is the stage of the parasite considered as the most relevant target for the primary screening of new compounds (de Muylder et al., 2011), and the most consistent indicator of *in vivo* activity (Croft, 1986), additionally considered "the gold standard" of in *in vitro* studies (Baek et al., 2020). It is therefore of interest to test the effectiveness of *A. chica* compounds in intracellular amastigotes.

It was possible to observe an improvement in the leishmancidal activity, being ACFF and luteolin 11.3 and 2.67 times more active against intracellular amastigote forms than to the promastigotes forms, respectively. A study performed by Wong et al. (2012) reported that luteolin exhibited promising activity only against the intracellular amastigote, but not for extracellular promastigotes, suggesting that its specific targets are present only in the intracellular phase. This activity is also observed in another study against *L. donovani*, according to which luteolin reduced intracellular amastigote load by 70% at a final concentration as low as 12.5 μM (Mittra et al., 2000). Therefore, the high concentration of luteolin in ACFF may be responsible for the increased inhibition against the intracellular forms of the parasite.

When comparing the results of the activity against the intracellular phase, ACFF was 3.2 times more active than luteolin. It is worth mentioning that another flavone present in the ACFF composition was apigenin, but this compound was not tested against the intracellular amastigote form due to its low yield. However, data from the literature show the significant inhibitory effect of apigenin against the intracellular amastigote forms of *L. donovani*, at IC_{50} values of 45.66 ± 0.01 μM (12.34 μg/mL). In addition, when the infected macrophages were treated with increasing concentrations of apigenin, there was a decrease in the number of infected cells (Antwi et al., 2019). Therefore, this leads us to infer that the presence of a couple of compounds in the ACFF can contribute to the leishmanicidal activity and to lower cytotoxic effect, favoring the greater selectivity to the parasite observed to ACFF when compared to luteolin. These findings are often considered to be the result of a synergistic or additive effect of the extract’s constituents (Dalby-Brown et al., 2005).

The inhibition of intracellular amastigote is directly related to the presence of nitric oxide (NO) in activated macrophages (Mukbel et al., 2007). Therefore, in an attempt to understand the leishmanicidal activity of ACFF and luteolin against intracellular amastigote forms, nitrite quantification was performed as an indirect way to determine NO levels (Almeida-Souza et al., 2016). However, there were no significant changes in this assessment parameter.

In a study with apigenin and luteolin, both inhibited NO production, considering that a C-2,3 double bond may be important, and that the patterns of substitution of flavonoid molecules can determine the potency of the inhibition in NO production (Kim et al., 1999). In addition, suppression in the production of NO and prostaglandin E2 (PGE2), without having cytotoxicity in RAW 264.7 mouse macrophage cells activated by bacterial lipopolysaccharide was also observed when exposed to flavones, luteolin and its luteolin-7-O-glucoside. The suppression of inducible nitric oxide synthase (iNOS) and the expression of cyclooxygenase-2 protein (COX-2) are responsible for the inhibitory effects, and not for the reduction of enzymatic activity (Hu and Kitts, 2004). Different culture conditions and cell types may also be responsible for some difference between data in the literature and the results of the present study. Therefore, other mechanisms may be involved in the leishmanicidal activity of *A. chica* flavonoids against intracellular amastigote.

Trying to understand the leishmanicidal effect of *A. chica* compounds directly on the parasite, ultrastructural evaluation of the promastigote forms of *L. amazonensis* was performed by transmission electron microscopy. The treatment with ACFF promoted ultrastructural alterations such as the vacuolization process of the cytoplasm, lipid and multivesicular bodies, swelling of the kinetoplast and mitochondria with the breaking of the mitochondrial ridges. Ultrastructural changes were also observed in the study by Rodrigues et al. (2014), with *L. infantum* promastigotes treated with fraction B2 (1:1 n-hexane/ethyl acetate) obtained from the crude hexane extract of *A. chica*. In this study, mitochondrial edema with loss of matrix content and the presence of vesicles within this organelle were observed.

Luteolin, the metabolite with the highest concentration in ACFF composition, was evaluated for ultrastructural alterations induced in *L. amazonensis* promastigotes. It was possible to observe vacuolization of the cytoplasm, change in nuclear chromatin, lipid bodies, with kinetoplast and fully degenerated mitochondria, and vacuoles similar to autophagosomes. Studies performed by Mittra et al. (2000) indicates that flavonoids may target the enzyme topoisomerase II in the kinetoplast of parasites, since it reports that luteolin and quercetin induce significant cleavage of the topoisomerase II-mediated kDNA minicircle in *Leishmania*, an inhibition similar to the well-known anti-*Leishmania* drug, pentamidine. Another study elucidates the mechanism of action of luteolin by analyzing mitochondrial and cytosolic changes associated with death similar to *L. donovani* cell apoptosis (Sen et al., 2006). In this work, Sen et al. (2006) reports that luteolin inhibition of the production of glycolytic ATP was an essential event responsible for the depolarization of the mitochondrial membrane in depleted mt-DNA cells to propagate apoptosis-like death in *Leishmania* cells.

Our results provide additional evidence on the antileishmanial activity induced by *A. chica*. However, based on the findings obtained, further studies should be carried out to elucidate the mechanisms of action, as well as *in vivo* studies are essential to assess the leishmanicidal activity of the ACFF against *Leishmania*.

CONCLUSION

Chromatographic techniques and analysis of the mass spectra obtained from the ACCE allowed the identification of compounds of the ACFF derived from *A. chica*. The flavones luteolin and apigenin were isolated from the ACFF using chromatographic techniques and identified by NMR spectrometry techniques. ACCE, ACFF and flavones showed leishmanicidal activity against the promastigotes of *L. amazonensis*. The antiparasitic effect of ACFF and luteolin was confirmed by the ultrastructural changes with induction of mitochondrial damage. ACFF also showed low cytotoxicity in host cells if compared with the isolated flavones. ACFF and luteolin showed leishmanicidal activity against the intracellular amastigotes, however, this activity is not related to the production of NO by host cells. Thus, ACFF is a suitable candidate for further *in vivo* investigations against *L. amazonensis*.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee on Animal Care and Utilization of Oswaldo Cruz Institute (CEUA-IOC L53/2016).

AUTHOR CONTRIBUTIONS

JS, CM, MC, PS, CS and DH performed the experiments. MB, KC and FA contributed to conception and design of the study. NT obtained TEM images. JS, DM and FA organized the database. JS and FA performed the statistical analysis. JS and CM wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.703985/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3.2 Carajurin: a anthocyanidin from *Arrabidaea chica* as a potential biological marker of antileishmanial activity

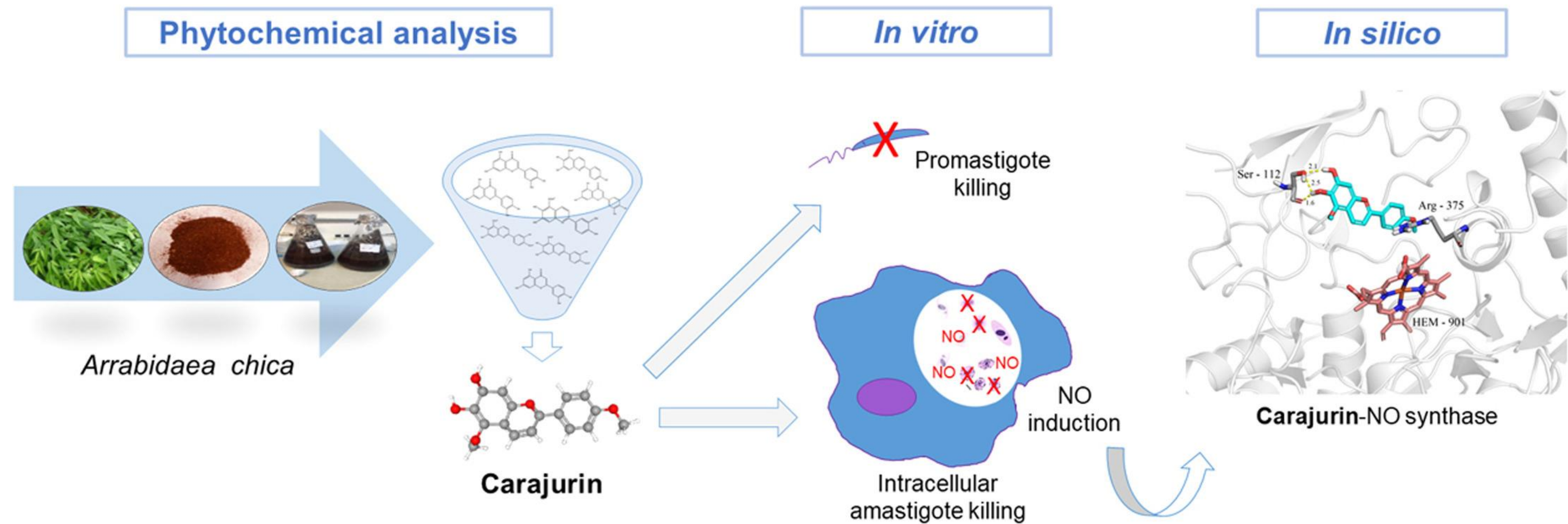

Figura 6 Resumo gráfico da ação da carajurina contra contra as diferentes formas de *L. amazonensis*.

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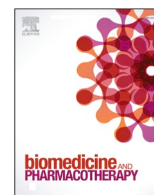

Carajurin: a anthocyanidin from *Arrabidaea chica* as a potential biological marker of antileishmanial activity

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A R T I C L E I N F O

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Macrophages
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Nitric oxide synthase

A B S T R A C T

Leishmaniasis is a group of neglected tropical diseases whose treatment with antimonials bears limitations and has changed little in over 80 years. Medicinal plants have been evaluated as a therapeutic alternative for leishmaniasis. *Arrabidaea chica* is popularly used as a wound healing and antiparasitic agent, especially as leishmanicidal agent. This study examined the leishmanicidal activity of a crude extract (ACCE), an anthocyanidin-rich fraction (ACAF), and three isolated anthocyanidins from *A. chica*: carajurin, 3′-hydroxy-carajurone, and carajurone. We evaluated the antileishmanial activity against promastigote and intracellular amastigote forms of *Leishmania amazonensis* and determined cytotoxicity in BALB/c peritoneal macrophages, as well as nitrite quantification, using the Griess method. Molecular docking was carried out to evaluate interactions of carajurin at the nitric oxide synthase enzyme. All compounds were active against promastigotes after 72 h, with IC_{50} values of 101.5 ± 0.06 μg/mL for ACCE and 4.976 ± 1.09 μg/mL for ACAF. Anthocyanidins carajurin, 3′-hydroxy-carajurone, and carajurone had IC_{50} values of 3.66 ± 1.16, 22.70 ± 1.20, and 28.28 ± 0.07 μg/mL, respectively. The cytotoxicity assay after 72 h showed results ranging from 9.640 to 66.74 μg/mL for anthocyanidins. ACAF and carajurin showed selectivity against intracellular amastigote forms (SI> 10), with low cytotoxicity within 24 h, a statistically significant reduction in all infection parameters, and induced nitrite production. Molecular docking studies were developed to understand a possible mechanism of activation of the nitric oxide synthase enzyme, which leads to an increase in the production of nitric oxide observed in the other experiments reported. These results encourage us to suggest carajurin as a biological marker of *A. chica*.

1. Introduction

Leishmaniasis are a complex of diseases caused by an intracellular protozoan of the genus *Leishmania*, which has more than 20 species, covering a broad clinical spectrum, from self-limited cutaneous forms to the fatal visceral form, depending on the immune response of the host and the *Leishmania* species [1].

Leishmaniasis are a serious public health problem and are classified

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as neglected tropical diseases [1,2], being associated with limited investments in diagnosis, treatment, and control, as well as the population's conditions of poverty [3]. For this reason, more than 1 billion people live in endemic areas at risk of infection, with 1 million cases of cutaneous leishmaniasis reported in the last 5 years, 300 thousand cases of visceral leishmaniasis estimated annually, and more than 20 thousand deaths per year [1]. Brazil was among the seven countries responsible for more than 90% of the new cases of visceral leishmaniasis notified to WHO in 2017, and among the nine countries in which most skin cases of leishmaniasis occur [1].

In addition to these circumstances, current chemotherapy for the treatment of leishmaniasis, in all clinical forms, has changed little in over 80 years and still depends on the use of pentavalent antimonials such as sodium stibogluconate (Pentostam) and meglumine antimonate (Glucantime®) [4]. Although there are alternatives in severe cases for those not responding to antimonials, such as amphotericin B liposomal and conventional, pentamidine, paromomycin, and miltefosine, they also involve toxicity problems, high costs, the resistance of parasites, and therapeutic failure [5]. Therefore, there is an urgent need for discovery or development of new and more effective drugs to replace or complement those in use.

The use of plants in the treatment of these diseases appears as an alternative, as it has long been considered a medicinal source to treat different diseases [6]. It is worth mentioning that, of the 16 antiparasitic drugs that were approved by the United States regulatory agency (FDA, USA), between January 1981 and December 2014, 43.75% are natural products or derivatives [7]. As a result, the search for new therapies has grown in recent years, being natural products — mainly those derived from plants — used in the study of therapeutic alternatives for leishmaniasis [8]. In Brazil, the National List of Medicinal Plants of Interest to the Unified Health System (RENISUS) was issued in 2009. In this list of 71 medicinal plants, the species *Arrabidaea chica* stands out, being considered by the Brazilian health authorities as one of the most important medicinal plants used in popular medicine, especially in the Amazon region [9].

Arrabidaea chica (Humb. & Bonpl.) B. Verlot belongs to the Bignoniaceae family and is found in the Cerrado (a sort of Brazilian savanna), Atlantic Forest, and in the Amazon biomes [10]. Pharmacological studies in the literature describe antimicrobial [11], anti-inflammatory, antiangiogenic and antiproliferative activities [12], cytotoxicity [13], genotoxicity [14], healing potential [15], photochemical-protective [16] and antioxidant activities [16–18]. Other studies also report antiparasitic activity against *Trypanosoma cruzi* [19,20] and *Leishmania* spp. [13,21,22]. Recent results by our research network shows that the variation in the anthocyanidin profile in extracts of four morphotypes of *A. chica* affected their leishmanicidal activity [23]. That study implies this activity might be related to the presence of carajurin and that this would, therefore, be a potential biological marker of the species. However, that was the first study on the characterization of the biological marker for the species. To date, there are no data on possible mechanisms of leishmanicidal action for extracts or isolated substances from *A. chica*.

Given that natural products are promising sources of new potential therapeutic agents, the widespread use of *A. chica* in popular medicine, as well as the advances in phytochemical studies of this plant, this study presents the evaluation of the leishmanicidal activity of the crude extract — through bioguided fractionation — of the anthocyanidin-rich fraction, and the isolated compounds, carajurin, 3′-hydroxy-carajurone, and carajurone. Its results contributed to confirm the importance of carajurin as a biological marker of the leishmanicidal activity of *A. chica*.

2. Materials and methods

2.1. Reagents

Brewer thioglycolate medium, RPMI 1640 medium, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), amphotericin B, Schneider's insect medium, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), and penicillin were acquired from Gibco (Gaithersburg, MD, USA).

2.2. Plant material

Leaves of *A. chica*, morphotype IV, were collected at Fiocruz Atlantic Forest Campus, Rio de Janeiro city, State of Rio de Janeiro, Brazil (S 22.9406° W 43.4046°) in February 2016. Voucher specimens were identified by Dr. Marcelo Galvão of Botanical Collection of Medicinal Plants (CBPM) of Farmanguinhos/Fiocruz. A sample (CPBM 668) was deposited at the CBPM.

2.3. Extraction, fractionation, and isolation

Leaves of *A. chica* were dried at 60 °C with air circulation and ground in a cutting mill. *Arrabidaea chica* crude extract (ACCE) was obtained by maceration in ethyl alcohol: water 70/30 (v/v) for seven days. The resulting extract was filtered and evaporated to dryness under reduced pressure at 30–40 °C. The reddish residue (15 g) was fractioned by liquid-liquid partition, with *n*-hexane (3 × 200 mL), dichloromethane (3 ×200 mL), ethyl acetate (3 ×200 mL) and *n*-butanol (3 × 200 mL). The dichloromethane fraction (5 g) (ACDF) was chromatographed by column chromatography on Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA) using methanol as eluent to produce an anthocyanidin-rich fraction named ACAF. Successive chromatographic fractionation steps on Sephadex LH-20 led to the isolation of three anthocyanidins: (**1**) carajurin (45 mg), (**2**) 3′-hydroxy-carajurone (12 mg), and (**3**) carajurone (3.5 mg). The identification of isolated compounds was achieved by direct infusion electrospray ionization mass spectrometry (ESIMS) and ^{1}H- and ^{13}C NMR spectrometry.

Carajurin (**1**) ^{1}H NMR (400 MHz-CDCl$_3$-d6) d: 6.98 (d, 1 H, H-3, J = 7.8 Hz); 7.99 (d, 1 H, H-4, J = 7.8 Hz); 7.01 (d, 2 H, H-3′and H-5′, J = 8.8 Hz); 7.89 (d, 2 H, H-2′ and H-6′, J = 8.8 Hz); 6.53 (s, 1 H, H-8); 3.90 (s, 3 H, 4′-O-CH$_3$); 4.10 (s, 3 H, 5-O-CH$_3$). ^{13}C NMR (100 MHz-CDCl$_3$-d6) d: 158.90 (C-2); 102.62 (C-3); 133.76 (C-4); 135.02 (C-5); 139.93 (C-6); 176.82 (C-7); 98.61 (C-8); 156.86 (C-9); 118.16 (C-10); 123.43 (C-1′); 127.68 (C-2′); 114.77 (C-3′); 162.50 (C-4′); 60.42 (C-5′); 55.58 (C-6′). Positive ESIMS *m/z*: 299.0905 [M + H]$^+$ (calc. *m/z* of 299.0924; Δ = −6.35), *m/z* 284.0671 M −[CH$_3$]$^+$ (calc. *m/z* of 284.0690; Δ = −6.68); *m/z* 269.0441 M −[CH$_3$] −[CH$_3$]$^+$ (calc. *m/z* of 269.0455; Δ = −5.20); *m/z* 256.0722 (M − [CH$_3$] − [C═O]$^+$) (calc. *m/z* of 256.0741; Δ = −7.41); 241.0491 M −[CH$_3$] −[CH$_3$] −[C═O]$^+$ (calc. *m/z* of 241.0506; Δ = −6.22).

3′-hydroxy-carajurone (**2**) ($C_{16}H_{13}O_6$) ^{1}H NMR (500 MHz-Methanol-d6) d: 7.27 (d, 1 H, H-3); 8.17 (d, 1 H, H-4, J= 8.0 Hz); 6.50 (s, 1 H, H-8); 7.44 (d, 1 H, H-2′, J= 2 Hz); 7.48 (dd, 1 H, H-5′, J= 8.5, J=2 Hz); 6.88 (d, 1 H, H-6′, J= 8 Hz), 4.04 (s, 3 H, 5-O-CH3). ^{13}C NMR (100 MHz-Methanol-d6) d: 166.05 (C-2); 103.41(C-3); 183.49 (C-4); 163.05 (C-5); 95.91(C-6); 166.05 (C-7); 101.31 (C-8); 159.72 (C-9); 104.27 (C-10); 123.43 (C-1′); 113.89 (C-2′); 147.31 (C-3′); 151.65 (C-4′); 116.86 (C-5′); 120.23 (C-6′). Positive ESIMS *m/z*: 301.0627 *m/z* [M + H]$^+$ (calc. *m/z* 301.0717; Δ = 29.8), *m/z* 286.0414 [(M)-CH$_3$]$^+$ and at *m/z* 256.0466 [(M) −CH$_3$ −CO]$^+$.

Carajurone (**3**) ($C_{16}H_{13}O_5$) ^{1}H NMR (400 MHz-Methanol-d6) d: 7.35 (d, 1 H, H-3, J= 8 Hz); 8.20 (d, 1 H, H-4, J= 8.0 Hz); 6.53 (s, 1 H, H-8); 7.94 (dd, 2 H, H-2′ and H6′, J= 2,0; J=7,0 Hz); 6,94 (dd, 2 H, H-3′and H-5′, J= 7,;0; J=2 Hz), 4.05 (s, 3 H, 4′-O-CH3). Positive ESIMS *m/z*: 285.0749 *m/z* [M + H] (calc. *m/z* of 285.0749; Δ = −4.56), *m/z* 270.0492 [(M − CH$_3$]$^+$ (calc. *m/z* of 270.0533; Δ = −15.44) and at *m/z* 242.0541 [(M − CH$_3$− CO)]$^+$ (calc. *m/z* of 242.0584; Δ = −17.76).

2.4. High-performance liquid chromatograph coupled to diode-array UV-vis detector (HPLC-DAD-UV)

Chromatographic analyses were performed on an HPLC-DAD-UV, using a Shimadzu Nexera XR® liquid chromatograph (Shimadzu, Kyoto, Japan) coupled to a UV detector with the diode array SPDM20A, a CBM20A controller, DGU20A degasser, LC20AD binary pump, CTO20A oven, and SILA20A auto-injector (Shimadzu, Kyoto, Japan). A Shimadzu LabSolutions Software Version 5.3 (Shimadzu, Kyoto, Japan) was used to analyze the chromatograms. DAD analysis was applied to select the optimized wavelength of anthocyanidins in this study. In a full-scan experiment, chromatograms at 480 nm show the maximum wavelength (λ_{max}) for the anthocyanidins. Combinations of acidified ultrapure water (pH 3.0, with anhydrous acetic acid, Merck, Darmstadt, Germany) (A) and acetonitrile (HPLC grade, Tedia, Rio de Janeiro, Brazil) (B) were used as the mobile phase (initially 5% A rising to 95% in 80 min). HPLC column was silica-based C18 (250 mm × 4.6 mm i.d. × 5 µm particle size, ODS Hypersil, Thermo, Waltham, MA, USA). The oven was set at 50 °C and the injection volume was 10 µL for all analyses.

2.5. Preparation of samples of the extracts

A total of 1000 µL of acetonitrile: methanol (both HPLC grade, Tedia, Rio de Janeiro, Brazil) mixture (75:25; v/v) was added to 10 mg of extracts and anthocyanidins-rich fraction, previously weighed in a 4 mL vial. The vial was sealed and the sample was sonicated for 10 min with occasional swirling. The sample was then vortexed to mix thoroughly, followed by filtering through a 0.45 µm PTFE filter (Merck Millipore, Darmstadt, Germany) before further analyses into an HPLC vial.

2.6. Quantification of anthocyanidins, using carajurin as standard

Quantification of anthocyanidins was performed using carajurin as the external standard. Since there are no standards available for all anthocyanidins, their content was expressed in milligrams of carajurin per gram of dry extract. A 200 µg/mL stock solution of the isolated carajurin (98%, chromatographic determined) was prepared in an acetonitrile:methanol (both HPLC grade, Tedia, Rio de Janeiro, Brazil) mixture (75:25; v/v) in a volumetric flask. Six concentrations of solutions (20; 40; 60; 100; 150 and 200 µg/mL) were done on the day, in order to prepare an analytical curve. The solutions were filtered in a 0.45 µm PTFE filter before analyses by HPLC-DAD-UV. Injections of 10 µL were performed in triplicate to obtain the analytical curve from the areas corresponding to the peaks of carajurin. The analytical curve (20–200 µg/mL) of the standard was plotted based on the UV-Vis signal at 480 nm for better selectivity: carajurin content (µg/mL) = (Abs (mAu) + 661228)/48694; $R^2 = 0.9993$). Carajurin and other anthocyanidin amounts (mg/g of dry extract) were calculated and expressed as carajurin content [23].

2.7. Animals and ethical statements

The study was approved by the local Ethics Committee on Animal Care and Utilization (CEUA-IOC L53/2016), following all procedures described by the Control of Animal Experimentation (CONCEA). Female BALB/c mice aged 4–6 weeks were purchased from the Institute of Science and Technology in Biomodels of the Oswaldo Cruz Foundation.

2.8. Parasites

Leishmania amazonensis strain MHOM/BR/76/MA-76 was maintained in promastigote form by culturing at 26 °C Schneider's Insect Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco (Gaithersburg, MD, USA), 100 U/mL penicillin (Gibco, Gaithersburg, MD, USA), and 100 µg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cultures with a maximum of 10 passages *in vitro* were used.

2.9. Peritoneal macrophage isolation and cell culture

Peritoneal macrophages were isolated from BALB/c mice administered with 3 mL thioglycolate 3% intraperitoneal for 72 h. Then, the animals were euthanized with ketamine 10% and xylazine 2% according to the weight of each animal and, after the death and exposure of the peritoneum, 10.0 mL of sterile phosphate buffered saline solution pH 7.2 was inoculated, followed by a light manual massage. Cells were recovered from peritoneum, cultured overnight and maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL), at 37 °C and 5% CO_2.

2.10. Activity against promastigote forms

The exponential phase of L. *amazonensis* promastigote forms were seeded into 96-well flat-bottomed plates at 2×10^6 parasites per well. Then, 100 µL of compounds solution, resuspended in dimethyl sulfoxide (DMSO) 1%, were mixed in the first well, proceeding to serial microdilutions in a 1:1 proportion ratio (compounds solution: Schneider's Insect Medium) until the penultimate cavity. Thus, different concentrations of the ACCE (1000–3.9 µg/mL), ACAF (1000–3.9 µg/mL), or isolated anthocyanidins (100–3.125 µg/mL) were obtained. After diluting the samples, the plates were incubated at 26 °C. Amphotericin B (2.5–0.07 µg/mL) was used as the reference drug, while wells without parasites were used as blanks, and wells with parasites and DMSO 1% only were used as untreated control. After 72 h of treatment, the plates were examined under an inverted microscope to assure the growth of the controls under sterile conditions, and viable promastigotes were counted in a Neubauer chamber [24]. The experiments were conducted in triplicate. The percentage of growth inhibition was calculated from the count of viable parasites relative to the untreated control, and 50% inhibitory concentration (IC_{50}) values were determined.

2.11. Cytotoxicity assay

Peritoneal macrophages were cultured in 96-well plates (5×10^5 cells/mL) and tested with different concentrations of ACCE and ACAF (7.81–1000 µg/mL), anthocyanidins (3.9–500 µg/mL) or amphotericin B (0.19–25 µg/mL), in a final volume of 100 µL/well, at 37 ºC and 5% of CO_2. Wells without cells were used as blanks and wells with cells and 1% DMSO were used as controls. After 24 and 72 h of treatment, cell viability was evaluated by the modified colorimetric method based on the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA). MTT (5 mg/mL) was added to each well in a volume equal to 10% of the total. After 2 h, the supernatant was completely removed and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was read on a spectrophotometer at a wavelength of 570 nm. Data were normalized according to the formula: % survival = (Abs. sample-Abs. blank) / (Abs. control-Abs. blank) × 100 [25].

2.12. Activity against intracellular amastigotes and selectivity index

Peritoneal macrophages of BALB/c mice were cultured in 24-well plates (5×10^5 cells/well) containing round coverslips, at 37 °C and 5% CO_2. The cells were infected with promastigote forms of *L. amazonensis* — in the parasite/cell ratio of 10:1 — for 6 h followed by washing with PBS to remove non-internalized parasites. Infected cells were treated with different concentrations of ACAF (2.5–0.078 µg/mL) or carajurin (25–0.78 µg/mL) for 24 h. The slides of the infected and treated cells were fixed with Bouin, stained with Giemsa, and examined by light microscopy. The IC_{50} was calculated from the intracellular amastigotes count in 200 cells. The percentage of infected cells was

obtained from the number of infected cells divided by two. The average number of amastigotes per cell was obtained from the number of intracellular amastigotes in 200 cells divided by the number of infected cells [25]. Amphotericin B (2.5–0.15 µg/mL) was used as a reference drug. The selectivity index (IS) was obtained from the ratio between the half-maximal cytotoxic concentration (CC_{50}) for BALB/c peritoneal macrophages and the IC_{50} for promastigote or intracellular amastigote.

2.13. Nitrite quantification

BALB/c peritoneal macrophages (5×10^6 cells/mL) were treated with ACAF (2.5 µg/mL) or carajurin (25 µg/mL) and stimulated with *L. amazonensis* (3×10^7 parasites/mL). After 48 h, the supernatant was collected and the nitrite quantification was performed with the Griess reagent. 50 µL of culture supernatant was added to 50 µL of Griess reagent (25 µL of 1% sulfanilamide in 2.5% H_3PO_4 solution and 25 µL of 0.1% N-(1-naphthyl) ethylenediamine solution) in 96-well plates. After 10 min, the plates were read at 570 nm on the spectrophotometer and the nitrite values were obtained from the standard sodium nitrite curve (1.5–100 µM) [26].

2.14. Molecular docking

Molecular docking simulations were performed with GOLD v.2020.2.0 [44] and the ChemPLP [27] scoring function. The receptor (PDB 1DF1, 2.35 Å) was treated as rigid, and the compounds were treated as fully flexible. The preparation of the receptors was made within the GOLD suite. Only was used the chain A of receptor. No crystallographic water molecules were considered. The binding site was defined as all the receptor atoms up to 6 Å of the reference crystallographic inhibitor. At least 10 poses were generated for ligand using the default parameters of the genetic algorithm. For analysis, the top-scoring conformations of the most populated clusters of poses/ligands were selected. Prior to the simulations, the ligands were optimized with a steepest descent algorithm (100 steps. FF: AM1BCC. The atomic charges were assigned with Ammp-Mom) in VEGA ZZ v.3.2 [28]. The propensity maps were generated with SuperStar, a module for knowledge-based pharmacophore generation and prediction of intermolecular interactions available within the GOLD suite. We worked with the default parameters for cavity detection and the PDB data, allowing R-H rotation and [O, N, S]-H bonds. The propensity map figure was generated with Hermes v.1.10.5 (also available within the GOLD suite). The receptor-ligand figures were generated with PyMOL v.1.8.

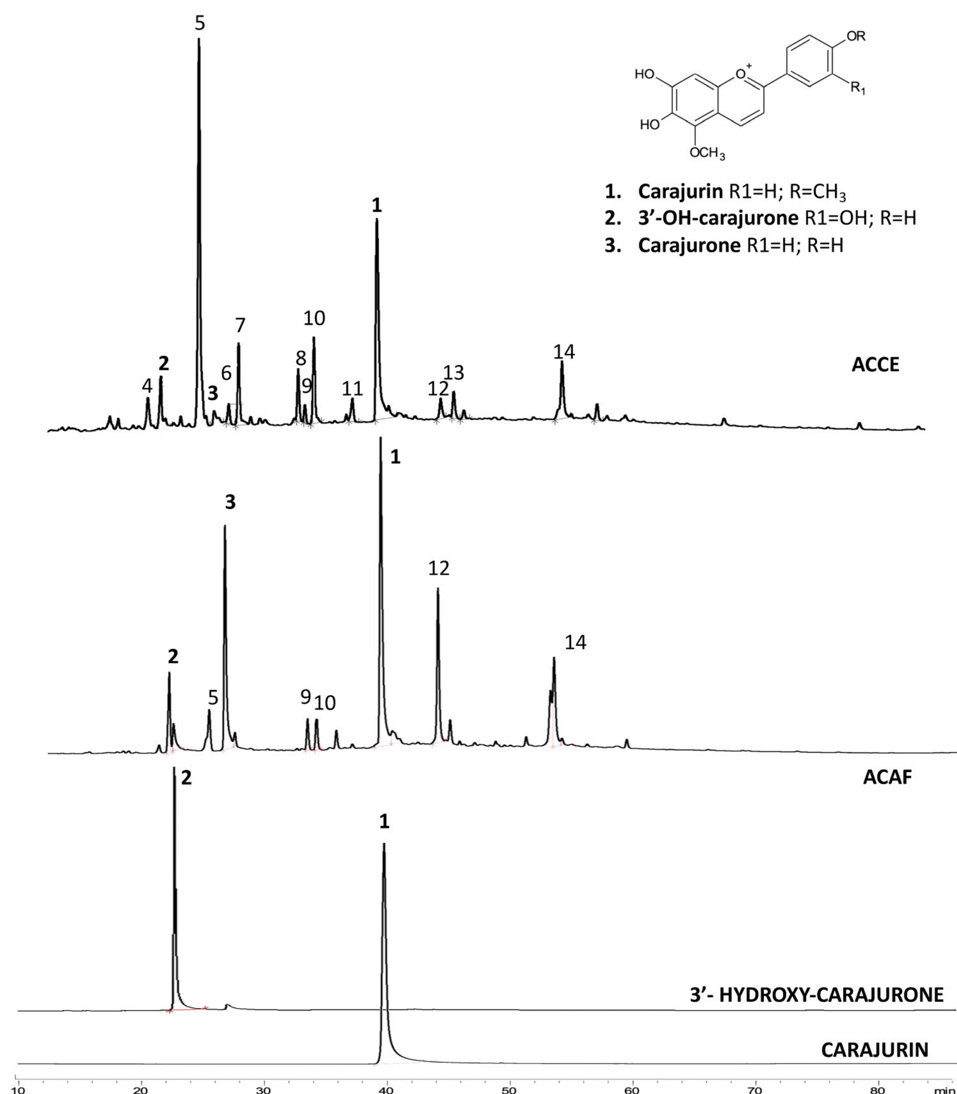

Fig. 1. HPLC-DAD-UV chromatograms of *Arrabidaee chica* crude extract (ACCE), anthocyanidins-rich fraction (ACAF) and isolated compounds carajurin (1, Rt= 38.96 min) and 3′-hydroxy-carajurone (2, Rt= 23.54 min). Note: 3′-OH-carajurone = 3′-hydroxy-carajurone. Rt = retention time.

2.15. Statistical analyses

The statistical analyses were conducted using the statistical software GraphPad Prism® version 7 (GraphPad Software Inc., San Diego, CA, USA). The numerical results were expressed as mean ± standard deviation and differences were considered significant when $p < 0.05$ by one-way analysis of variance (ANOVA) or Mann-Whitney test.

3. Results

3.1. Phytochemical analysis

The bioguided fractionation of *A. chica* crude extract (ACCE) was carried out in this study, leading to an anthocyanidin-rich fraction (ACAF) and the isolation of three known anthocyanidins: carajurin **(1)**, 3′-hydroxy-carajurone **(2),** and carajurone **(3).**

HPLC-DAD-UV analyses were performed to determine the ACCE and ACAF profile. The isolated anthocyanidins carajurin **(1)**, 3′-hydroxy-carajurone **(2),** and carajurone **(3)** were found in both ACAF and ACCE (Fig. 1). The UV data and retention times (Rt) were important to tentatively identify some other compounds (Table 1).

ACCE chromatogram (Fig. 1) showed 14 peaks, five of them presented UV λ_{max} in the range of 474–485 nm, characteristic of anthocyanidin compounds (peaks 1, 2, 3, 12, and 14). The elution order and the retention times of these compounds followed their structural and polarity characteristics, as had been previously discussed [23]. All five anthocyanidins identified at ACCE were also present at ACAF. Three of them have already been described in the literature: 3´-hydroxy-carajurone **(2)**, the first anthocyanidin to be eluted (Rt = 23.54 min) due to its more polar structure, features four hydroxyl groups; carajurone **(3)** (Rt = 27.35 min), which contains three hydroxyl groups; and carajurin **(1)** (Rt = 38.96 min), the least polar of the four known anthocyanidins from *A. chica* (two hydroxyl and two methoxyl groups) [29,30]. The other two anthocyanidins have already been reported by Moragas-Tellis et al. [23] but although they have a proposed structure, they have not yet been confirmed and are still here designated as anthocyanidins A2 (**12**, Rt= 43.51 min) and A3 (**14**, Rt= 52.17 min), in accordance with previous studies [23].

Among the nine non-anthocyanidin compounds found in the ACCE only one — in minor concentration (peak 6) — did not have in its UV spectrum the broad bands of absorption with maximum peaks in the range of 338 and 348 nm (band I) and 256 and 283 nm (band II), which are characteristic of flavonoids [31].

Table 1
Comparative composition in area percentage (area %) of anthocyanidins and other constituents in *Arrabidaea chica* crude extract (ACCE) and the anthocyanidins-rich fraction (ACAF).

Peak	HPLC-DAD-UV		Area %		Tentative Identification
	Rt (min)	UV data (nm)	ACCE	ACAF	
4	22.63	344, 281	3.44	–	flavonoid
2	**23.54**	**484, 295, 275, 243**	**4.48**	**6.73**	**3′-hydroxy-carajurone**
5	26.27	338, 283	33.67	3.15	flavonoid
3	**27.35**	**475, 293, 246**	**1.89**	**21.92**	**carajurone**
6	28.39	262, 234	1.46	–	unknown
7	29.10	347, 292	6.64	–	luteolin
8	33.35	337, 267	3.71	–	apigenin
9	33.82	340, 281	1.21	2.58	flavonoid
10	34.47	345, 274	6.70	3.25	flavonoid
11	37.22	334, 279	1.99	–	flavonoid
1	**38.96**	**475, 294, 245**	**24.19**	**36.80**	**carajurin**
12	43.51	485, 290, 240	1.79	14.86	Anthocyanidin A2
13	44.44	330, 268	2.28	–	flavonoid
14	52.17	480, 292, 240	6.51	10.63	Anthocyanidin A3

Rt = retention time.

The fractionation steps applied to the ACCE were effective in increasing the content of anthocyanidins (Tables 1 and 2). Luteolin (**7**) and apigenin (**8**) could be identified because they have already been isolated and identified by our group and we could compare their Rt and UV spectral data. Both were present only in the ACCE. According to our analyses, the composition of ACCE and the ACAF showed a flavonoid **(5)** and the anthocyanidin carajurin (**1**), respectively, as the compounds found in major amount.

3.2. Characterization and quantitation of anthocyanidins (mg/g of dry weight) expressed as carajurin content in ACCE and ACAF

As the anthocyanidins of *A. chica*, especially carajurin, had already been pointed out by our group as probably responsible for the leishmanicidal activity of the extracts of *A. chica*, we evaluated the quantitative variations on their content between ACCE and ACAF. The results of the quantitative determination of the 5 anthocyanidins identified (mg/g of dry extract calculated as carajurin content) are presented in Table 2.

Quantitative comparative analyses among anthocyanidins of the ACCE and ACAF showed that all anthocyanidins present in the extract are found in the rich fraction in higher quantities. Thus, the successive steps of fractionation were effective to produce a fraction rich in anthocyanidins.

3.3. Anti-Leishmania activity and cytotoxicity

The effect of the ACCE, the ACAF, and isolated compounds on the promastigote forms of L. *amazonensis* was monitored for 72 h (Table 3). It was possible to observe that the fractionation contributed to enhance the antipromastigote activity, having the ACAF a 20-fold higher level of activity than the ACCE. The evaluation of the antipromastigote activity of the isolated substances showed that 3′-hydroxy-carajurone and carajurone, although being more active than ACCE, were not as effective as the ACAF and the isolated carajurin, which was the most active of all. Additionally, 3′-hydroxy-carajurone exhibited the highest cytotoxicity, being followed by isolated carajurin and carajurone. However, considering the ratio between highest promastigote inhibition and lowest cytotoxicity, ACAF yielded the best results.

Carajurin, which had the highest level of activity against promastigote forms, and the ACAF, which has carajurin as its main component, were selected to further examination of their effects against intracellular amastigote forms. In this evaluation, the rich fraction presented a lower IC_{50} compared to carajurin (Table 4). However, carajurin showed less cytotoxicity, making it possible to obtain greater selectivity (IS 34.8). Such result is similar when compared with the drug used as reference, amphotericin B (SI 32.9). Amphotericin B showed leishmanicidal activity and cytotoxicity as expected. Carajurin was 26.4-fold less cytotoxic than amphotericin B. The SI showed that carajurin was 34.8 times more selective for *L. amazonensis* intracellular amastigote than for BALB/c peritoneal macrophage.

Table 2
Quantification of anthocyanidins content (mg/g of dry extract) calculated as carajurin in the crude extract of *Arrabidaea chica* Verlot (ACCE) and the anthocyanidin rich fraction (ACAF).

Anthocyanidin	Rt (min)	Anthocyanidin Content (mg/g of dry weight calculated as carajurin)	
		ACAF	ACCE
3′-hydroxy-carajurone	23.5	4.78 ± 0.028	1.97 ± 0.005
Carajurone	27.5	18.84 ± 0.193	3.54 ± 0.039
Carajurin	39.0	24.96 ± 0.355	17.26 ± 0.011
A2	43.5	7.30 ± 0.029	2.10 ± 0.006
A3	52.1	7.44 ± 0.047	3.89 ± 0.022

Note: Values are expressed as the mean ± SD ($n = 3$). A2 and A3 are unidentified anthocyanidins. Rt = retention time.

Table 3
Activity against promastigote forms of *Leishmania amazonensis* and cytotoxicity in peritoneal macrophages of BALB/c after treatment with compounds of *Arrabidaea chica* for 72 h.

Compounds	Promastigote IC_{50} (μg/mL)	Peritoneal macrophage CC_{50} (μg/mL)
ACCE	101.5 ± 0.06	39.39 ± 1.158
ACAF	4.976 ± 1.09	44.90 ± 1.14
Carajurin (1)	3.662 ± 1.16	16.48 ± 1.10
3′-Hydroxy-carajurone (2)	22.70 ± 1.20	9.640 ± 1.2
Carajurone (3)	28.28 ± 0.07	66.74 ± 0.10
Amphotericin B	0.0345 ± 1.14	9.984 ± 1.12

ACCE: *A. chica* crude extract. ACAF: *A. chica* anthocyanidins-rich fraction. IC_{50}: half-maximal inhibitory concentration. CC_{50}: half-maximal cytotoxic concentration. Data are presented as the mean ± SD of three independent experiments performed in triplicate.

Table 4
Leishmanicidal activity against intracellular amastigote forms of *Leishmania amazonensis*, cytotoxicity in BALB/c peritoneal macrophages, and selectivity index after 24 h of treatment with *Arrabidaea chica* constituents and amphotericin B.

Compounds	Intracellular amastigote IC_{50} (μg/mL)	Peritoneal macrophage CC_{50} (μg/mL)	SI
ACAF	5.925 ± 1.29	60.60 ± 1.15	10.2
Carajurin	7.065 ± 1.19	264.1 ± 0.32	34.8
Amphotericin B	0.302 ± 1.23	9.984 ± 1.12	32.9

IC_{50}: half-maximal inhibitory concentration; CC_{50}: half-maximal cytotoxic concentration; SI: selectivity index; ACAF: *A. chica* anthocyanidins-rich fraction. Data are presented as the mean ± SD of three independent experiments performed at least in triplicate.

The analysis of the leishmanicidal activity showed that, in infected cells treated with ACAF, a statistically significant reduction was observed only at the highest concentration evaluated (2.5 μg/mL), according to the following infection parameters: intracellular amastigote number ($p = 0.0202$, Fig. 2(a)), percentage of infected cells ($p = 0.0286$, Fig. 2(b)), and the mean of amastigotes per infected cell ($p = 0.0285$, Fig. 2(c)). The concentrations used were selected from the intracellular amastigote assay, therefore they did not show cytotoxicity against peritoneal macrophages. At 12.5 and 25 μg/mL, carajurin exhibited a statistically significant reduction in the intracellular amastigote number ($p = 0.0169$ and $p = 0.0011$, respectively) (Fig. 2(d)), percentage of infected cells ($p = 0.0292$ and $p = 0.0029$, respectively) (Fig. 2(e)) and the mean of amastigotes per infected cell ($p = 0.0476$ and $p = 0.0029$, respectively, Fig. 2(f)).

3.4. Nitrite quantification in L. amazonensis-infected peritoneal macrophages treated with ACAF and carajurin

We analyzed whether the treatment with ACAF and carajurin increased the production of nitric oxide (NO) by measuring the nitrite levels according to the Griess method. We measured nitrite levels in the supernatant of BALB/c peritoneal macrophages and they were higher in cells treated with ACAF (5.02 ± 0.68 μM $NaNO_2$) and carajurin (5.76 ± 0.57 μM $NaNO_2$, $p = 0.0476$), with an enhancement of 66.22% and 90.72%, respectively, when compared to untreated-unstimulated cells (3.02 ± 0.74 μM $NaNO_2$). However, this difference was statistically significant only for carajurin. The same high nitrite levels pattern was observed in *L. amazonensis* infected macrophages treated with ACAF (5.52 ± 1.93 μM $NaNO_2$) and carajurin (7.10 ± 1.08 μM $NaNO_2$, $p = 0.0286$), with an increase of 35.62% and 74.44%, respectively, when compared with stimulated-untreated cells (4.07 ± 1.39 μM $NaNO_2$) (Fig. 3).

3.5. Molecular docking

In order to discuss a possible pathway that would lead to an increase in NO production, without disregarding other possibilities due to the complexity of this system, studies of enzyme regulation were developed through molecular docking. Data are presented in Table 5. Of the compounds tested, carajurin had the best score, indicating greater ability to activate the nitric oxide synthase enzyme (NOS). Redocking is reported in Fig. 4. Interactions in the binding mode of carajurin at the activation site of NOS are presented in Fig. 5.

4. Discussion

The crude extract obtained from *A. chica* Verlot (ACCE) is rich in flavonoids [17] including anthocyanidins [29,30,32,33], the chemical markers of the species, as well as flavones [19,34] and flavonols [19]. Previous results of our research network indicated that the leishmanicidal activity of *A. chica* is associated with their anthocyanidin profile, especially the content of carajurin [23]. The extract was therefore fractionated to provide an anthocyanidins-rich fraction, containing the five anthocyanidins with carajurin as the major component. The successive fractionation steps also led to the isolation of carajurin **(1)**, 3′-hydroxy-carajurone **(2)**, and carajurone **(3)**, which allowed the evaluation of the leishmanicidal activity of these isolated anthocyanidins. Some flavones, although it was not isolated in this study, could be identified from the comparison with their authentic samples or isolated compounds, as well as two other anthocyanidins (A2 and A3) for which structures have been presented but remain to be confirmed [23].

The antiparasitic activity of the hydro-alcoholic extract of *A. chica* has been previously reported against *Trypanosoma cruzi* [19,20] and *Leishmania* [13,22]. These previous studies have also shown that the bioguided fractionation process of extracts can promote a progressive increase in activity against *T. cruzi* and *Leishmania*, demonstrating the potential of this plant as a source of biologically active compounds. The evaluation of the effect of the extract, anthocyanidin-rich fraction, and isolated compounds from *A. chica* against the promastigote forms of *L. amazonensis* monitored for 72 h showed that the fractionation also contributed to the improvement of the antipromastigote activity. The increase in leishmanicidal activity of the ACAF may be related to the increase in the concentration of these compounds relative to ACCE or to a possible increase in the synergistic effect between them [35].

Anthocyanidins are active compounds with remarkable biological activities [36]. 3-Deoxyanthocyanidin named carajurin (6,7-dihydroxy-5,4′-dimethoxy-flavylium) [30] is the main constituent of *A. chica* [37]. Thus, in an attempt to identify the component responsible for the antipromastigote activity of the fractions, biological tests were performed with the isolated anthocyanidins. Purified 3′-hydroxy-carajurone and carajurone showed less promising antipromastigote activity than the fraction, but carajurin exhibited an inhibitory capacity greater than 6 times among the tested anthocyanidins. The difference of leishmanicidal activity observed is possibly related to the different patterns of hydroxylation and methoxylation at the flavylium cation (phenyl-2-benzopyrylium) of these substances [38,39].

It has been shown that flavonoids in general are compounds possessing various biological activities. Those characterized by the presence of hydroxyl groups have several biological activities on tumor cells, such as apigenin (4′,5,7-trihydroxyflavone) that induce apoptosis in human hepatoma cell lines, notably Hep G2 [40]. Besides, among the flavonoids active against different species of *Leishmania*, those containing methoxyl groups in one of the rings are referred to as very promising molecules [38,39]. Thus, the antipromastigote results suggest that the presence of a methoxyl group in ring B favors activity expressed by carajurin, since both carajurone and 3′-hydroxy-carajurone, which has one and two hydroxyl groups, respectively, and no methoxyl in ring B, showed lower leishmanicidal activity. 3′-hydroxy-carajurone was the most cytotoxic among the evaluated compounds, suggesting that the presence of the

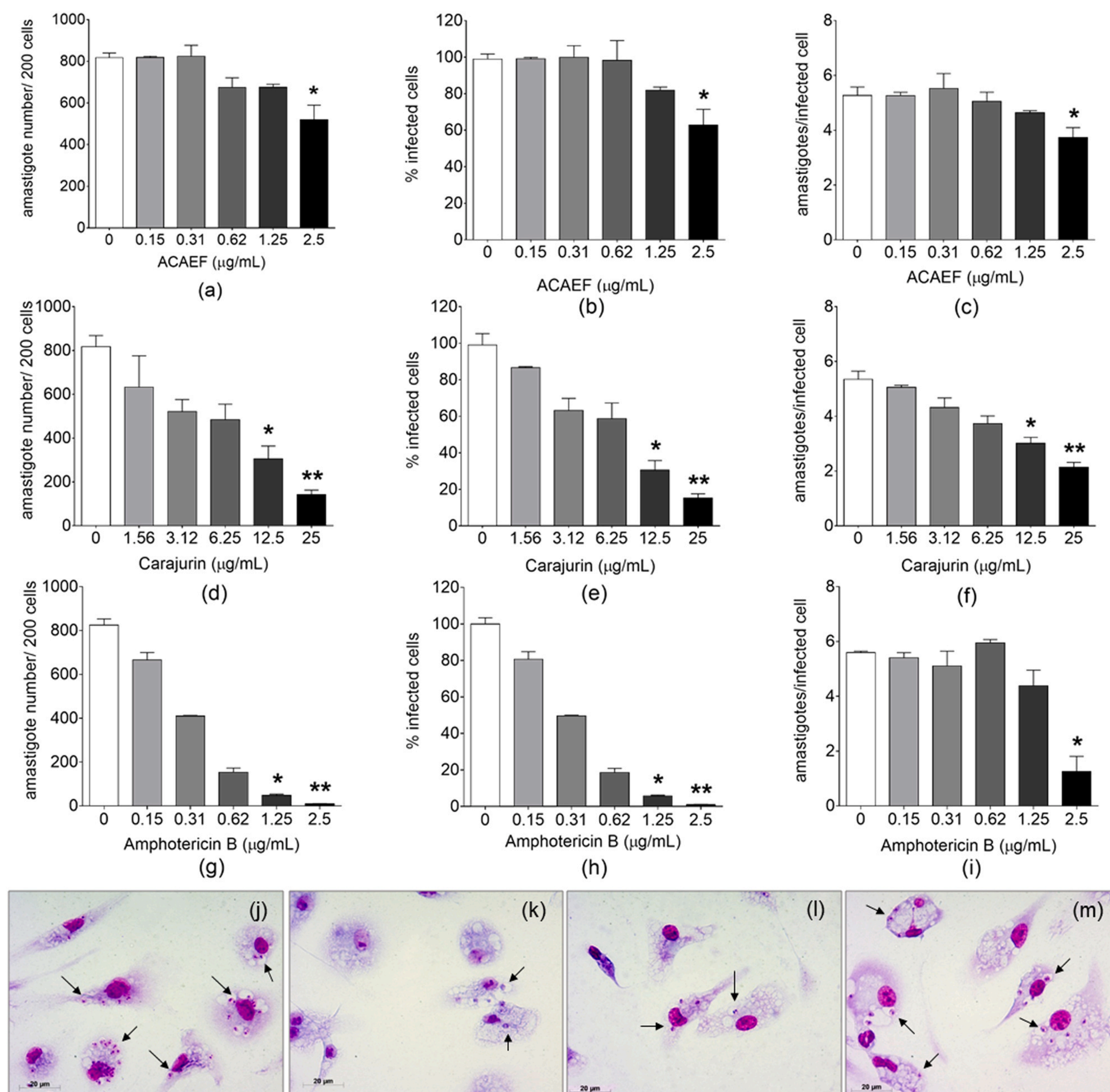

Fig. 2. Infection parameters of BALB/c peritoneal macrophages infected with *Leishmania amazonensis* and treated for 24 h with: anthocyanidins rich fraction of *Arrabidaea chica* (ACAF) (a-c), carajurin (d-f) or amphotericin B (g-i). (j) Untreated infected macrophages; (k) infected macrophages treated with amphotericin B (0.31 μg/mL); (l) infected macrophages treated with ACAF (2.5 μg/mL); (m) infected macrophages treated with carajurin (25 μg/mL). The treatment decreased the number of intracellular amastigotes (arrows). The data represent the mean ± standard deviation. * $p < 0.05$ and ** $p < 0.001$, when compared to the untreated group by the Mann-Whitney test.

hydroxyl group in 3′ increases the harmful effect on the macrophage. This observation is confirmed by the low cytotoxicity presented by carajurone (without hydroxyl group in 3′), but in this case the absence of the methoxy group also leads to lower leishmanicidal activity.

In the activity against promastigote forms, performed at different times of exposure, carajurin showed a time-dependent effect. However, treatment time has also contributed to increased cytotoxicity. Some reports demonstrated that anthocyanins in time-dependent studies markedly induces strong growth inhibitory effects against human hepatoma HepG2 [41], and causes apoptosis in human promyelocytic leukemia cells (HL-60) [42]. Thus, when assessing the selectivity index, we noticed that carajurin has better selectivity at 24 h of treatment.

For *Leishmania*, the intracellular amastigote form of the parasite provides the ideal conditions for carrying out an *in vitro* drug screening procedure, since it requires conditions that mimic the environment found by the target cell [43]. Therefore, carajurin and ACAF were selected to evaluate the activity against intracellular amastigote forms, for presenting the best activity against promastigote forms of *L. amazonensis* . Moreover, even with the indication of possible synergistic action of the compounds presented in the fraction, it was important to evaluate the activity of the isolated majority compound in an attempt to characterize the possible biological marker of the species *A. chica*. However, there was no significant difference between carajurin and ACAF against intracellular amastigote forms, just a difference in the analysis of cytotoxicity data, resulting in greater selectivity of carajurin. Such results are similar when compared with the drug used as a reference. Furthermore, the infection parameters showed a statistically significant reduction for the treatment with ACAF and carajurin.

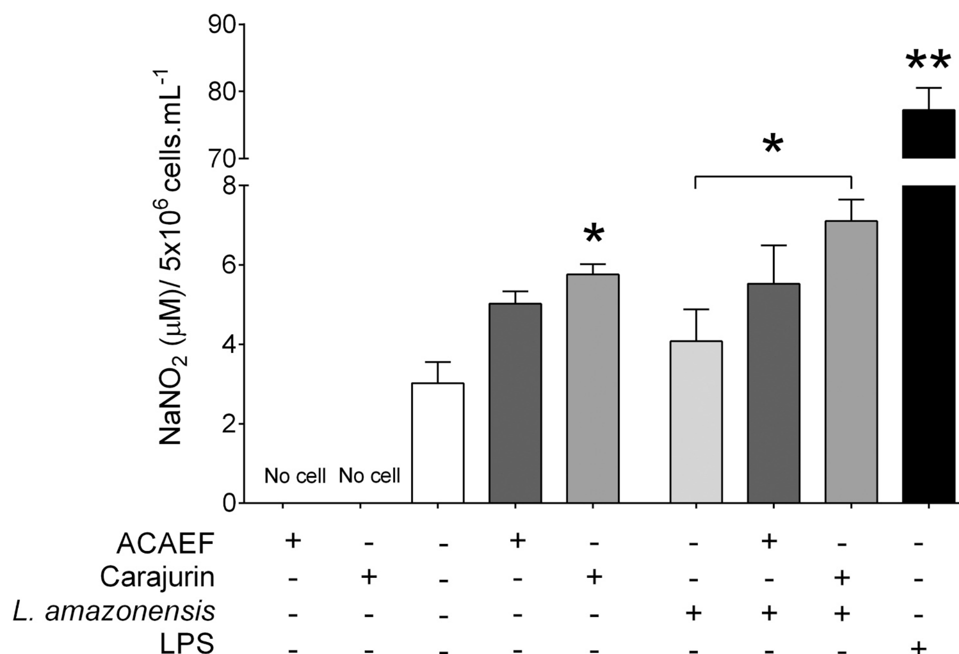

Fig. 3. Nitrite quantification in the supernatant of BALB/c peritoneal macrophages treated with anthocyanidins rich fraction of *Arrabidaea chica* (ACAF) at 2.5 μg/mL or carajurin at 25 μg/mL, stimulated or not with *Leishmania amazonensis*. * $p < 0.05$ when compared with the untreated group by Mann-Whitney test.

Table 5

Scores of the predicted binding modes of anthocyanidins from *Arrabidaea chica* after docking in the murine inosoxy dimer with isothiourea bound in the active site (PDB ID 1DF1).

Compounds	ChemPLP Score
Carajurin	53.800
3′-Hydroxy-carajurone	52.440
Carajurone	50.551
2-amino-6-(1,2-dihydroxypropyl)octahydropteridin-4(1 H)-one[a]	54.126

[a] cocrystallized ligand.

Only a few species from the *Arrabidaea* genus have been investigated for their antiprotozoal activity. Dimeric flavonoids purified from *Arrabidaea brachypoda* presented anti-leishmanial activity [44]. By quantifying the *in vitro* infected macrophages, it was evidenced that the compound brachydin B was the most active against intracellular amastigotes of *L. amazonensis*, without exhibiting host cell toxicity. Brachydin B was possibly more active due to the presence of the methoxyl group, which is necessary to improve membrane penetration [44]. Therefore, this leads us to infer that the presence of two methoxyl groups in the structure of carajurin may possibly be responsible for its higher activity against promastigote forms of *L. amazonensis*.

Some studies have shown that *A. chica* extracts and fractions possess antileishmanial activity against different *Leishmania* species [13,22]. Nevertheless, in the light of our knowledge, our study is the first to show an evaluation of the bioguided antileishmanial activity of ACCE, ACAF, and isolated compounds (carajurin, 3′-hydroxy-carajurone, and carajurone) against forms of *L. amazonensis*. Our findings confirm the results in a previous study [23], pointing out that the variation of the anthocyanidin profile in the extracts of four morphotypes of *A. chica*, especially the content of carajurin, was responsible for the variation of its leishmanicidal activity. In that study, it was also suggested that the presence of anthocyanidin carajurin would enhance this activity. The results of the present study, especially of the intracellular amastigote assays, in addition to the results by Moragas-Tellis et al. [23], allow us to designate carajurin as a biological marker for the leishmanicidal activity of the species.

The differences observed in the leishmanicidal activity of the samples against *Leishmania* promastigotes and intracellular amastigotes may be due to dissimilar biochemical or metabolic characteristics of the two stages of the parasite [45]. Besides a direct effect on intracellular amastigotes, this finding may also be indicative of activation of leishmanicidal macrophage functions, especially inducing NO [46]. Therefore, we evaluated ACAF and carajurin for the production of nitrite, an indirect measure to quantify NO, observing induced NO production by both compounds.

Literature on *A. chica* related to NO and its effects on *Leishmania* confirms our results [22]. After treatment with a fraction hexane:ethyl acetate obtained from *A. chica* hexane extract, the nitrite contents detected in the supernatant of macrophages infected by *L. amazonensis* and *L. infantum* was higher than those found on infected and untreated cell cultures [22]. These results help to contribute to a better understanding of induction of death by macrophage treated with *A. chica* against the intracellular form of *Leishmania*.

The secretion of inflammatory mediators by macrophages, including NO, is important for the success in controlling *Leishmania* multiplication [47]. Thus, the leishmanicidal activity of ACAF and carajurin would be associated with the ability to induce activation of the microbicidal response in macrophages and promote NO production, both of which lead to amastigote death.

NO is produced from L-arginine by a reaction catalyzed by the enzymes constitutive nitric oxide synthase, which is dependent on the interaction with calmodulin and calcium ions, and is involved in cell signaling, and oxide inducible nitric synthase (iNOS), produced by macrophages and other cells activated by cytokines [48].

The NOS functions as a dimer, consisting of two identical monomers, which, in turn, can be divided functionally and structurally into two main domains: a C-terminal reductase domain, homologous to cytochrome P450 and containing binding sites for NADPH, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and an N-terminal oxidase domain, which abstracts one electron from the substrate L-arginine and has binding sites for heme iron, for the cofactor tetrahydrobiopterin (BH4) and for L-arginine [49]. The catalysis reaction of the constitutive NOS involves two oxidation stages: the hydroxylation of L-arginine to NG-hydroxy-L-arginine, followed by the oxidation of this intermediate using an electron from NADPH, forming

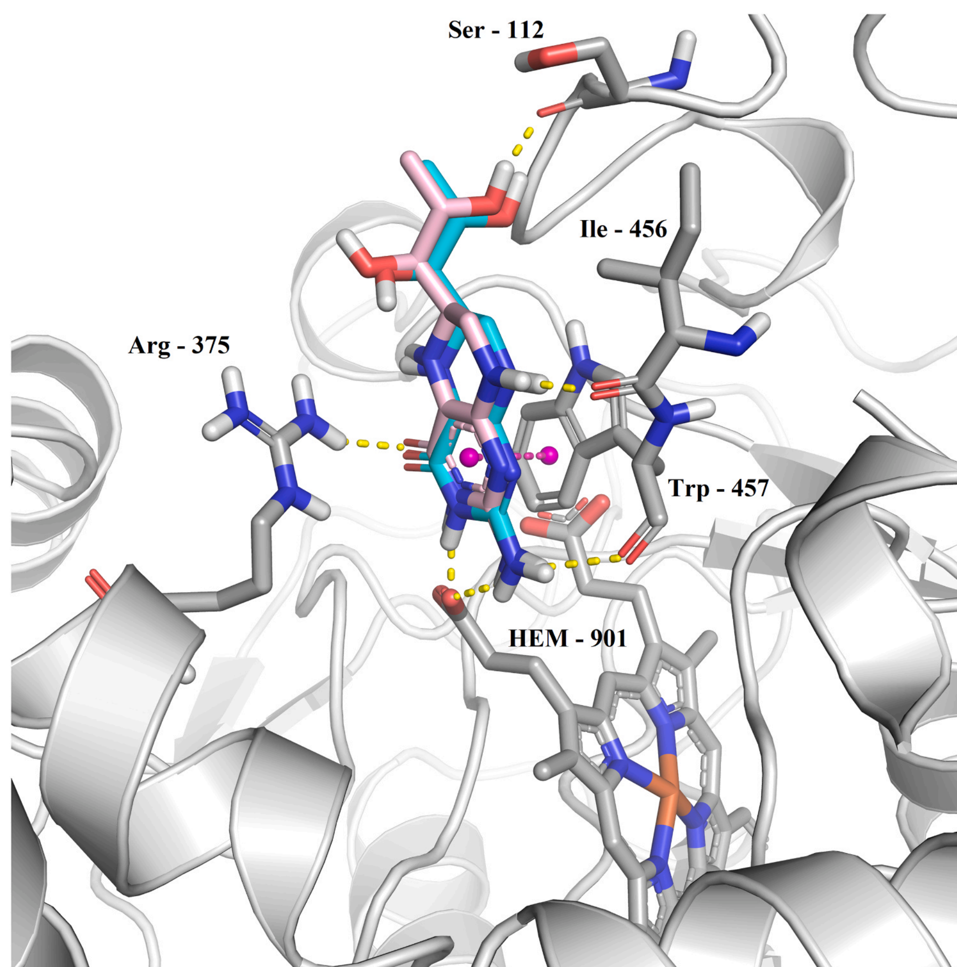

Fig. 4. Redocking image for 2-amino-6- (1,2-dihydroxypropyl) octahydropteridin-4 (1 H) -one (PDB ID 1DF1). The structure of the crystallographic image's binder has a cyan color and the structure resulting from the calculation is represented in pink.

L-citrulline and NO [50]. This reaction consumes 1.5 mol of NADPH and 2 mol of oxygen per mol of L-citrulline formed [51]. Cofactors such as heme iron, BH4 and L-arginine have been particularly studied, and their low bioavailability induces the phenomenon of dysfunctional endothelial NOS (eNOS) [52]. Heme iron is essential for the dimerization of the three isoforms [52], low concentrations or absence of L-arginine catalyze the reduction of oxygen into superoxide ($O_2^{\cdot-}$), and decreased levels of BH_4 lead to simultaneous production of NO and $O_2^{\cdot-}$, products that react with each other to form peroxynitrite ($ONOO^-$) [53].

Therefore, and considering that all NOS isoenzymes are hemodimeric, we investigated whether the increase in NO levels investigated by the Griess method, which corresponds to the reported leishmanicidal activity, may correspond to an activation mechanism of one of the isoforms of this enzyme. In this sense, molecular docking studies were directed to the analysis of possible interactions of carajurin with enzyme activation sites, especially in the important region in the dimerization process as reported in the literature.

A more accurate analysis of the interaction shows that carajurin occupies the binding site of 2-amino-6-(1,2-dihydroxypropyl) octahydropteridin-4(1 H)-one (co-crystallized ligand), for which the redocking studies showed an excellent alignment pattern.

In the predicted binding mode on the 1DF1 structure, carajurin interacts with the activation site of the NOS, especially through two non-covalent hydrogen bond-type interactions between the hydrogens of the hydroxyl groups of carujirin and the oxygens of the hydroxyl group and the carbonyl group, present in the serine residue (112). In addition, there is interaction with residue 375 and spatial proximity with the heme group (901), which is of great importance in the process of dimerization and activation of that enzyme. iNOS or isoform II is not constitutively expressed, this means, it is not normally present, being induced in macrophages and other cells by bacterial lipopolysaccharides and/or cytokines. This isoenzyme can also be called macNOS (macrophage NO synthase). Once induced, iNOS is capable of producing NO for a long time, and this characterizes its involvement in various pathological processes.

5. Conclusion and perspective

This is the first study on bioguided assay and characterization of a biological marker for antileishmanial activity of *A. chica*. The compounds were evaluated against *L. amazonensis*, having ACAF and carajurin the highest activity against the promastigote and intracellular amastigote forms, altering all parameters of *in vitro* infection. Both also exhibited a high selectivity index to parasites over cells. Carajurin was able to enhance nitrite levels in the macrophage stimulated or not with *L. amazonensis* , whose docking studies showed a possible interaction of carajurin at the activation site of NOS, the hypothesis that corroborates the *in vitro* results obtained. Underpinned by a set of intertwined pieces of evidence from our experimental results and literature reports, our findings substantiate our proposition that carajurin is a biological marker of the species *A. chica* for antileishmania activity. Nevertheless, further studies are needed to better elucidate the mechanism of action and determine this effect in the experimental murine model of leishmaniasis infection.

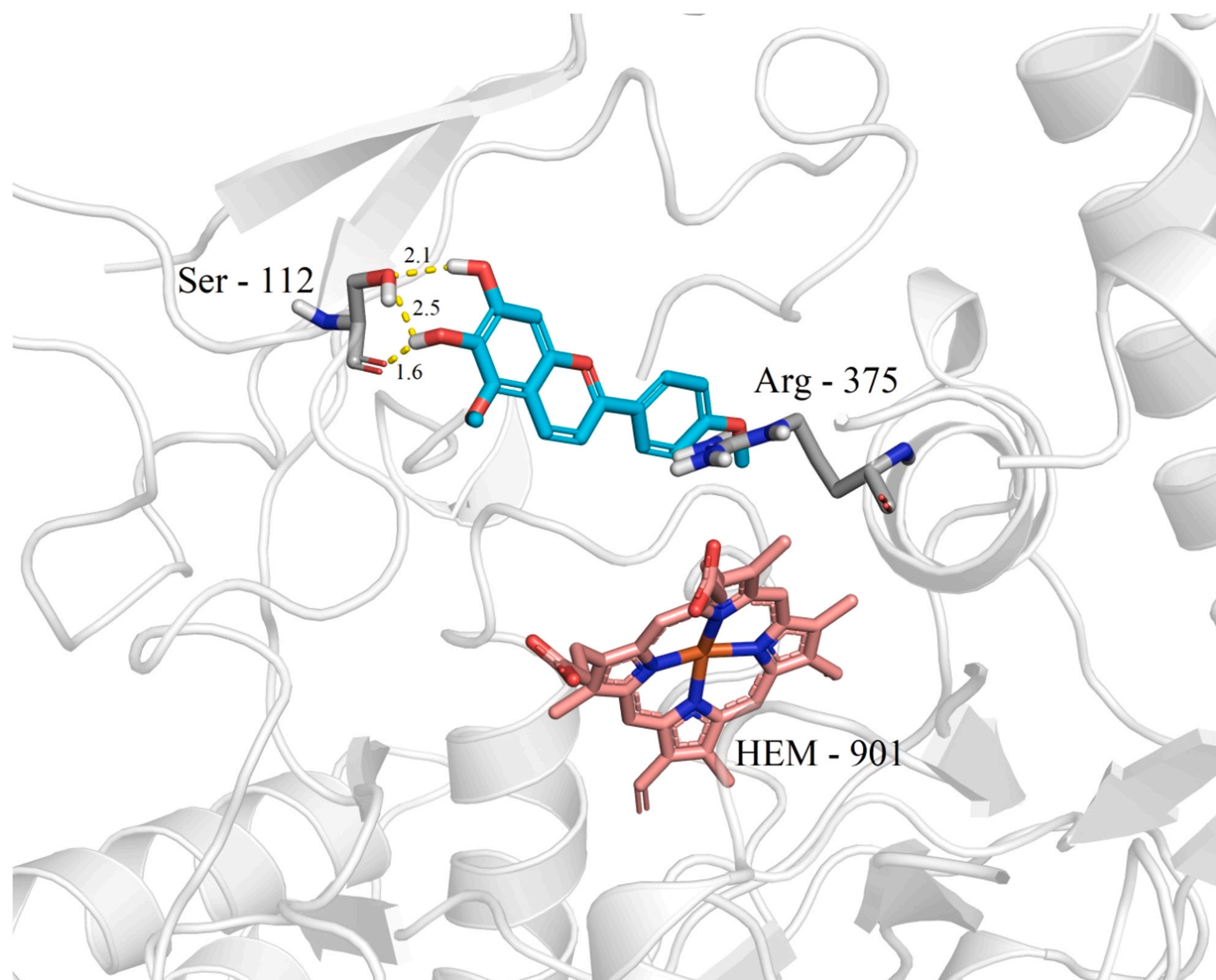

Fig. 5. Noncovalent interactions observed in the predicted binding mode of carajurin at the activation site of nitric oxide synthase. For the sake of clarity, only polar hydrogen atoms are shown.

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CRediT authorship contribution statement

João Victor Silva-Silva: Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Carla J. Moragas-Tellis**: Methodology, Formal analysis, Data curation, Writing – original draft. **Maria S. S. Chagas**: Methodology, Formal analysis. **Paulo Victor R. Souza**: Methodology, Visualization, Formal analysis. **Davyson L. Moreira**: Conceptualization, Data curation, Visualization, Formal analysis. **Celeste S. F. de Souza**: Methodology, Visualization. **Kerolain F. Teixeira**: Methodology, Visualization. **Arthur R. Cenci**: Methodology, Visualization. **Aldo S. de Oliveira**: Conceptualization, Data curation, Formal analysis, Writing – original draft, Visualization, Supervision, Writing – review & editing, Funding acquisition. **Fernando Almeida-Souza**: Conceptualization: Methodology, Formal analysis, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Funding acquisition. **Maria D. Behrens**: Conceptualization, Formal analysis, Data curation, Supervision, Funding acquisition. **Kátia S. Calabrese**: Conceptualization, Formal analysis, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Funding acquisition.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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3.3 Carajurin Induces Apoptosis in *Leishmania amazonensis* Promastigotes through Reactive Oxygen Species Production and Mitochondrial Dysfunction

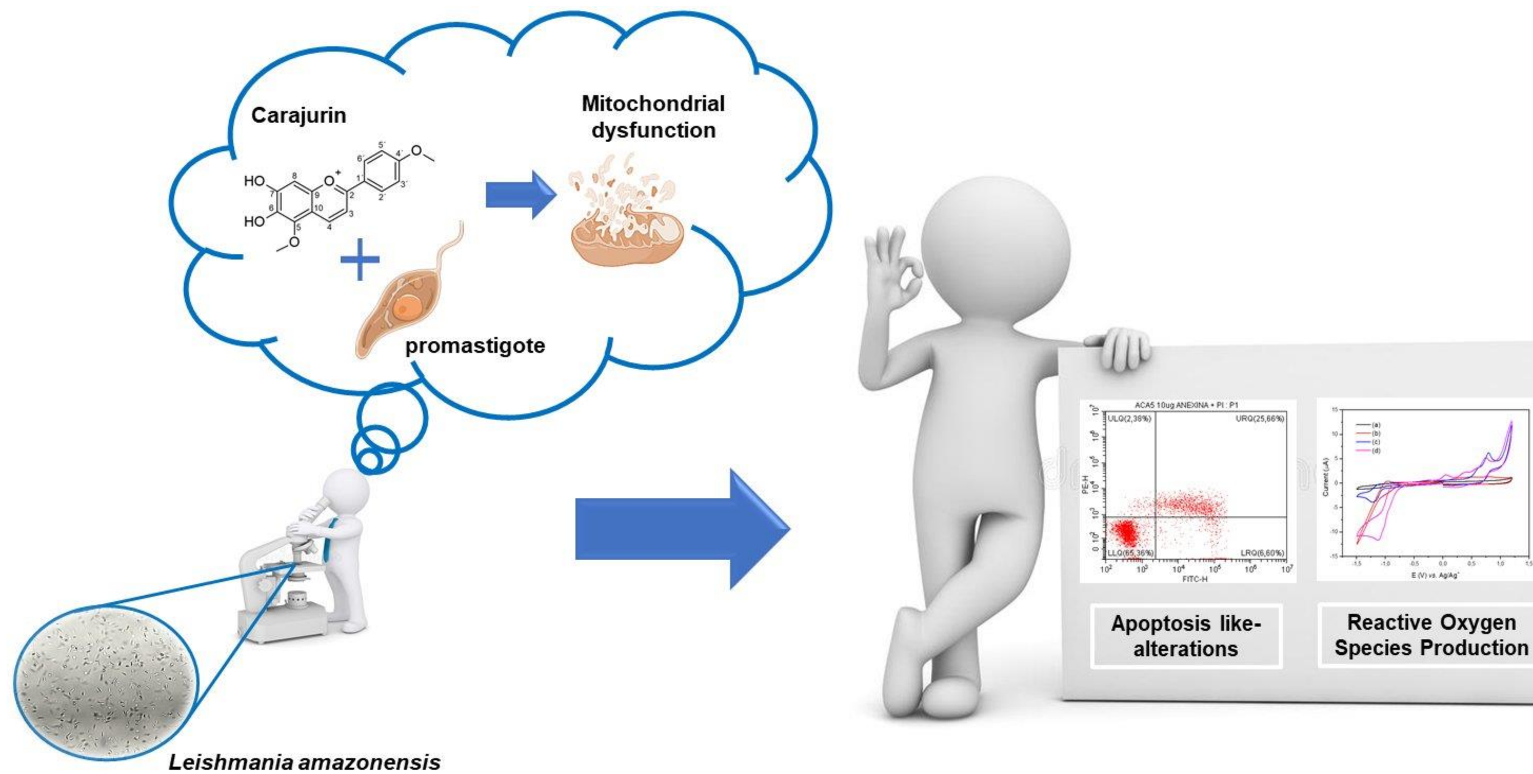

Figura 7 Resumo gráfico do mecanismo de ação da carajurina contra formas prosmatigota de *L. amazonensis.*

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Carajurin Induces Apoptosis in *Leishmania amazonensis* Promastigotes through Reactive Oxygen Species Production and Mitochondrial Dysfunction

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Abstract: Carajurin is the main constituent of *Arrabidaea chica* species with reported anti-*Leishmania* activity. However, its mechanism of action has not been described. This study investigated the mechanisms of action of carajurin against promastigote forms of *Leishmania amazonensis*. Carajurin was effective against promastigotes with IC_{50} of 7.96 ± 1.23 μg.mL^{-1} (26.4 μM), and the cytotoxic concentration for peritoneal macrophages was 258.2 ± 1.20 μg.mL^{-1} (856.9 μM) after 24 h of treatment. Ultrastructural evaluation highlighted pronounced swelling of the kinetoplast with loss of electron-density in *L. amazonensis* promastigotes induced by carajurin treatment. It was observed that carajurin leads to a decrease in the mitochondrial membrane potential ($p = 0.0286$), an increase in reactive oxygen species production ($p = 0.0286$), and cell death by late apoptosis ($p = 0.0095$) in parasites. Pretreatment with the antioxidant NAC prevented ROS production and significantly reduced carajurin-induced cell death. The electrochemical and density functional theory (DFT) data contributed to support the molecular mechanism of action of carajurin associated with the ROS generation, for which it is possible to observe a correlation between the LUMO energy and the electroactivity of carajurin in the presence of molecular oxygen. All these results suggest that carajurin targets the mitochondria in *L. amazonensis*. In addition, when assessed for its drug-likeness, carajurin follows Lipinski''s rule of five, and the Ghose, Veber, Egan, and Muegge criteria.

Keywords: carajurin; *Leishmania amazonensis*; mitochondria; cell death; apoptosis

1. Introduction

Leishmaniasis is one of the world's most neglected diseases, with more than 1 billion people living at risk of infection in around 92 countries or territories where the disease is considered endemic [1]. Leishmaniasis treatment is not totally successful and vaccine candidates in humans still need to be evaluated by further clinical trials [2]. The available chemotherapeutic options have serious limitations, such as high costs, limited efficacies, and high toxicities. In addition, prolonged parenteral administration hinders patient adherence to treatment, impacting the appearance of drug-resistant strains [3].

Acknowledging the need to overcome the limitations of anti-leishmanial chemotherapy, plant-derived natural products have shown promising results for new antiprotozoal therapies, due to their vast chemical diversity. These products may be useful as an alternative and safe approach against leishmaniasis [4,5].

Mitochondria play a central role in generation cellular energy production and the survival of any cell depends on the proper function of these organelles [6]. The fact that kinetoplastids have a single mitochondrion indicates that this organelle is a potential candidate for the development of drugs [7]. The mitochondrial ultrastructural changes of *Leishmania* are associated with apoptosis-like death by the potential impairment of the mitochondrial membrane and/or by reactive oxygen species (ROS) production [8].

Arrabidaea chica (Humb. & Bonpl.) B. Verlot, a plant popularly known as crajiru that is native to the Amazon rainforest [9], has been used in folk medicine for wound healing, treatment of inflammation, and antioxidant activities, possibly related to the presence of anthocyanidins [10]. Analyzing different morphotypes of *A. chica*, we observed that its hydro-alcoholic extract is rich in anthocyanidins, mainly carajurin [11]. We recently demonstrated, through the bioguided fractionation of *A. chica* extract, that carajurin favored its leishmanicidal activity [12], validating our earlier identification of carajurin as a pharmacological marker for the anti-leishmanial potential of *A. chica*. However, the mechanism of action of carajurin on *L. amazonensis* has not been previously studied. Thus, this work aimed to demonstrate the mechanisms involved with cell death induced by carajurin in promastigote forms of *L. amazonensis*.

2. Results

2.1. Phytochemical Analysis

The molecular formula $C_{17}H_{15}O_5$ was established by the positive mode quasi-molecular ion peaks at m/z 299.0905 for $[M + H]^+$ (calcd. 299.0924 for $C_{17}H_{15}O_5$) in combination with its NMR data as previously described [12]. Carajurin purity was previously determined by HPLC-DAD-UV as 98%, as well as by data from ESI-MS experiments and by NMR ^1H and ^{13}C data [11]. The compound was initially described by Chapman et al. [13] and characterized by Zorn et al. [14] and Devia et al. [15]. Unequivocal characterization of carajurin (Table 1; Figure 1) was confirmed by other structural experiments, such as distortionless enhancement by polarization transfer (DEPT) and NMR-2D, homonuclear correlated spectroscopy (COSY), heteronuclear multiple bond coherence (HMBC), and heteronuclear single quantum correlation (HSQC) (Supplementary Materials S1-S4). The ^1H NMR, together with HMBC spectra, confirmed the presence of two methoxyl groups [δH 4.10 (s) and 3.90 (s)] that showed correlation with the aromatic carbons at δ162.50 and d 135.02, respectively, which were assigned as carbons 4′and 5. Protons at δ7.89 and 7.01 ppm could be attributed to the aromatic protons of B rings H2′/H6′ and H3′/H5′, due to the correlations observed with C2′/C6′(δ127.68) and C3′/C5′(δ114.77) at the HSQC spectra. The position of protons 3 and 4 was confirmed by the correlations observed, respectively, with the H4 and H3 in the COSY spectrum, and by the observed correlations of the HMBC spectra for H3 long-distance interactions with C10, C1′, and C2, and for H4 with C5, C2, and C9.

Table 1. NMR 1D and 2D data for carajurin..

Position	^{1}H [a,b] 400 MHz (δ) in ppm ($CDCl_3$)	Cosy (H/h correlation)	^{13}C [b] 100 MHz (δ) in ppm ($CDCl_3$)	DEPT	HSQC (H/C Correlation)	Hmbc (H/C Correlation)
2	-		158.90	Q	-	-
3	6.98 (*d*, J = 19.5 Hz)	H4	102.62	CH	C3	C10; C1′; C2
4	7.99 (*d*, J = 19.5 Hz)	H3	133.76	CH	C4	C5; C2; C9
5	-	-	135.02	Q	-	-
6	-	-	139.93	Q	-	-
7	-	-	176.82	Q	-	-
8	6.53 (*s*)	-	98.61	CH	C8	C10; C6; C9
9	-	-	156.86	Q	-	-
10	-	-	118.16	Q	-	-
1′	-	-	123.43	Q	-	-
2′/6′	7.89 (*d*) J = 22.3 Hz	H3′e H5′	127.68	CH	C2′/C6′	C2′; C6′; C2, C4′
3′/5′	7.01 (*d*) J = 22.2 Hz	H2′e H6′	114.77	CH	C3′/C5′	C3′; C5′; C1′, C4′
4′	-	-	162.50	Q	-	-
OCH_3-5	4.10 (*s*)	-	60.42	CH3	OCH_3-5	C5
OCH_3-4`	3.90 (*s*)	-	55.58	CH3	OCH_3-4′	C4′

[a] Multiplicities and coupling constants in Hz are shown in parentheses. [b] Data previously presented in Silva-Silva et al. [12]

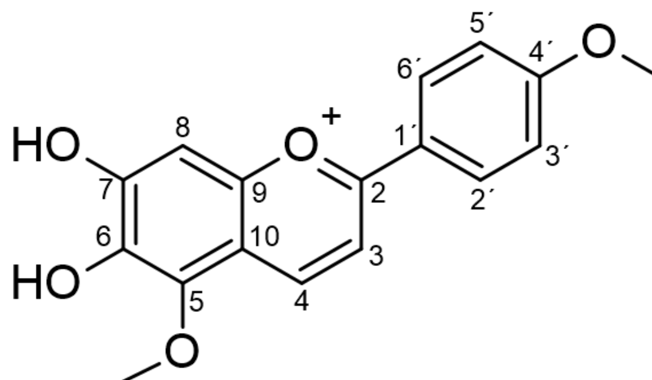

Figure 1. Chemical structure of carajurin.

2.2. Anti-leishmanial Activity and Cytotoxicity

The anti-leishmanial activity of carajurin was evaluated in promastigote forms of *L. amazonensis*. Viable promastigotes were counted in a Neubauer chamber according to Rottini et al. [16], with the percentage of growth inhibition calculated from the count of viable parasites in relation to the untreated control to determine the values of 50% of inhibitory concentration (IC_{50}). The results showed a significant concentration-dependent decrease ($p < 0.0001$) in parasite viability (Figure 2), with IC_{50} at 7.96 ± 1.23 μg.mL^{-1} (26.4 μM). In the evaluation of carajurin against peritoneal macrophage cells, the CC_{50} was approximately 33-fold higher when compared to the IC_{50} (Table 2), indicating that carajurin was more toxic to the parasites than to the cells. Amphotericin B was active against the promastigotes and peritoneal macrophages.

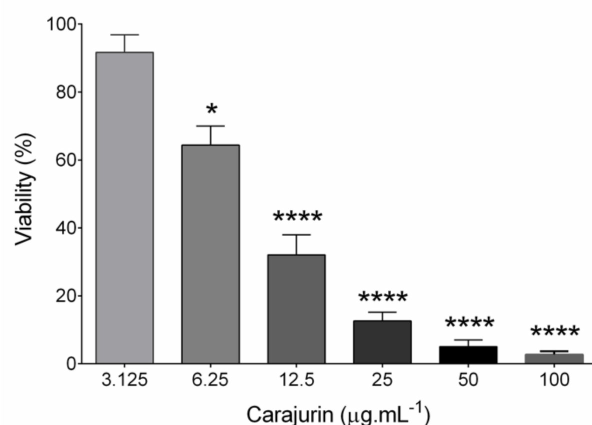

Figure 2. Effects of carajurin on growth of *Leishmania amazonensis* promastigote forms. Parasites in log-phase (2×10^6 mL^{-1}) were incubated in different concentrations of carajurin. Data represent the mean ± standard error of three independent experiments carried out in triplicate. (*) $p < 0.05$; (****) $p < 0.0001$, when compared to untreated parasites by Mann-Whitney test.

Table 2. Antileishmanial activity, cytotoxicity, and selectivity index of carajurin for 24 h of treatment.

Compounds	Peritoneal Macrophages	*L. amazonensis* Promastigotes	
	CC_{50} (µg mL^{-1})	IC_{50} (µg mL^{-1})	SI
Carajurin	258.2 ± 1.20 (856.9 µM)	7.96 ± 1.23 (26.42 µM)	32.4
Amphotericin B	8.740 ± 1.08 (9.458 µM)	0.0299 ± 1.18 (0.03236 µM)	292.3

Data represent mean ± SD. CC_{50}: cytotoxic concentration for 50% of cells; IC_{50}: inhibitory concentration for 50% of parasites. SI: selectivity index.

2.3. Ultrastructural Changes

Transmission electron microscopy analyses were performed to evaluate ultrastructural alterations caused on *L. amazonensis* promastigote forms treated with the IC_{50} of carajurin. Figure 3A shows the well-preserved cell morphology of nontreated parasites, with characteristic elongated fusiform shape, and all its organelles with typical morphology. Figure 3B–E shows numerous and large vesicles in cytoplasm, some of them with electron-dense content, electron-dense corpuscles, and pronounced swelling of the kinetoplast with loss of electron-density.

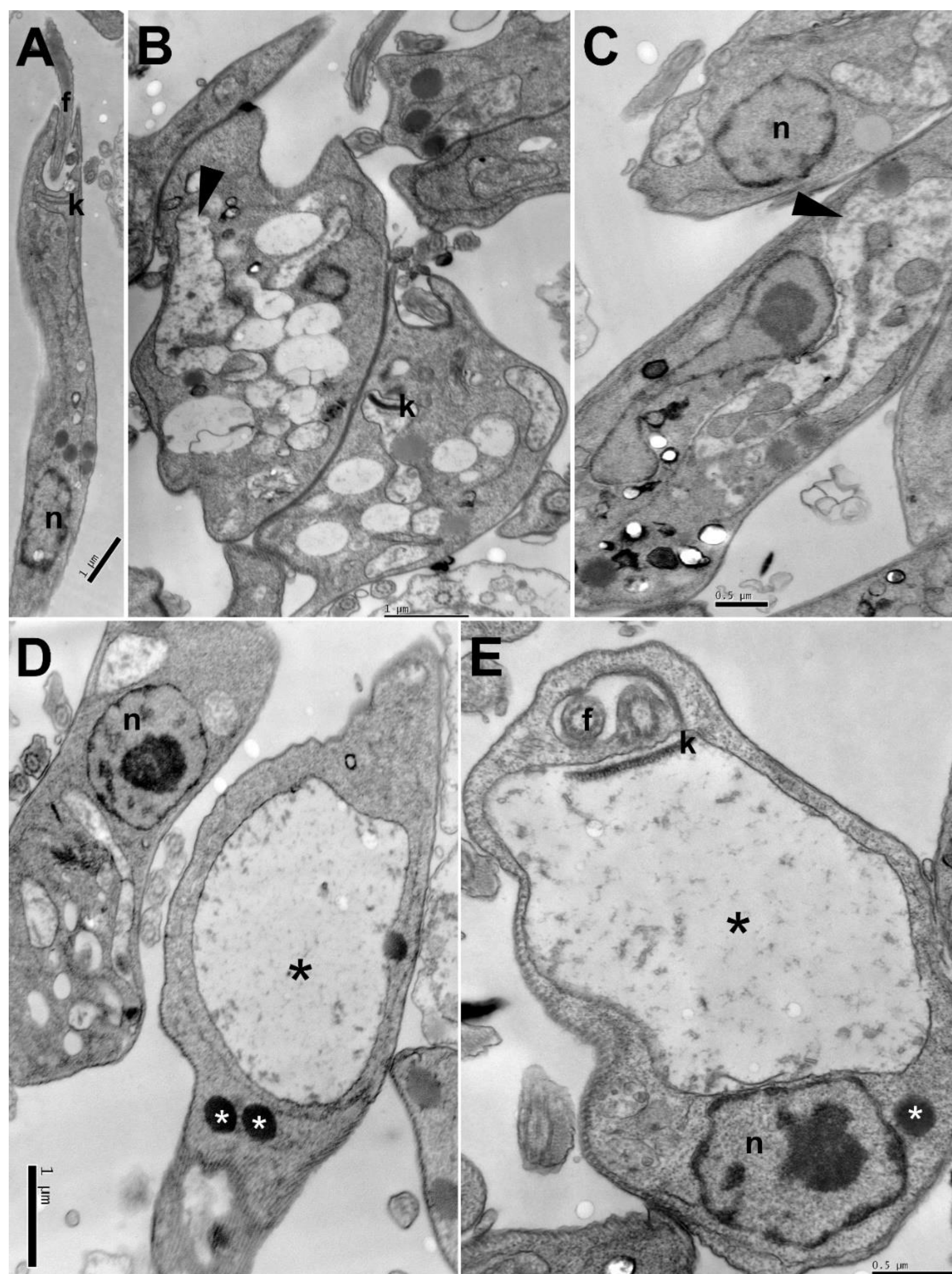

Figure 3. Ultrastructural changes in promastigote forms of *Leishmania amazonensis* treated with carajurin: (**A**) untreated parasites; (**B**–**E**) parasites treated for 24 h with carajurin (IC_{50}). Electron-dense corpuscles (white asterisks), vesicles with electron-dense content (arrowhead), kinetoplast swelling (black asterisks). N = nucleus, f = flagellum, k = kinetoplast.

2.4. Mitochondrial Membrane Potential ($\Delta\psi m$)

Mitochondrial membrane potential ($\Delta\psi m$) plays a key role in vital mitochondrial functions, as it is directly linked to ATP synthesis and, as such, its regulation is essential for cell viability. As mitochondria damage was observed in the ultrastructural analysis, flow cytometry analysis was performed to confirm carajurin-induced damage to the mitochondria. Flow cytometric analysis used tetramethylrhodamine ethyl ester (TMRE), a cell-permeant fluorescent dye that is readily sequestered by active mitochondria. Statistically significant changes in mitochondrial membrane potential were observed after treatment with carajurin ($p = 0.0286$). TMRE labeling after 24 h-treatment with carajurin was 20.98 ± 3.70%, while non-treated parasites' labeling was 89.22 ± 0.67% (Figure 4).

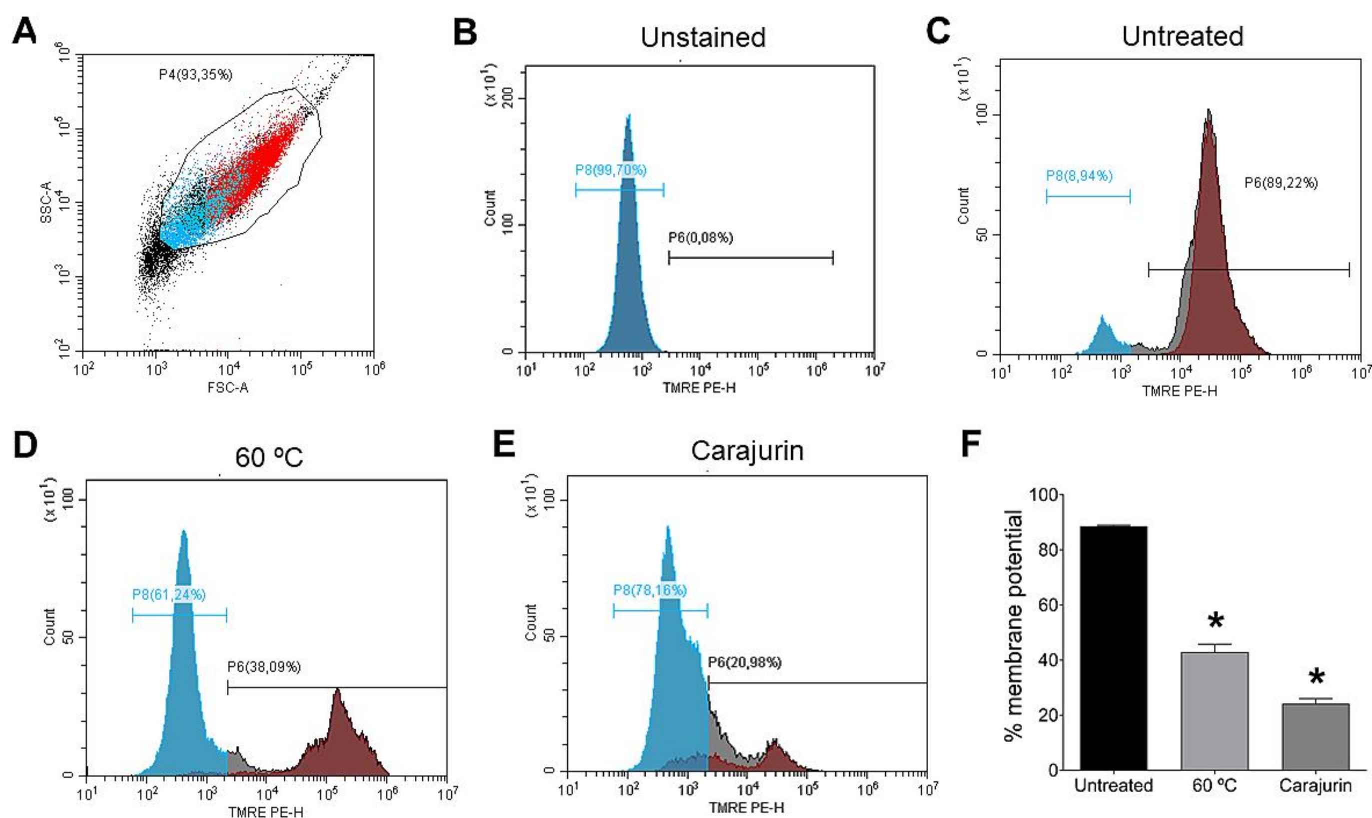

Figure 4. Flow cytometry of *Leishmania amazonensis* treated with IC_{50} of carajurin to assess the potential of the mitochondrial membrane ($\Delta\Psi m$). (**A**) Promastigotes captured in the gated region and representative histogram. (**B**) Unstained parasites. (**C**) Untreated parasites. (**D**) Promastigote forms of *L. amazonensis* killed by heat. (**E**) Histogram representative of promastigotes treated with carajurin. (**F**) Statistically significant differences were observed between the percentages of cells marked with TMRE in the untreated group and the groups treated with carajurin, at the IC_{50} concentration (26.4 μM). (*) $p < 0.05$, when compared with the untreated group by Mann-Whitney test.

2.5. Measurement of ROS

To investigate whether the leishmanicidal effect of carajurin is due to the production of ROS in *L. amazonensis* promastigote forms, ROS levels were measured using the cell-permeable dye H_2DCFDA. Carajurin induced ROS production in parasites, with ROS levels increased to 58.9 ± 1.65% ($p = 0.0286$), in comparison to untreated parasites (Figure 5). Hydrogen peroxide (H_2O_2) and miltefosine were used as positive controls and resulted in increased ROS levels to 74.1 ± 0.86% ($p = 0.0286$) and 65.1 ± 3.11% ($p = 0.0286$), respectively. Furthermore, we evaluated whether the pre-incubation of *L. amazonensis* promastigotes with NAC could prevent the inhibitory effect of carajurin, and it was observed that NAC protected promastigotes from carajurin anti-leishmanial activity, enhancing the percentage of viable parasites (Figure 5C) by the reduction of the levels of ROS (Figure 5D).

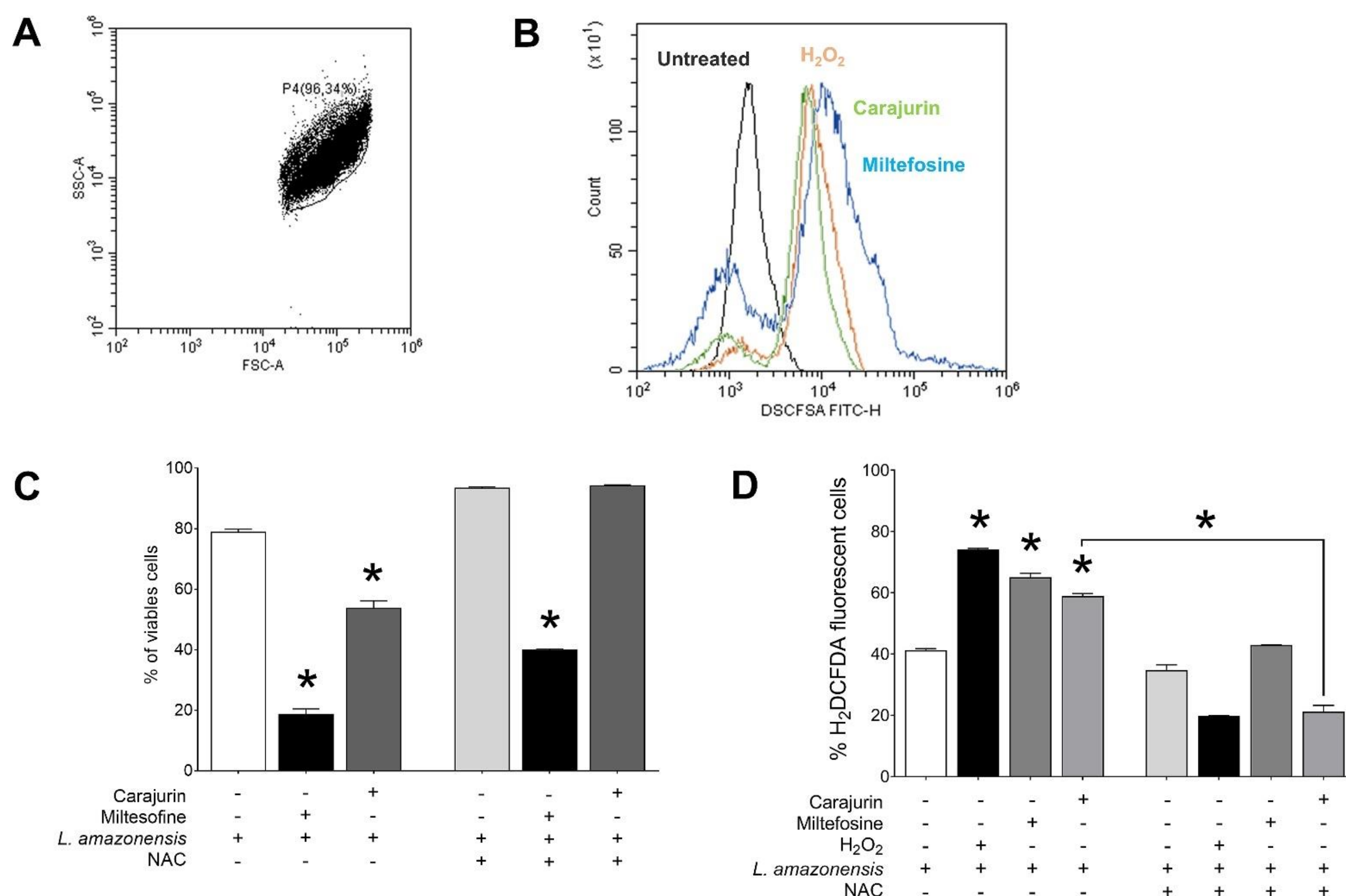

Figure 5. Evaluation of intracellular ROS levels in *Leishmania amazonensis* promastigotes and effect of N-Acetyl-L-cysteine on carajurin-induced cell death after incubation with carajurin for 24 h. (**A**) Promastigotes captured in the gated region and representative histogram. (**B**) The green line shows increased ROS production in parasites treated with carajurin, at the IC_{50} concentration (26.4 μM), when compared to control parasites (black line). The same was observed in the group treated with H_2O_2 (orange line) and miltefosine (blue line). (**C**,**D**) Promastigotes were cultivated in the presence of N-Acetyl-L-cysteine (NAC, 300 μM) and carajurin (IC_{50} concentration, 26.4 μM). Miltefosine (50μM) was used as a cell death control, and H_2O_2 (50 μM) as a natural inducer of ROS. Generation of cell death and ROS was measured using fluorescent dye propidium iodide (PI) and H_2DCFDA, respectively. (*) $p < 0.05$, when compared to untreated parasites by Mann-Whitney test.

2.6. Evaluation of Phosphatidylserine Exposure and Cell Membrane Integrity

To determine the mechanism of cell death triggered by carajurin, promastigote forms were evaluated using Annexin V-FITC and PI staining to distinguish the necrotic or late apoptotic cells from the early apoptotic ones. After treatment with carajurin for 24 h, promastigotes captured in the closed region and the representative histogram (Figure 6A), as well as unstained parasites (Figure 6B), were observed, and the number of viable parasites decreased from 94.7 ± 3.32% to 65.36 ± 0.56% (lower left quadrant; $p = 0.0238$, Figure 6C). The percentage of parasites staining positive for PI but negative for Annexin V (upper left quadrant, Figure 6C) increased to 2.38 ± 1.53% ($p = 0.0159$, Figure 6D), and the intensity of Annexin-V and PI fluorescence (upper right quadrant) increased up to 25.66 ± 1.54% ($p = 0.0095$, Figure 6E), compared to untreated parasites. Carajurin also induced early-stage apoptosis with the percentage increased to 6.60 ± 2.52% ($p = 0.0119$, Figure 6F) (lower right quadrant). These results suggest that carajurin induces late apoptosis in *L. amazonensis* promastigotes.

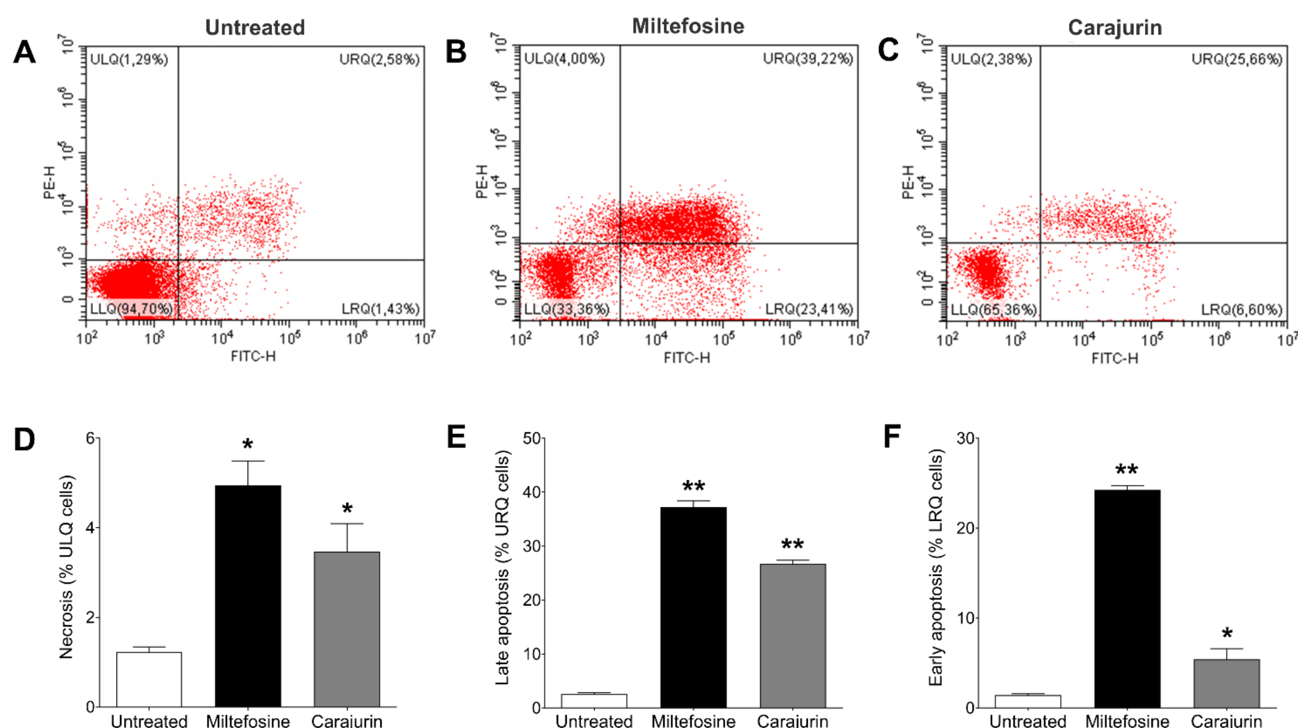

Figure 6. Changes in phosphatidylserine exposure and plasma membrane integrity in *Leishmania amazonensis* promatigotes treated with IC_{50} of carajurin for 24 h. Parasites were labeled with Annexin V-FITC and PI. (**A**) Untreated promastigotes used as control. (**B**) Parasites treated with miltefosine, an apoptosis-inducing drug, at a concentration of 50 µM. (**C**) Promastigotes treated with carajurin, at the IC_{50} concentration (26.4 µM). In (**D**,**E**,**F**), statistical differences between the percentage of necrotic, late apoptotic, and early apoptotic cells, respectively, are shown. In all, the results obtained in the groups treated with carajurin and miltefosine were statistically different when compared to untreated parasites. (*) $p < 0.05$; (**) $p < 0.01$, when compared to untreated parasites by Mann-Whitney test. ULQ: = upper left quadrant; URQ = upper right quadrant; LRQ = low right quadrant.

2.7. Electrochemical Tests/Cyclic Voltammetry

The potential was applied to the working electrode at a constant rate (50 mV s^{-1}), and for evaluation of oxidizing species the potential was swept in the window from 0 to 1.2 V vs. Ag/Ag^+. To evaluate the reduction processes, the potential was swept in the window from 0 to −1.5 V vs. Ag/Ag^+). Carajurin presented the first oxidation process at 0.47 V vs. Ag/Ag^+ (0.42 V vs. NHE) (Figure 7, green line), which can be attributed to the oxidation of the hydroxy substituent; the second oxidation peak is probably due to the formation of an intermediate radical produced during the oxidation process. Both oxidation processes showed some reversibility; however, the cathodic peaks showed slower processes, resulting in lower peak currents [17]. As shown in Figure 7 (green line), when scanning at more negative potentials, the carajurin in oxygen absence showed a reversible process in potential,1.07 V vs. Ag/Ag^+ (–1.12 V vs. NHE), which indicated the compound's ability to capture electrons forming a radical anion, a factor that influences in the compound's properties [18,19]. Figure 7 (red line) shows the voltammetric response in an air-saturated electrolyte solution, associated with superoxide radical generation, in the absence of carajurin. The possible interaction of the radical anion with oxygen was studied from the carajurin response in the presence of dissolved oxygen (Figure 7, green line), showing a shift from the reduction process to more positive potential values. The energies of HOMO and LUMO were calculated from the first oxidation and the reduction process, respectively. The experimental energy values of the HOMO and LUMO levels for carajurin were –4.86

eV and −3.32 eV, respectively; therefore, the gap estimated from the electrochemical data was 1.54 eV.

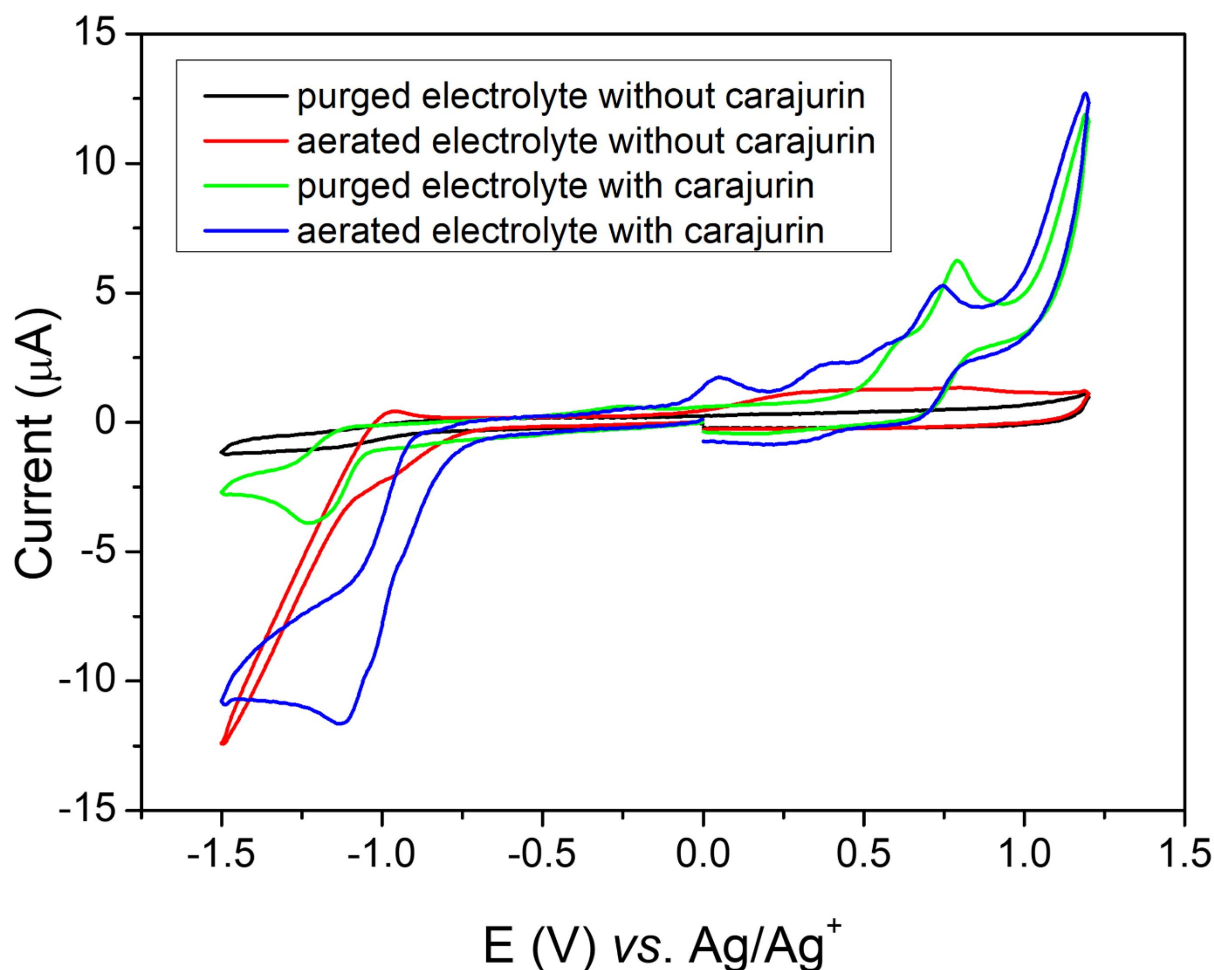

Figure 7. Cyclic voltammograms obtained at glassy carbon electrode in dichloromethane containing 0.05 mol L^{-1} of $TBAPF_6$ (electrolyte) at scan rate of 50 mV s^{-1}, where the black line shows purged electrolyte (without oxygen) in the absence of carajurin, the red line shows aerated electrolyte (with oxygen) in the absence of carajurin, the green line shows electrolyte with carajurin in the absence of oxygen, and the blue line shows electrolyte with carajurin in the presence of oxygen.

2.8. Quantum Studies

Molecular orbitals play a crucial role in understanding chemical reactivity at the atomic level and are important descriptors for the rationalization of various chemical reactions, in addition to comprising a wide range of biological activities. In this study, we performed the calculation of the energies of the frontier orbitals (LUMO and HOMO) for carajurin (Figure 8), with results that are compatible with the observations from the cyclic voltammetry experiments.

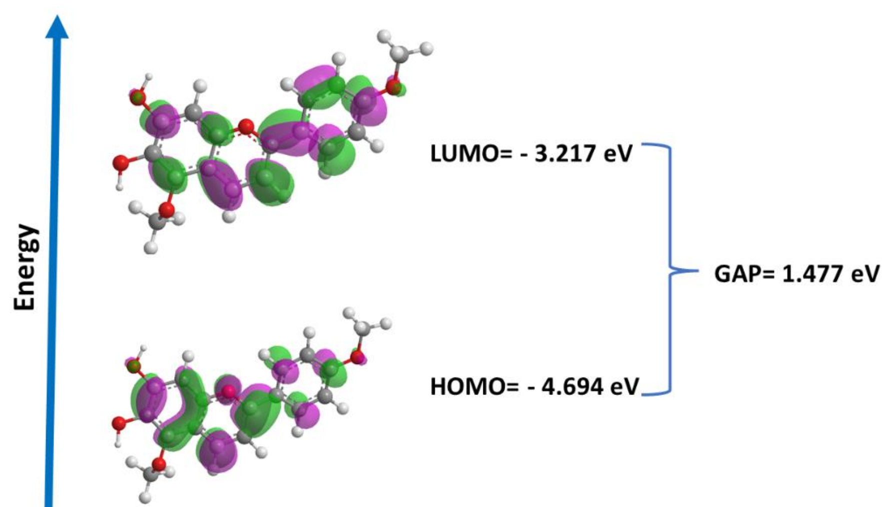

Figure 8. Energies and surfaces of the frontier orbitals (LUMO and HOMO) and GAP (LUMO-HOMO) for carajurin.

2.9. In Silico Prediction Physico-chemical

To analyze the profile of cajurin as a prototype for drugs in preclinical stages of development, the Swiss ADME® platform (http://www.swissadme.ch/index.php, accessed on 16 February 2022) was used, from which were extracted information about: physico-chemical properties, Drug-likeness, and Medicinal chemistry (Table 3).

Table 3. Predicted physicochemical, drug-likeness, and medicinal chemistry properties for carajurin.

Property/Model Name	Carajurin
Physico-chemical	
Molecular weight	301.31
# Rotatable bonds	3
# H-bond acceptors	5
# H-bond donors	2
Surface area	126.520
TPSA (Å2)	75.99
Lipophilicity (log Po/w)	0.68
Drug-likeness	
Lipinski	Yes; 0 violation
Ghose	Yes
Veber	Yes
Egan	Yes
Muegge	Yes
Medicinal chemistry	
PAINS	0 alert
Brenk	1 alert: charged oxygen sulfur
Lead-likeness	Yes
Syntheticaccessibility	4.24

#: number, TPSA: topological polar surface area; PAINS: pan-assay interference compounds; MW: molecular weight.

3. Discussion

Previous studies demonstrated a direct effect of *A. chica* against *L. amazonensis* and *L. infatum* promastigote forms [20,21]. Our research group reported that *A. chica* was able to

inhibit the proliferation of promastigote forms of *L. amazonensis* [11,12,22]. Moreover, we verified a superiority of carajurin among the other anthocyanidins in inhibiting *Leishmania* promastigotes, demonstrating the direct action of this natural compound on this parasite [12]. Data described in the previously published article [12], emphasize the inhibition of intracellular amastigote forms, indicating activation of leishmanicidal macrophage functions, especially the induction of NO. These results showed that carajurin can act indirectly by activation of mechanisms in macrophage, such as an increased release of NO. However, in the present study we discuss a possible mechanism of inhibition against the free forms of the parasite (extracellular evolutionary form, promastigote), so the data presented here refer to the direct effect on the parasite.

Carajurin stood out as the most active anthocyanidin, with IC_{50} less than 4 μg.mL^{-1} after 72 h of treatment [12]. However, in the present study, using a 24-h treatment, an increase in selectivity by 7 times (SI = 32.4) was observed, compared to the 72-h treatment. Compounds with SI ≥ 10 are considered effective anti-leishmanial compounds, compared with in vitro cytotoxicity [23]; thus, carajurin deserves to be considered as a good candidate for further experimental chemotherapy studies against *Leishmania*.

Observations of ultrastructural and morphological alterations are used to elucidate the mechanisms of action of new compounds and to investigate the cell death mechanism involved [24]. Consequently, to investigate and identify which organelles are the targets of carajurin and the damages within the parasite, ultrastructural analyses of *L. amazonensis* promastigotes were performed using transmission electron microscopy. In promastigotes treated for 24 h with the IC_{50} of carajurin, we observed vesicles with electron-dense content, electron-dense corpuscles, and pronounced swelling of the mitochondria.

There is little information available on the leishmanicidal activity of anthocyanidins for comparison with the results presented herein. Previous studies by our group suggested that the leishmanicidal activity of carajurin would be associated with the ability to induce the activation of the microbicidal response in macrophages and promote the production of NO [12]. Furthermore, data from the literature report that flavonoids can target the kinetoplast of parasites, as they induce significant cleavage of the topoisomerase II-mediated kDNA minicircle in *Leishmania* [25].

Important alterations on the ultrastructure of *L. infantum* promastigotes were also observed in parasites treated with the fraction obtained with an increasing gradient of polarity (hexane:ethyl acetate) from *A. chica* hexane extract [20]. In that study, parasites exposed to the active fraction (18.6 μg/mL, 24 h) showed abnormal cell body shapes. Mitochondrial dilatation with loss of matrix contents and Golgi complex alterations, followed by a cytoplasm vacuolization process and an intense exocytic process of cytoplasmic content into the flagellar pocket, were also observed [20]. The experiments with dimeric flavonoid (braquidina 2) from *Arrabidaea brachypoda* also demonstrated alterations in the Golgi complex and the accumulation of vesicles inside the flagellar pocket in *L. amazonensis* amastigotes [26]. In addition, other drugs directly interfere with mitochondrial physiology in parasites such as *Leishmania* [27,28]. The mitochondria of protozoan are considered an ideal drug target, while minimizing toxicity [6,29]. Anti-trypanosomal compounds, such as pheophorbide A [30] obtained from leaves of *A. chica*, also affected the parasite's mitochondrion. The ultrastructural alterations induced by pheophorbide A in trypomastigotes of *T. cruzi* were similar to those observed in the present study for carajurin in *L. amazonensis*. These results suggest mitochondrial collapse as part of the mechanism of action of carajurin and demonstrate its leishmanicidal effect.

To confirm drastic damage to the mitochondrion of the parasite evidenced by transmission electron microscopy, the $\Delta\Psi m$ was evaluated by flow cytometry using TMRE. We observed that carajurin induced the depolarization of the mitochondrial membrane of the promastigote parasite, showing that this compound is capable of crossing the plasma membrane and causing a collapse of the mitochondrial membrane of the parasite. Several plant compounds that cause mitochondrial damage and parasite death have their

mechanisms of action attributed mainly to the potential dysfunction of the mitochondrial membrane [20,31].

Knowing that the production of ROS in promastigotes is one of the possible events triggered by the loss of mitochondrial integrity [32,33], we investigated whether carajurin could act through this process. Treatment of *Leishmania* promastigotes with carajurin resulted in a significant increase of ROS levels and demonstrated that N-acetylcysteine (NAC) protected *L. amazonensis* from inhibition by carajurin, in addition to reducing the ROS levels in carajurin-treated cells. NAC is a thiol compound that increases the levels of glutathione [34]; it is an important molecule for protecting kinetoplastids from ROS or toxic compounds, acting as an antioxidant [35]. This result indicated that the inhibition of growth promoted by carajurin in *L. amazonensis* is mediated by ROS production. This might explain the depolarization of the mitochondrial membrane for this parasite stage. Studies conducted by Fonseca-Silva, et al. [6] reported that the mitochondrial dysfunction observed in *L. amazonensis* promastigote treated with the flavonoid quercetin is promoted by ROS production, in the same way as *L. amazonensis* promastigote treated with the flavonoid apigenin [36] is promoted, for the same parasites. Furthermore, the results of these compounds suggest the involvement of ROS in leading to an alteration of the mitochondrial membrane potential as part of the mechanism of action.

Mitochondrial ROS production followed by the depolarization of the mitochondrial membrane can trigger parasite death through an apoptosis-like mechanism [29,32,37,38]. Promastigotes of *L. amazonensis* treated with carajurin IC_{50} were double stained with annexin V and PI to evaluate cell death induction, and it was observed that carajurin induced late apoptosis in parasites.

Similar to our findings, previous studies reported cell death induction in *L. amazonensis* promastigotes induced by compounds isolated from natural products [39,40]. In addition, luteolin and quercetin inhibited DNA synthesis in *L. donovani* promastigotes and promoted topoisomerase-II mediated linearization of kDNA minicircles, leading to apoptosis [25]. In other studies, the flavonoids fisetin, quercetin, and luteolin inhibited the arginase enzyme from *L. amazonensis* [41,42]. L-arginine deprivation promotes an externalization of phospholipids that bind to annexin V, signaling apoptosis-like cell death in *L. donovani* promastigotes [43].

Cyclic voltammetry (CV) is a simple method for screening active redox compounds and estimating electrochemical activity in different samples, such as medicinal plants [17]. To analyze the mode of action of carajurin, the electrochemical behavior of the compound was analyzed by CV in an aprotic organic environment (dichloromethane), in order to mimic the nonpolar cellular environment [18,19]. Under aerobic conditions, the compound reduction mechanism predominates, resulting in the radical anion intermediate, which, when undergoing a retro-oxidation process in the presence of oxygen, releases ROS, similar to the process described for other compounds in actions against parasites [18,19,44,45]. When analyzing the electroactivity of carajurin, it can be observed that in the absence of molecular oxygen (Figure 7, green line) the compound shows a reduction process, which can lead to the generation of the radical intermediate. As seen in Figure 7 (blue line), in the presence of molecular oxygen, the voltammogram shows a change in the profile, indicating a possible interaction of carajurin electroreduction products with dissolved oxygen in the electrochemical cell [18,19]. These effects include a shift from the position of the peak of carajurin reduction, for more positive potentials.

As the electronic level is an inherent characteristic of a substance, the redox potential is also a unique value of the substance; thus, the electrochemical data obtained by voltammetry were used to determine the energy of the boundary orbital, which data were similar to the values obtained by DFT. HOMO energy is a better indicator of antioxidant activity than LUMO energy; in general, it is possible to relate HOMO energy values and scavenging activities. On the other hand, the energy of LUMO is a better indicator in relation to antiparasitic activity, as it is associated with the molecule's reduction process. Furthermore, the HOMO and LUMO values obtained from voltammetry and quantum studies

suggest that carajurin can acquire an electron more easily than donating an electron, favoring its action in the generation of ROS over an antioxidant action, in agreement with the results obtained in computational studies [46].

From the data in Table 3, it is possible to observe that carajurin has a set of physicochemical properties (molecular weight, rotational bonds, H-bond acceptors, H-bond donors, surface area TPSA-Å2, and lipophilicity (log Po/w) according withwhat is expected for a drug, since it does not contradict any of the rules established by Lipinski, Ghose, Veber, Egan, and Mueggue. Furthermore, carajurin did not present pan-assay interference compounds (PAINS). These data encourage further research with carajurin with an in-depth analysis of pharmacokinetic parameters (ADME) and toxicity, using a combination of in silico and in vitro strategies. Finally, these results advance our knowledge on the mechanisms involved in the leishmanicidal effect of carajurin, building solid foundations for drug discovery and opening new opportunities for research in this significant area of human health.

Taken together, our results are consistent with findings with respect to *L. amazonensis* promastigotes, that the presence of ROS causes mitochondrial depolarization and that this can trigger parasite death through an apoptosis-like mechanism. In addition, further in silico and in vitro enzymatic evaluation tests of carajurin are being developed to achieve a better understanding of the mechanism by which carajurin acts in promoting a leishmanicidal effect.

4. Materials and Methods

4.1. Reagents

The reagents 2,7-dichlorodihydro-fluorescein (H_2DCFDA), Brewer thioglycolate medium, RPMI 1640 medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), EPON 812 resin, glutaraldehyde, amphotericin B, osmium tetroxide, Schneider's insect medium, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), and penicillin were acquired from Gibco (Gaithersburg, MD, USA). Tetramethylrhodamine ethyl ester (TMRE) was obtained from Molecular Probes (Carlsbad, CA, USA).

4.2. Plant Material

Arrabidaea chica, morphotype IV, were collected in February 2016 from the Fiocruz Atlantic Forest Campus, Rio de Janeiro city, State of Rio de Janeiro, Brazil (S 22.9406° W 43.4046°). The leaves were identified by Dr. Marcelo Galvão, and voucher specimens were deposited at the Botanical Collection of Medicinal Plants (CBPM) of Farmanguinhos/Fiocruz (CPBM 668).

4.3. Isolation and Structural Characterization of Carajurin

Carajurin was isolated as a red amorphous powder from *A. chica* hidroalcoholic extract after successive fractionation steps, such as liquid-liquid partition and column chromatography using Sephadex as the stationary phase and dichlorometane:metanol (1:1) as the eluent. The present work shows other structural characterization results of carajurin, in addition to those recently shown in a previous paper [12]. Distortionless enhancement by polarization transfer (DEPT) NMR-1D and NMR-2D as homonuclear correlated spectroscopy (COSY), heteronuclear multiple bond coherence (HMBC), and heteronuclear single quantum correlation (HSQC) were useful to confirm the unequivocal identification of anthocyanidin carajurin. NMR 1D and 2D analyses were recorded with a Bruker 400 (Wissembourg, France),400.15 MHz (^1H) and 100.62 MHz (^{13}C). The chemical shifts were determined relative to $CDCl_3$ at 0 ppm.

4.4. Ethical Statements and Animals

Female BALB/c mice aged 4 to 6 weeks were purchased from the Institute of Science and Technology in Biomodels of the Oswaldo Cruz Foundation. The Ethics Committee on Animal Care and Utilization reviewed and approved the animal protocol (CEUA-IOC L53/2016). All procedures described by the Control of Animal Experimentation (CONCEA) were strictly followed.

4.5. Peritoneal Macrophage Isolation and Parasite Cultures

Peritoneal macrophages were isolated from BALB/c mice administered with 3 mL thioglycolate 3% intraperitoneal for 72 h. Then, cells were cultured overnight and maintained in RPMI 1640 medium, at 37 °C and 5% CO_2. *Leishmania amazonensis* strain MHOM/BR/76/MA-76 was maintained in promastigote form by culturing at 26 °C Schneider's Insect Medium. All media were supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} of streptomycin.

4.6. In Vitro Cytotoxicity Assay of Carajurin on Peritoneal Macrophages (CC_{50})

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Peritoneal macrophages were plated in 96-well plates at 5×10^5 cells.mL^{-1}. After cell adherence, the medium was removed and replaced by carajurin (3.9–500 μg.mL^{-1}) or amphotericin B (0.19–25 μg.mL^{-1}), in a final volume of 100 μL per well, at 37 °C and 5% of CO_2. The plates were incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2. Wells without cells were used as blank and wells with cells and 1% DMSO were used as controls. MTT (5 mg/mL) was added to each well in a volume equal to 10% of the total. After 2 h, the supernatant was completely removed and 100 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was read on a spectrophotometer at a wavelength of 570 nm. Data were normalized according to the following formula: % survival = (Abs. sample-Abs. blank)/(Abs. control-Abs. blank) × 100 [47].

4.7. In Vitro Inhibition Assay of Carajurin on Promastigotes (IC_{50}) and Selectivity Index

The susceptibility of promastigotes was carried out according to the method described by Silva-Silva, et al. [12]. Promastigote forms of *L. amazonensis* harvested at the log phase were seeded into 96-well flat-bottomed plates at 2×10^6 parasites per well. Then, serial dilutions of carajurin (100–3.125 μg mL^{-1}) were obtained. After diluting, the plates were incubated at 26 °C for 24 h. The plates were examined under an inverted microscope to assure the growth of the controls under sterile conditions, and viable promastigotes were counted in a Neubauer chamber [16]. Amphotericin B (2.5–0.07 μg mL^{-1}) was used as the reference drug, while wells without parasites were used as blanks, and wells with parasites and DMSO 1% only were used as an untreated control. The experiments were conducted in triplicate. The percentage of growth inhibition was calculated from the count of viable parasites relative to the untreated control, and 50% inhibitory concentration (IC_{50}) values were determined. The selectivity index (SI) was obtained from the ratio between the half-maximal cytotoxic concentration (CC_{50}) for BALB/c peritoneal macrophages and the IC_{50} for promastigote.

4.8. Transmission Electron Microscopy

Promastigote forms of *L. amazonensis* were treated with an IC_{50} carajurin concentration for 24 h, according to the calculated index for carajurin. Nontreated parasites were used as a control. After 24 h-incubation at 26 °C, promastigotes were collected by centrifugation at 1500×g for 5 min. The parasites were fixed with 2.5% glutaraldehyde in a 0.1 M sodium-cacodylate buffer, pH 7.2, overnight. Then, the parasites were washed three times with the 0.1 M sodium-cacodylate buffer and postfixed in a solution containing 1% osmium tetroxide, 0.8% ferrocyanide, and 5 mM calcium chloride, washed in the 0.1 M

sodium-cacodylate buffer, dehydrated in graded acetone, and embedded in EMbed 812 resin. Ultrathin sections were obtained from 100 nm cuts in Sorvall MT 2-B (Porter Blum) ultramicrotome (Sorvall, Newtown, CT, USA) stained with a 5% uranyl acetate aqueous solution and lead citrate (1.33% lead nitrate and 1.76% sodium citrate), and examined with a transmission electron microscope, JEM-1011 (JEOL, Tokyo, Japan), operating at 80 kV [8].

4.9. Determination of Mitochondrial Membrane Potential (MMP)(ΔΨm)

To measure the mitochondrial membrane potential, promastigote forms of *L. amazonensis* (2×10^6 parasites mL^{-1}) were treated with carajurin for 24 h with calculated IC_{50}, in Schneider's Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Heat-killed parasites (60 °C bath for 30 min) were used as positive control and nontreated parasites were used as a negative control. Subsequently, the parasites were incubated for 30 min at 26 °C with 50 nM tetramethylrhodamine, followed by ethyl ester (TMRE) for 15 min at room temperature, and submitted to flow cytometric analysis through a CytoFLEX flow cytometer (Beckman Coulter Life Sciences, Inc., Brea, CA, USA). TMRE fluorescence was excited through a 488 nm-blue laser and their fluorescence was collected at 585/42 bandpass filter. CytExpert software version 2.1 (Beckman Coulter Life Sciences, Inc., Brea, CA, USA) was used for flow cytometric analyses.

4.10. Measurement of Reactive Oxygen Species (ROS)

The ROS production was evaluated using cell permeable oxidative fluorescent dye 2′,7′dichlorodihydrofluorescein diacetate (H_2DCFDA). *Leishmania amazonensis* promastigotes (2×10^6 parasites mL^{-1}) were treated with a IC_{50} carajurin concentration for 24 h at 26 °C. ROS production was also monitored in *Leishmania* promastigotes pretreated with N-acetylcysteine (NAC, 300 μM) for 60 min, followed by treatment with carajurin (IC_{50} concentration). Hydrogen peroxide (50 μM) and nontreated parasites were used as positive and negative controls, respectively. Then, the parasites were centrifuged, washed with PBS, and incubated with 150 μL of H_2DCFDA (5 μM) for 30 min in the dark, at room temperature. The H_2DCFDA-fluorescence intensity was measured by flow cytometry [8].

4.11. Detection of L. amazonensis Apoptosis by Flow Cytometry

Following the 24 h treatment with carajurin (IC_{50}) at 26 °C, *L. amazonensis* promastigotes apoptosis and necrosis were analyzed—phosphatidylserine (PS) externalization; and plasma membrane integrity, respectively—using annexin V-FITC and propidium iodide (PI) / Dead Cell Apoptosis Kit (Invitrogen™), according to the manufacturer's instructions, and followed by flow cytometry analysis. After the incubation time, the parasites were centrifuged at 1500 rpm for 5 min at room temperature, washed in PBS, and resuspended in a 100 μL 1X annexin-binding buffer, 5 μL annexin V and 1 μL PI (100 μg mL^{-1}). Cell death was also monitored in *Leishmania* promatigotes after carajurin (IC_{50}) treatment using only PI (1 μL, 100μg mL^{-1}). As control procedures, we used miltefosine (50 μM) (antileishmanial reference drug) and untreated parasites. After 15 min incubation protected from light at room temperature, a 400 μL 1X annexin-binding buffer was added to each sample. For analytical purposes, promastigotes were classified according to their staining as apoptotic parasites (annexin V+; PIneg), late apoptotic/necrotic parasites (annexin V+; PI+), and viable parasites (annexin Vneg; PIneg).

4.12. Electrochemical Tests/Cyclic Voltammetry

The cyclic voltammetry (CV) measurements were taken using a potentiostat/galvanostat Autolab PGSTAT 204 (Metrohm). CV was performed using 1.6×10^{-4} mol L^{-1} carajurin and 0.05 mol L^{-1} tetra-n-butyl ammonium hexafluorophosphate ($TBAPF_6$) in dichloromethane as the supporting electrolyte. The experiments were performed using a

standard three-electrode cell with a glassy carbon electrode, a Pt-wire counter electrode, and Ag/Ag^+ ($AgNO_3$ 0.01 mol L^{-1} in acetonitrile) as the reference electrode. For experiments in the absence of oxygen, before each measurement the cell was deoxygenated by purging with argon. The energies of the highest occupied molecular orbital (E_{HOMO}) and the lowest unoccupied molecular orbital (E_{LUMO}) can be related to the potential of the first oxidation and the reduction process of the molecule, respectively. To obtain the values of E_{HOMO} and E_{LUMO}, initially the oxidation-reduction potentials were recalculated for the NHE scale, using the redox pair ferrocene/ferrocene (0.45 V vs. Ag/Ag^+) as an internal standard. Using the corrected potentials for the NHE scale and considering the potential of the NHE on the absolute scale equal to 4.44 eV, we calculated the values of E_{HOMO} and E_{LUMO} using the following empirical formulas [48]: $E_{LUMO} = -(E^{red}_{onset} + 4.44)$ eV and $E_{HOMO} = -(E^{oxi}_{onset} + 4.44)$ eV, where E^{red}_{onset} and E^{oxi}_{onset} are the onset potentials (vs. NHE) of reduction and oxidation, respective-ly.

4.13. Quantum Studies

DTF calculations to estimate all energy values of the highest occupied molecular orbitals (HOMO) and the lowest unoccupied molecular orbitals (LUMO performed on Gaussian v.09 program package with B3LYP level and 6–311++G(d,p) basis sets [16], were applied in these molecular systems' gas phase, considering the singlet and neutral structures. The calculations were run subject to the grid method and the Slater exchange potential correlations. Next, the Hückel [49] method generated an initial estimate of molecular orbitals and electronic density. Subsequently, the convergence of the self-consistent field (SCF) [50] was determined by the restricted Hartree-Fock (RHF) algorithm, which was limited to 30 iteration cycles [51].

4.14. In Silico Prediction Physico-chemical

The structure of carajurin was drawn using ChemDraw software (version Ultra 12.0, PerkinElmer Informatics, Waltham, MA, USA) and was converted into a single database file, SMILES. In silico prediction of physico-chemical properties was made using SwissADME, a tool to increase reliability [52].

4.15. Statistical Analyses

The statistical analyses were conducted using the statistical software GraphPad Prism® version 7 (GraphPad Software Inc., San Diego, CA, USA). The numerical results were expressed as mean ± standard deviation and differences were considered significant when $p < 0.05$.

5. Conclusions

The results obtained in this study show that the lethal effect of carajurin on the promastigote forms of *L. amazonensis* was the result of ultrastructural changes, mitochondrial membrane potential decrease, and increased ROS production, which together induced cell death by late apoptosis. In this context, our work helps to achieve a better understanding of the mechanism of action of this anthocyanidin against *L. amazonensis*.

Supplementary Materials: The following materials are available online at file:///D:/Pharmaceuticals/Documentos%20para%20submissao/Supplementary%20material.pdf. Figure S1 –[1] H-[13]C Heteronuclear Multiple Bond Correlation (HMBC) Spectroscopy for Carajurin. Figure S2 - Expansion of the [1]H-[13]C Heteronuclear Multiple Bond Correlation (HMBC) Spectroscopy for Carajurin. Figure S3 - [1]H-[13]C Heteronuclear Single Quantum Correlation (HSQC) Spectroscopy for Carajurin. Figure S4 - [1]H-^{1}H Homonuclear Correlation (COSY) Spectroscopy for Carajurin.

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and C.J.M.-T.; writing—review and editing, all authors; visualization, J.V.S.-S.; supervision, K.S.C., and F.A.-S.; funding acquisition, K.S.C. and F.A.-S. All authors have read and agreed to the published version of the manuscript.

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3.4 Carajurin-rich fraction from *Arrabidaea chica* Verlot (Bignoniaceae) reduces parasite load in BALB/c mice infected with *Leishmania amazonensis*

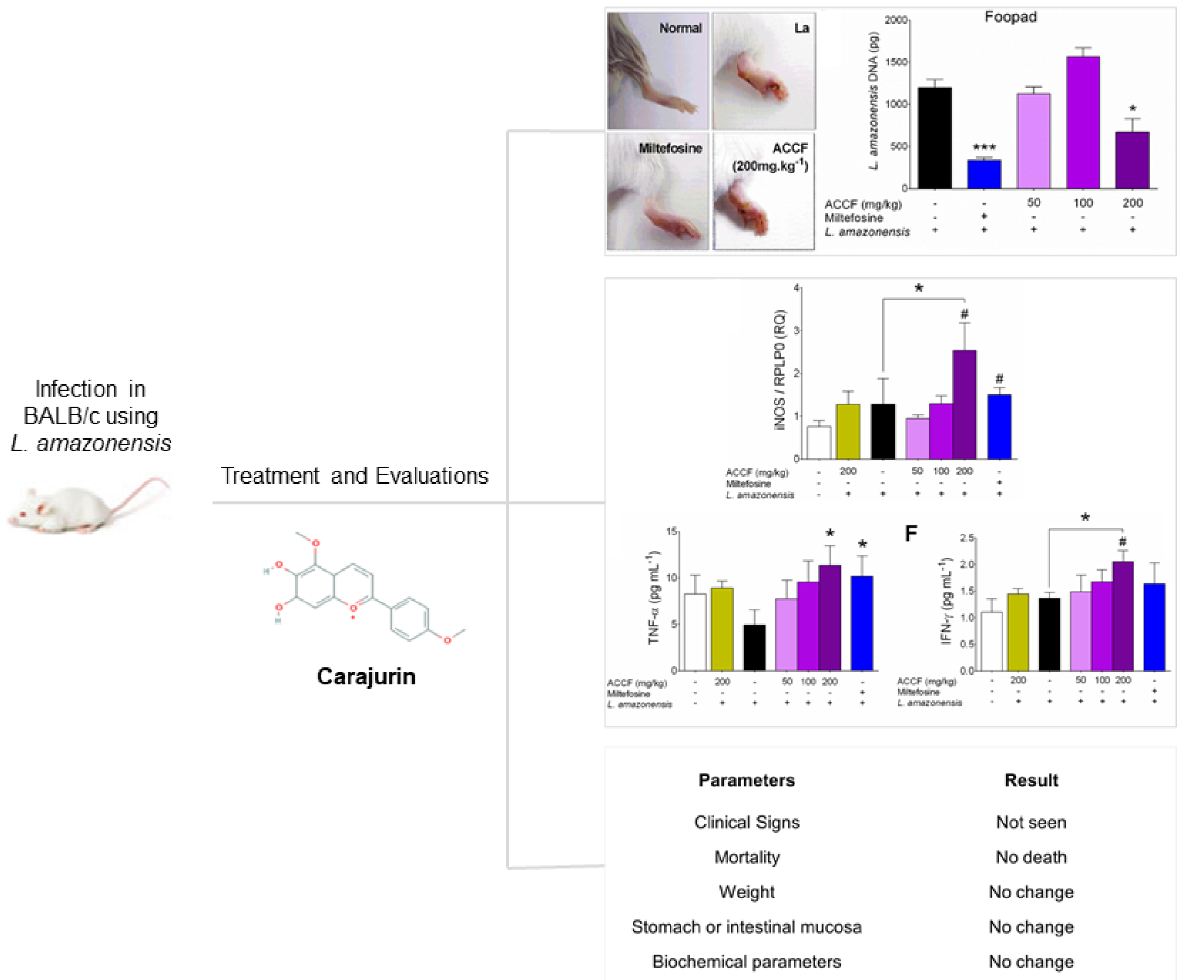

Figura 8 Resumo gráfico da fração rica em carajurina em ratos BALB/c infectados com *L. amazonensis*.

1 Carajurin-rich Fraction From *Arrabidaea chica* Verlot (Bignoniaceae)
2 Reduces Parasite Load in BALB/c Mice Infected with *Leishmania*
3 *amazonensis*
4
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20
21 **ABSTRACT**
22 This study was developed to investigate the activity of *Arrabidaea chica* carajurin-rich
23 fraction (ACCF) in BALB/c mice infected with *Leishmania amazonensis*. The
24 chromatographic profile of the ACCF was determined by high-performance liquid
25 chromatography coupled with a diode-array UV-Vis detector (HPLC-DAD-UV) and
26 carajurin was quantified by construction of an analytical curve obtained from the isolated
27 carajurin. Thirty days after *L. amazonensis* infection in BALB/c mice, intragastric
28 treatment with ACCF at 200, 100 and 50 $mg.kg^{-1}.day^{-1}$ was performed for 30 days. Parasitic
29 loads and cytokine expressions of the lesion site were analyzed by qPCR. Levels of
30 cytokines and biochemical parameters of toxicity were measured in serum.
31 Chromatographic and spectrometric methods identified carajurin as the main component

of ACCF. Treatment with ACCF at 200 mg.kg^{-1}.day^{-1} significantly decrease parasitic load in the footpad of mice infected with *L. amazonensis*. ACCF treatment showed that upregulated of iNOS and IFN-γ cytokine expression at the site of infection, as well as demonstrated that upregulated of TNF-α and IFNγ followed by downregulated of IL-4 and IL-10 in serum cytokine levels, when compared with group infected and mock-treated. No toxicity was observed. Overall, the treatment of *L. amazonensis*-infected BALB/c mice with ACCF showed parasite burden decrease without showing toxicity.

Keywords Cutaneous Leishmaniasis, Phytotherapy, Carajurin, Oral Route, Cytokines

INTRODUCTION

The use of medicinal plants has been the focus of several studies (da Silva E Silva et al. 2019; Tasneem et al. 2019; Benarba and Pandiell, 2020). The Bignoniaceae *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot is a climbing plant popularly known as crajiru or pariri (Behrens et al. 2012), native to tropical America (Rodrigues et al. 2014), considered the most known species of the genus *Arrabidaea* in Colombia (Torres, 1983), in addition to appearing on the National List of Medicinal Plants of Interest to the Unified Health System (RENISUS), in Brazil since 2009 (Brasil, 2009). *Arrabideae chica* leaves are employed in traditional medicine as an anti-inflammatory (Evangelista et al. 2013), clean wounds and ulcers to aid in healing (Lorenzi and Matos, 2002; Bieski et al. 2012), and cleanse the blood (Bieski et al. 2012). *In vivo* studies describe its effect on wound healing (Aro et al. 2013; Cortez de Sá et al. 2015).

Medicinal plants with a healing effect are used in studies against cutaneous leishmaniasis (da Silva E Silva et al. 2019; Gharirvand Eskandari et al. 2020). According to the world health organization, leishmaniasis is considered a group of diseases caused by parasitic protozoa of more than 20 species of *Leishmania*, divided into three main forms of the disease: cutaneous leishmaniasis, visceral leishmaniasis, also known as kala-azar, and mucocutaneous leishmaniasis. Cutaneous leishmaniasis is the most common form, considered endemic in 92 countries or territories, with an estimation of more than 1 million new cases of cutaneous leishmaniasis occur annually (WHO, 2021). Added to this scenario, the treatment of leishmaniasis has many drawbacks, such as toxicity, high costs, the

63 resistance of parasites, and therapeutic failure (Ponte-Sucre et al. 2017; Sundar et al., 64 2019). Thus, the search for new therapeutic alternatives becomes urgent.

65 The antileishmanial effect of *A. chica* extract and fractions against leishmania parasites 66 has been shown in a few studies (Rodrigues et al. 2014, Cortez de Sá et al. 2015, Moragas-67 Tellis et al. 2020). However, no *in vivo* studies to verify an effective antileishmanial 68 activity have been performed with *A. chica*. Our previous studies has shown that fractions 69 rich in flavones or anthocyanins, as well as isolated compounds from *A. chica* have 70 significant antileishmanial activity against *L. amazonensis* strains (Silva-Silva et al. 2021a, 71 Silva-Silva et al. 2021b), being possible to observe in the bioguided phytochemical study 72 of the leaves of *A. chica* a better *in vitro* leishmanicidal response for the anthocyanidins 73 fraction (Silva-Silva et al. 2021a). Based on these data, the aim of this study was evaluating 74 the antileishmanial activity of anthocyanidin-rich fraction from *A. chica*, after their 75 administration by gavage, in a model of cutaneous leishmaniasis in BALB/c mice 76 subcutaneously infected with *L. amazonensis*.

77

78 **MATERIALS AND METHODS**

79 **Plant material**

80 *Arrabidaea chica* (Humb. Bonpl.) B. Verlot leaves, morphotype IV, was cultivated at 81 Fiocruz Atlantic Forest Campus, Rio de Janeiro city, State of Rio de Janeiro, Brazil (S 82 22.9406◦ W 43.4046◦) and was collected in February 2016. The authentic sample was 83 identified by Dr. Marcelo Galvão of Botanical Collection of Medicinal Plants (CBPM) of 84 Farmanguinhos/Fiocruz, and a voucher specimen deposited under code: CPBM 668.

85 **Obtaining of carajurin-rich fraction from *A. chica* (ACCF)**

86 *Arrabidaea chica* carajurin-rich fraction (ACCF) was obtained by successive 87 chromatographic steps from *A. chica* crude extract (ACCE). To reach this fraction, the 88 dried and ground leaves of *A. chica* was extracted by maceration in ethyl alcohol: water 89 70/30 (v/v) for seven days. The resulting extract was filtered and evaporated until dryness 90 producing a reddish residue that was partitioned using n-hexane, dichloromethane and 91 ethyl acetate. The dichloromethane fraction (ACDF) was successively chromatographed 92 using Sephadex LH-20 as stationary phase (Sigma-Aldrich, St. Louis, MO, USA) and 93 methanol as mobile phase. This procedure was systematically repeated and all fractions

94 containing anthocyanidins were gathered. After successive stages of fractionation,
95 gathering of fractions rich in anthocyanidins and separation of non-containing
96 anthocyanidins fractions, a fraction concentrated in anthocyanidins, especially carajurin,
97 was obtained. The resultant fraction was named *A. chica* carajurin-rich fraction (ACCF)
98 because it presents the carajurin as the most concentrated anthocyanidin.

99 **Analysis of ACCF profile by high-performance liquid chromatograph coupled to**
100 **diode-array UV- vis (HPLC-DAD-UV)**

101 A total of 1000 µL of acetonitrile: methanol (both HPLC grade, Tedia, Rio de Janeiro,
102 Brazil) mixture (75:25; v/v) was added to 1 mg of ACCF, previously weighed in a 4 mL
103 vial. The vial was sealed and the sample was sonicated for 10 min with occasional swirling.
104 The sample was then vortexed to mix thoroughly, followed by filtering through a 0.45 µm
105 PTFE filter (Merck Millipore, Darmstadt, Germany) before further analyses into an HPLC
106 vial. Chromatographic analysis was performed on an HPLC-DAD-UV, using a Shimadzu
107 Nexera XR® liquid chromatograph (Shimadzu, Kyoto, Japan) coupled to a UV detector
108 with the diode array SPDM20A, a CBM20A controller, DGU20A degasser, LC20AD
109 binary pump, CTO20A oven, and SILA20A auto-injector (Shimadzu, Kyoto, Japan). A
110 Shimadzu LabSolutions Software Version 5.3 (Shimadzu, Kyoto, Japan) was used to
111 analyze the chromatograms. DAD analysis was applied to select the optimized wavelength
112 of anthocyanidins in this study. In a full-scan experiment, chromatogram at 480 nm show
113 the maximum wavelength (λmax) for the anthocyanidins. Combinations of acidified
114 ultrapure water (pH 3.0, with anhydrous acetic acid, Merck, Darmstadt, Germany) (A) and
115 acetonitrile (HPLC grade, Tedia, Rio de Janeiro, Brazil) (B) were used as the mobile phase
116 (initially 5% A rising to 95% in 80 min). HPLC column was silica-based C18 (250 mm ×
117 4.6 mm i.d. × 5 µm particle size, ODS Hypersil, Thermo, Waltham, MA, USA). The oven
118 was set at 50 ◦C and the injection volume was 10 µL for all analyses.

119 **Quantification of Anthocyanidins Using Carajurin as the Standard**

120 An analytical curve of carajurin was done to quantify all anthocyanidins at ACFF.
121 Since there are no standards available for all anthocyanidins, their content was expressed
122 in milligrams of carajurin by grams of dry extract. Carajurin, previously isolated by our
123 group, was used as external standard. A 400 µg/mL stock solution of the isolated carajurin
124 (98%, chromatographic determined) was prepared in an acetonitrile:methanol (both HPLC

125 grade, Tedia, Rio de Janeiro, Brazil) mixture (75:25; v/v) in a volumetric flask. Ten
126 concentrations of solutions (1, 10, 20; 40; 60; 100; 150, 200, 300 and 400 µg/mL) were
127 done on the day, in order to prepare an analytical curve. The solutions were filtered in a
128 0.45 µm PTFE filter before analyses by HPLC-DAD-UV. Injections of 10 µL were
129 performed in triplicate to obtain the analytical curve from the areas corresponding to the
130 peaks of carajurin. The analytical curve (1–400 µg/mL) of the standard was plotted based
131 on the UV-Vis signal at 480 nm for better selectivity: carajurin content (µg/mL) = (Abs
132 (mAu) + 406935)/47045; R2 = 0.9991). Carajurin and other anthocyanidin amounts (mg/g
133 of dry extract) were calculated and expressed as carajurin content.

134 **Animals and Ethical statements**

135 Female BALB/c mice of 6–8 weeks of age were purchased from the Institute of
136 Science and Technologies in Biomodels (ICTB)/FIOCRUZ. Animal procedures described
137 by the Control of Animal Experimentation (CONCEA), were conducted in strict
138 accordance with the recommendations by the guidelines and protocols reviewed and
139 approved by the Ethics Committee for Animal Experimentation (CEUA-IOC L53/2016).

140 **Parasite strain and mice infection**

141 *L. amazonensis* (MHOM/BR/1976/MA-76) was sustained in the laboratory through
142 sequential passages in BALB/c mice, and reinoculation were performed with 10^4
143 amastigote forms were subcutaneously into the right footpad (Almeida-Souza et al. 2016).

144 **ACCF treatment in mice infected by *L. amazonensis***

145 Treatment protocol was performed with 7 groups of 10 animals, with infection of mice
146 with the parasite *L. amazonensis*. The daily treatment was carried out with 100 µL of ACCF
147 by gavage. The seven groups were organized as follows: normal (mock-infected and mock-
148 treated); infected and mock-treated (100µL of PBS daily by gavage); infected and reference
149 drug-treated (Miltefosine 20 $mg.kg^{-1}$ by intramuscular injection, twice a week); infected
150 and ACCF-treated (100 µL of 50 $mg.kg^{-1}$ daily by gavage); infected and ACCF-treated
151 (100 µL of 100 $mg.kg^{-1}$ daily by gavage) and infected and ACCF-treated (100 µL of 200
152 $mg.kg^{-1}$ daily by gavage) and mock-infected and treated with ACCF (100µL of 200 $mg.kg^{-1}$
153 daily by gavage). Thirty days after infection, treatment was started for all groups. Lesion
154 kinetics was evaluated weekly using a Schnelltaster dial gauge caliper (Kröplin GRBH),
155 and the lesion thickness was obtained from the thickness difference, in millimeters,

156 between the inoculated footpad and the contralateral non-inoculated footpad. Thirty days
157 after treatment, the animals were euthanized with Xylazine Hydrochloride (30 mg/kg,
158 Syntec) associated with Ketamine Hydrochloride (300 mg/kg, Syntec). Tissue and blood
159 samples were collected for subsequent analysis.

160 **Detection and quantification of *Leishmania* by quantitative real-time PCR**

161 DNA was extracted from the footpad and draining lymph nodes of 3 animals per group
162 by a routine phenol-chloroform technique (Sambrook and Russel, 2001). After DNA
163 extraction, 10 ng of total DNA were amplified using a specific primer for *Leishmania* sp.
164 kDNA3 (Weirather et al., 2011), as well as for the mouse β-actin endogenous control (S1
165 Table) (Giulietti et al., 2001). Amplification was performed in a QuantStudio 3 equipment
166 (Applied Biosystems) using GoTaq® PCR Master Mix (Promega), with 250 nM of kDNA3
167 or 100 nM of β-actin primers per reaction. PCR conditions were as follows: hold at 95°C
168 for 2 min, followed by 40 cycles of 95°C for 15 s and 62°C for 1 min followed by a
169 dissociation curve. Standard curves were generated from 10-fold serial dilutions (100 ng–
170 1 pg) of axenic *Leishmania* DNA and used to calculate parasite concentration in the
171 samples (Rizk et al. 2021).

172 **Cytokine gene expression at the lesion site by RT-PCR**

173 Total RNA from tissues was extracted using the TRIZOL reagent (Invitrogen,
174 Karlsruhe, Germany) following the manufacturer's instructions. cDNA synthesis was
175 performed with 1 µg of total RNA using iScript cDNA Synthesis kit (Bio-Rad Laboratories,
176 Hercules, CA), according to the manufacturer's recommendations. Primers targeting the
177 mouse genes, Nos2 – nitric oxide synthase 2, inducible, transcript variant 1 (iNOS); Il10 –
178 interleukin 10 (IL-10); Il12a – interleukin 12a, transcript variant 1 (IL-12); Tnf – tumor
179 necrosis factor, transcript variant 1 (TNF-α); Ifng – interferon gamma (IFN-γ); Tgfb1 –
180 transforming growth factor, beta 1 (TGF-β); and Rplp0 – ribosomal protein, large, P0
181 (Rplp0), were designed using the Primer Express software version 3.0 (Applied
182 Biosystems, 2004), and manufactured by Invitrogen (S1 Table). Real Time PCR assays
183 were performed using Power SYBR Green Master Mix. The relative quantification ($2^{-\Delta\Delta Ct}$)
184 and calibration of the mRNA levels was performed using the mouse gene Rplp0 as the
185 endogenous control. Results were analyzed with the StepOne Software v2.3 (Applied
186 Biosystems).

187 **Quantification of cytokine production by ELISA**

188 Serum samples were obtained from the blood of five mice per group was measured
189 using CBA kit for IL-2, IL-4, IL-10, TNF-α, IFNγ (BD Bioscience) and ELISA kit for IL-
190 12 and TGF-β (R&D System) according to the manufacturer's instructions.

191 **Toxicity analysis parameters**

192 Treatment-related clinical signs of toxicity, such as piloerection, diarrhea, salivation,
193 convulsions or changes in mobility, respiration rate or muscle tone, were evaluated. Animal
194 weight was measured on an analytical balance weekly. At necropsies, serum was obtained
195 to analyze the levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline
196 phosphatase (ALP), total protein, direct bilirubin, indirect bilirubin, total bilirubin,
197 albumin, globulin, urea and creatinine performed at the ICTB platform (Fiocruz/RJ). In
198 addition, stomach and gut mucosa were macroscopically evaluated for abnormal findings.

199 **Statistical analyses**

200 The numerical results were expressed as mean ± standard deviation, with statistical
201 analyses were conducted using the statistical software GraphPad Prism® version 7
202 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant
203 when $p < 0.05$ by one-way analysis of variance (ANOVA) or Mann-Whitney test.

204

205 **RESULTS**

206 **Phytochemical analysis**

207 The successive fractionation steps and the gathering of fractions rich in
208 anthocyanidins, in order to eliminate as much as possible, the non-anthocyanidins
209 components of the extracts, led to the obtaining of an carajurin-rich fraction named ACCF.
210 In addition to carajurin, other four anthocyanidins identified in ACCF had already been
211 previously identified by our research group (Moragas-Tellis et al. 2020; Silva-Silva et al.
212 2021a). All of them were previously quantified both in the crude extract, ACEE (Moragas-
213 Tellis et al. 2020), in an anthocyanidin-rich fraction (ACAF), which was tested against the
214 promastigote and intracellular amastigote forms of *L. amazonensis* in an *in vitro* approach
215 (Silva e Silva, 2021a) and compared, in this work, with the carajurin-rich fraction (ACCF)
216 (Table 1). In comparison with quantification data presented previously for crude extract
217 (ACCE) (Moragas-Tellis et al., 2020) and anthocyanidins-rich fraction (ACAF), all

218 anthocyanidins showed an increase in their final concentrations and their content were
219 expressed in mg/g of carajurin. Among all the anthocyanidins present in the ACCF (Figure
220 1), carajurin presented a higher concentration increase in comparison to the crude extract
221 ACEE (16.77 times) and the anthocyanidin-rich fraction (ACAF) (11.60 times) (Table 1).
222 Although all anthocyanidins showed an increase in their concentration, in the order of 2.9,
223 carajurone (ACAF/ACCF) to 16 times, carajurin (ACAF/ACCE), carajurin showed the
224 highest increase in its total concentration which allowed us to name it as *A, chica* carajurin-
225 rich fraction (ACCF).
226
227 Table 1. Quantification of anthocyanidins content in carajurin-rich fraction (ACCF)
228 expressed in milligrams by grams of dry extract and their comparison with the content of
229 anthocyanidins previously showed in the crude extract of *Arrabidaea chica* Verlot (ACCE)
230 (Moragas-Tellis et al., 2020) and anthocyanidin-rich fraction (ACAF) (Silva-Silva et al.
231 2021).
232

Peak	Identification	Rt (min)	ACEE[(a)]	ACAF[(b)]	ACCF
1	3'-hydroxy-carajurone	23.5	n.d.	4.78 ± 0.028	22.95 ± 0.38
2	carajurone	27.3	3.54 ± 0.039	18.84± 0.193	56.31 ± 1.32
3	carajurin	39.0	17.26 ± 0.11	24.96 ± 0.35	289.59 ± 2.28
4	A2	43.5	2.10 ± 0.0062	7.30 ± 0.029	25.12 ± 0.18
5	A3	52.1	3.89 ± 0.5748	7.44 ± 0.047	44.87± 0.77

233 Note: Values are expressed as the mean ± SD (n = 3). A2 and A3 are unidentified
234 anthocyanidins. Rt = retention time.
235 [a.] Moragas-tellis et al., 2020
236 [b.] Silva-Silva et al., 2021
237

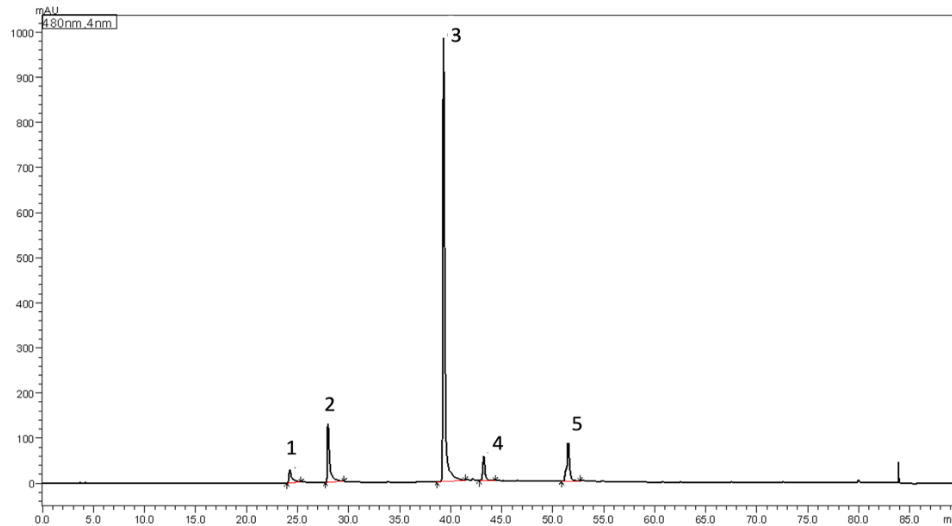
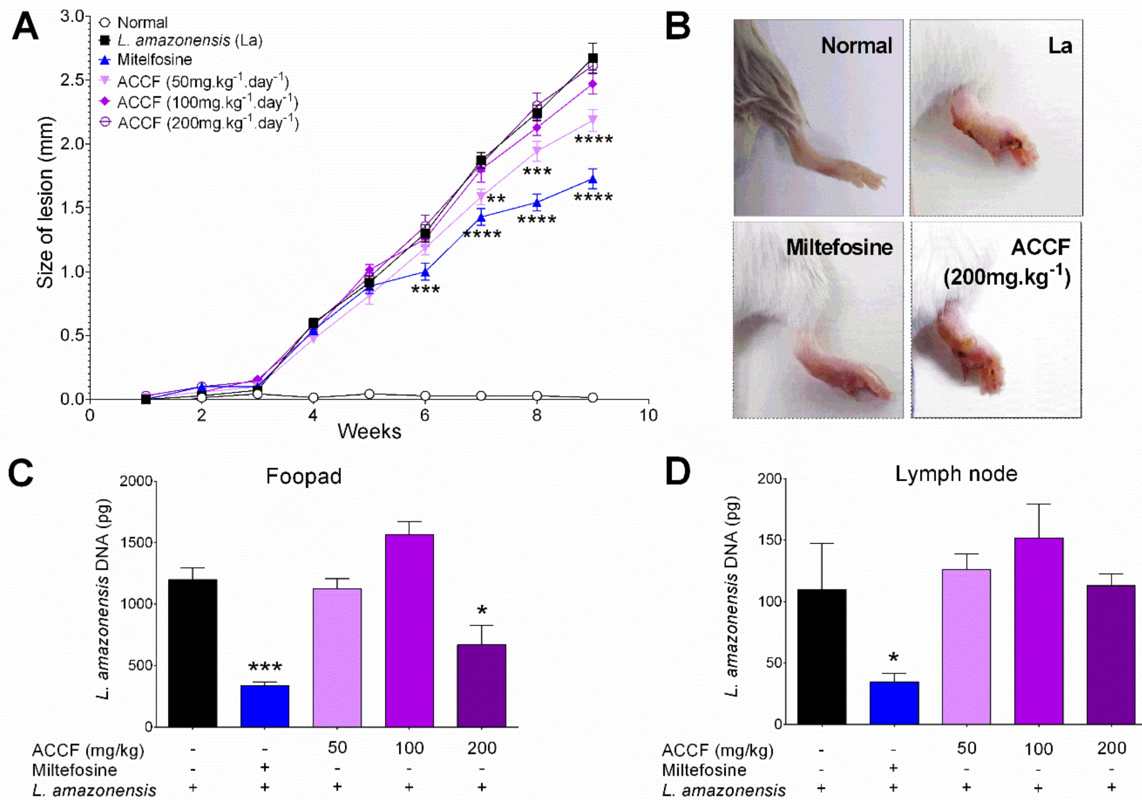


Figure 1. HPLC-DAD-UV chromatogram of *Arrabidaea chica* carajurin-rich fraction (ACCF), showing 3′-hydroxy-carajurone (1, Rt= 23.5 min), carajurone (2, Rt= 27,3 min) carajurin (3, Rt = 39,0 min), A1 (4, Rt= 43,5 min) and A2 (5, Rt= 52,1 min). Note: 3′-OH-carajurone = 3′-hydroxy-carajurone. Rt = retention time.

ACCF treatment decreases the parasite load

The ability to significantly reduce the size of the lesion was only observed in the treatment with ACCF at 50mg.kg^{-1}.day^{-1} from the seventh week onwards, when compared to the untreated infected group (Fig. 2A and Fig. 2B). The control drug, Miltefosine, was able to significantly reduce lesion size as of the sixth week. After 30 days of ACCF treatment there was no change in the draining lymph node parasite quantification, but in the treatment with ACCF at 200 mg.kg^{-1}.day^{-1}, the parasite loads in the footpad exhibited significantly lower level ($p = 0.0428$, Fig. 2C) in comparison to the untreated infected group. Elseways, Miltefosine was able to significantly lower level parasite loads for the footpad ($p = 0.0002$, Fig. 2C), and lymph node ($p = 0.0281$, Fig. 2D), when compared to the untreated infected group.



256

257 **Fig 2. Efficacy of carajurin-rich fraction (ACCF) treatment in BALB/c mice infected**
258 **with *Leishmania amazonensis*.** (A) Kinetic of lesion of infected footpads treated with
259 ACCF (50, 100 or 200 $mg.kg^{-1}.day^{-1}$) or Miltefosine (20 $mg.kg^{-1}$.twice a $week^{-1}$). (B)
260 Representative images of footpads referring to the Normal group, infected (La), treated
261 with Miltefosine (20 $mg.kg^{-1}$.twice a $week^{-1}$) or ACCF (200 $mg.kg^{-1}.day^{-1}$). (C-D) Parasite
262 loads in footpad and draining lymph node after 30 days of ACCF treatment. Data represent
263 mean ± SD of two independent experiments realized at least in triplicate. *p<0.05,
264 **p<0.01, ***p<0.001 ****p<0.0001 in relation to the infected and mock-treated animals
265 by Kruskal–Wallis and Dunn's multiple comparison test. La+ACCF: group infected and
266 treated with ACCF; La+Miltefosine: group infected and treated with Miltefosine; La:
267 group infected and mock-treated; Normal: mock-infected and mock-treated group.

268

269 **ACCF reduces *L. amazonensis*-induced inflammation in BALB/c mice**

270 The expressions of iNOS, IL-10, IL-12, TNF-α, and IFN-γ at the lesion site were
271 evaluated (Fig. 3). Analyses of footpads showed that *L. amazonensis* infection upregulated
272 TNF-α (p =0.0238, Fig. 3C) and IFN-γ expression (p =0.0159, Fig. 3D), when compared

273 with mock-infected and mock-treated group. Changes was also observed in the treated
274 group with ACCF at 50, 100 and 200 $mg.kg^{-1}.day^{-1}$ with IFN-γ expression (p =0.0028,
275 p =0.0015 and p =0.0011, respectively; Fig. 3D), as well as in animals treated with
276 Miltefosine (p =0.0159, Fig. 3D), when compared with mock-infected and mock-treated
277 group. No changes in IL-12 expression were observed. In infected mice treated with ACCF
278 at 200 $mg.kg^{-1}.day^{-1}$ there was induction of IFN-γ (p =0.0356, Fig. 3D) and iNOS
279 (p =0.0462, Fig. AD) expression, when compared with group infected and mock-treated.
280 The IL-10 expressions were upregulated in the treated group with ACCF at 50, 100 and
281 200 $mg.kg^{-1}.day^{-1}$ (p =0.0238, p =0.0238 and p =0.0167, respectively; Fig. 3B) or
282 Miltefosine (p =0.0167, Fig. 3B) treated mice. This upregulation was not observed in mock
283 treated infected mice.
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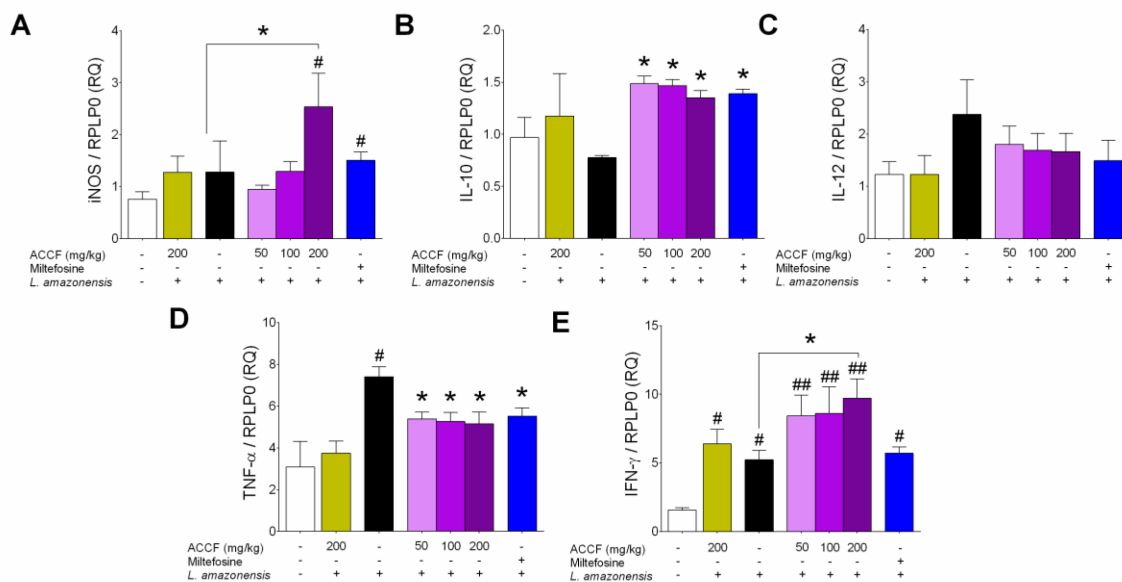

285
286 **Fig 3. Cytokine gene expression in BALB/c mice footpads infected with Leishmania**
287 **amazonensis and treated with carajurin-rich fraction (ACCF).** Relative quantification
288 of iNOS (A), IL-12 (B), TNF-α (C), and IFN-γ (D) with RPLP0 as endogenous control.
289 ACCF group treated at 50, 100 and 200 $mg.kg^{-1}.day^{-1}$; and Miltefosine group treated at 20
290 $mg.kg^{-1}.day^{-1}.twice\ a\ week^{-1}$, both for 30 days. Data represent mean ± SD of two
291 experiments realized in duplicate. #p<0.05, ##p<0.01 in relation to the mock-infected and
292 mock-treated, and *p<0.05, **p<0.01 in relation to the infected and mock-treated control
293 by the Mann-Whitney test. RQ: relative quantification; RPLP0: ribosomal protein large P0.

294 Cytokine levels in the serum of *L. amazonensis*-infected and mock-treated animals
295 showed upregulated of IL-4 at 30 days (p =0.0357, Fig 4B) when compared with mock-
296 infected and mock-treated group. On the other hand, animals treated with ACCF at 200
297 mg.kg^{-1}.day^{-1} and Miltefosine downregulated IL-4 (p =0.0238 and p=0.0357, respectively;
298 Fig 4B) and IL-10 (p =0.0390 and p=0.0016, respectively; Fig 4C) production, when
299 compared with infected and mock-treated group. ACCF at 200 mg.kg^{-1}.day^{-1} was able to
300 induce the production of TNF-α (p =0.0260, Fig 4E) and IFN-γ (p =0.0485, Fig 4F). The
301 upregulated production of TNF-α was also observed in the infected groups treated with
302 Miltefosine (p =0.0411, Fig 4E). Animals treated with ACCF at 200 mg.kg^{-1}.day^{-1} also
303 showed upregulated production of IL-2 (p = 0.0159, Fig. 4A) and IFNγ (p = 0.0127, Fig.
304 4F), in comparison with the mock-infected and mock-treated group. The group infected
305 and treated with Miltefosine also showed upregulated production of IL-2 (p =0.0317, Fig.
306 4A). The IL-12 and TGF-β production were not observed in infected and ACCF or
307 Miltefosine treated mice (Fig 4).
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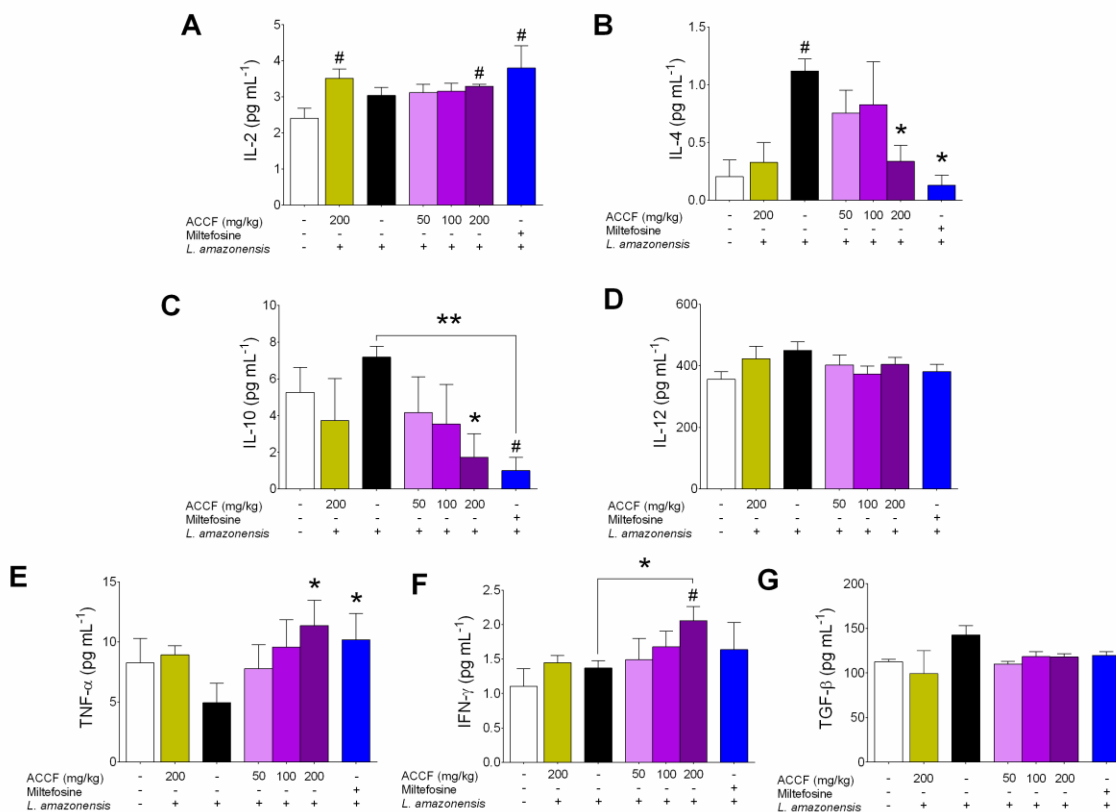

309

310 **Fig 4. Quantification of serum cytokines from BALB/c mice infected with *Leishmania***
311 **amazonensis and treated with carajurin-rich fraction (ACCF).** Cytokines levels of IL-
312 2 (A), IL-4 (B), IL-10 (C), IL-12 (D), TNF-α (E), IFNγ (F), and TGF-β (G). ACCF group
313 treated at 50, 100 and 200 $mg.kg^{-1}.day^{-1}$; and Miltefosine group treated at 20 $mg.kg^{-1}.day^{-}$
314 ^{1}.twice a $week^{-1}$, both for 30 days. Data represent mean ± SD of two experiments realized
315 in duplicate. #$p<0.05$ in relation to the mock-infected and mock-treated, and *$p<0.05$,
316 **$p<0.01$ in relation to the infected and mock-treated control by the Mann-Whitney test.
317
318 **ACCF treatment showed no toxicity in BALB/C mice**
319 Over the 30 days of treatment, no clinical signs of toxicity were observed and there
320 was no mortality. No statistically significant weight loss (S2 Table) was recorded during
321 treatment. During necropsy, no changes were observed in the stomach (hyperemia) or gut
322 mucosa of the animals treated with ACCF. Regarding the change in serum biochemical
323 parameters of hepatic and renal functions, changes were observed in Urea, Albumin and
324 ALT parameters, however, no statistically significant changes were observed when
325 compared to the normal group or to the infected and untreated group (Table 2).
326
327

328 Table 2. Biochemical quantification of liver and kidney parameters in the sera of BALB/c mice treated with ACCF by gavage for 30
329 days.

Parameters	Unit	Normal		*Leishmania amazonensis*-infection				
		Mock-treated	ACCF (200 mg.kg^{-1}.day^{-1})	Mock-treated	Miltefosine (20 mg.kg^{-1}.day^{-1})	ACCF (mg.kg^{-1}.day^{-1})		
						50	100	200
Urea	mg/dL	28.83 ± 0.96	38.80 ± 0.29	27.40 ± 1.85	29.30 ± 1.17	31.83 ± 1.84	32.17 ± 2.17	31.70 ± 0.29
Uric acid	mg/dL	3.70 ± 0.10	4,0 ± 0.23	3.750 ± 0.05	2.400 ± 0.17	2.833 ± 0.17	2.950 ± 0.55	4.400 ± 0.20
Albumin	g/dL	2.50 ± 0.00	2.30 ± 0.06	2.533 ± 0.03	2.467 ± 0.03	2.533 ± 0.03	2.433 ± 0.03	2.467 ± 0.03
AST	U/L	221.0 ± 5.00	197.0 ± 2.89	281.0 ± 25.00	166.0 ± 4.00	222.3 ± 11.22	135.0 ± 1.00	164.7 ± 3.48
ALT	U/L	97.67 ± 3.67	137.0 ± 9.24	152.5 ± 24.50	69.00 ± 10.00	82.67 ± 7.69	78.33 ± 9.60	98.00 ± 8.00
LDH	U/L	1488.0 ± 68.74	1006.0 ± 1.16	1823.0 ± 81.00	1569.0 ± 397.00	1090.0 ± 34.41	1045.0 ± 193.6	947.3 ± 64.34
CK	U/L	314.5 ± 31.50	132.0 ± 31.18	421.0 ± 16.00	263.0 ± 107.00	369.0 ± 6.66	179.0 ± 36.00	268.0 ± 27.00
ALP	U/L	143.7 ± 6.17	127.0 ± 4.62	116.5 ± 0.50	122.0 ± 6.00	135.0 ± 2.31	128.7 ± 5.49	128.0 ± 4.00
Total proteins	g/dL	5.10 ± 0.06	5.10 ± 0.06	5.233 ± 0.09	5.033 ± 0.03	5.250 ± 0.05	5.233 ± 0.07	5.150 ± 0.05
Total bilirubin	mg/dL	0.60 ± 0.00	0.50 ± 0.06	0.6333 ± 0.03	0.6000 ± 0.06	0.575 ± 0.03	0.6667 ± 0.03	0.5667 ± 0.03

330 AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, CK: Creatine kinase, ALP: alkaline phosphatase, N: Normal.
331 Data represent mean ± standard error of the mean of two experiment performed in quintuplicate. Data represent mean ± SD of two independent experiments realized
332 in duplicate.

333 **DISCUSSION**

334 *Arrabidaea chica* has various pharmacological studies described in the literature 335 including antioxidant activities (Ribeiro et al. 2018; Siraichi et al. 2013; Martins et al. 336 2016), anti-inflammatory, antiangiogenic and antiproliferative (Michel et al. 2015), healing 337 potential (Aro et al. 2013) and leishmanicidal activity (Cortez de Sá et al. 2016; Estevez et 338 al. 2007; Rodrigues et al. 2014; Moragas-Tellis et al. 2020; Silva-Silva et al. 2021a, Silva-339 Silva et al. 2021b).

340 Taking into account the results previously presented by our research group that 341 carajurin is the pharmacological marker of leishmanicidal activity of *A. chica,* our purpose 342 was to obtain a fraction concentrated in anthocyanidins that had the carajurin as the major 343 component. This type of sample, carajurine-rich fraction, instead of the isolated carajurin 344 is technically easier to obtain and consequently economically more viable. The successive 345 fractionation, with gathering of only fractions containing anthocyanidins, led to a much 346 more concentrated fraction of the substances of interest, the ACCF, specially carajurin, 347 than the anthocyanidin-rich fraction obtained earlier (Silva-Silva, 2021a).

348 The use of plant extracts has been supported by the WHO as a path to the discovery of 349 new safe and cost-effective antileishmanial compounds (WHO, 2010; Chouhan et al. 350 2014). Previous studies published by our research group have demonstrated *in vitro* 351 leishmanicidal activity of *A. chica* (Moragas-Tellis et al. 2020; Silva-Silva et al. 2021a; 352 Silva-Silva et al. 2021b). Encouraged by these results, we investigated the antileishmanial 353 activity of carajurin-rich fraction from *A. chica* under *in vivo* conditions, using BALB/c 354 mice subcutaneously infected with *L. amazonensis*.

355 Our model, which used a treatment with different doses of ACCF, showed therapeutic 356 efficacy at the highest dose tested to control the parasite load in infected mice. Compared 357 with group infected and mock-treated, mice treated with ACCF at 20 $mg.kg^{-1}.day^{-1}$ 358 displayed significantly lower parasite loads. However, we did not find a correlation 359 between the size of the lesion and the parasite load. Animals treated with ACCF, at a dose 360 of 50 $mg.kg^{-1}.day^{-1}$, for example, despite having smaller lesions when compared to animals 361 treated with the other doses, or with untreated animals, presented a parasite load very 362 similar to the infected and untreated groups. On the other hand, treatment performed with 363 a dose of 200 $mg.kg^{-1}.day^{-1}$ of ACCF did not show any difference in the size of lesions

364 when compared to untreated animals, but with a significant reduction in the parasite load.
365 Patrício et al. (2008) reported that animals infected with *L. amazonensis* and treated with a
366 crude hydroalcoholic extract of *Chenopodium ambrosioides* presented an increase in the
367 thickness of the foot pad, despite the reduction in the parasite load. Marques (1993)
368 correlatesthe increase in the size of the lesions with the presence of edema and the influx
369 of inflammatory cells to the site of primary infection. In addition, the formation of edema
370 in the animal's foot pad can be maintained even without the presence of the amastigote
371 (Marques, 1993), which justifies the size of the lesion.

372 Previously, our group demonstrated by *in vitro* and *in silico* study, an increase of nitric
373 oxide production and iNOS expression in the peritoneal macrophages infected with *L.*
374 *amazonensis* and treated with carajurin (Silva-Silva et al. 2021a). Induced nitric oxide
375 synthase (iNOS), which is the most effective mechanism for killing macrophage-mediated
376 intracellular parasites (Murray and Nathan, 1999; Murray et al. 2006), may explain the
377 decrease in parasite load.

378 The lower parasite load in the skin after ACCF administration at 20 $mg.kg^{-1}.day^{-1}$ was
379 related with a significant reduction in paw skin content of pro-inflammatory cytokines such
380 as TNF-α. Furthermore, the association of high levels of IFN-γ and iNOS was observed in
381 ACCF treatment. The most efficient mechanism of parasite killing involves the production
382 of IFN-γ and TNF-α, which stimulate the synthesis of inducible nitric oxide synthase
383 (iNOS), generating the production of nitric oxide (NO), a potent cytotoxin involved in the
384 inhibition of *Leishmania* parasites (Mauël, 1990; Liew et al. 1997; Murray and Delph-
385 Etienne, 2000). Therefore, these results corroborate our in vitro findings, since the carajurin
386 was able to enhance nitrite levels in the macrophage stimulated or not with *L. amazonensis*
387 (Silva-Silva et al. 2021a).

388 As the spread of infection by *L. amazonensis* is not limited to the skin, but can reach
389 the lymph nodes, spleen and liver (de Oliveira Cardoso et al. 2010), the quantification of
390 cytokines in the serum allowed to verify the immunomodulatory effect of the evaluated
391 compound (Almeida-Souza et al. 2016).

392 The susceptibility of BALB/c mice to infection with *L. amazonensis* and *L. major*
393 infection appears to be dependent of Th2 cytokines like IL-4 and IL-10 (Guimarães et al.
394 2006). Our results revealed an increase in IL-4 and IL-10 in the serum of animals infected

395 with *L. amazonensis* that were not observed after treatment with ACFF or Miltefosine. This
396 decrease in the levels of IL-4 and IL-10 contribute to maintaining a Th1 response in the
397 treated groups. No change was observed in IL-2, IL-12 or TGF-β, except for a slight
398 increase in IL-2 production for ACCF (group treated at 200 $mg.kg^{-1}$ and normal treated
399 with ACCF) and Miltefosine treated infected mice. However, increased levels of IFN-γ and
400 TNF-α were observed due to treatment with ACCF. The resistance to leishmaniasis is
401 related to Th1 development and production of pro-inflammatory cytokines as IL-2, IFN-γ
402 andTNF-α that lead to activation of macrophages and parasite killing (Von Stebut et al.
403 2003; Sacks and Noben-Trauth, 2002). Therefore, the increase in these cytokines
404 contributes to the effectiveness of macrophages by inducing iNOS and, consequently,
405 decreasing the parasite load.
406 Furthermore, our study did not demonstrate alterations in the toxicity parameters
407 analyzed, not being found alterations in the stomach or gut mucosa, showing that the
408 carajurin-rich fraction does not irritate the digestive system. There was also no weight loss
409 during treatment, and no changes in serum biochemical parameters of liver and kidney
410 function were observed. Therefore, ACCF treatment has no toxic effect on mice.
411
412 **CONCLUSION**
413 Our data has proved the efficacy of ACCF in reducing the parasite burden, it has shown
414 its modulatory effects on cytokine expressions, without showing toxicity. Together, these
415 results establish the therapeutic benefit of gavage treatment with a carajurin-rich fraction
416 in a murine model of cutaneous leishmaniasis and constitute an important step in the search
417 for a new orally active antileishmania therapeutic alternative.
418
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581

4 DISCUSSÃO

O extrato bruto obtido de *A. chica* Verlot (ACCE) é rico em flavonoides[98], incluindo antocianidinas[80,91,103,104], consideradas como marcadores químicos da espécie, bem como flavonas[16,27], e flavonóis[27]. O fracionamento realizado neste estudo teve como objetivo principal fornecer uma fração rica em antocianidinas (ACAF) e o isolamento desses metabólitos. Entretanto, devido à alta concentração de flavonas presente em ACCE, também foi realizado o fracionamento para obtenção de uma fração rica em flavonas (ACFF), sem antocianidinas e o isolamento de alguns compostos majoritários (Figura 9).

As etapas sucessivas de fracionamento a partir de ACAF levaram ao isolamento das antocianidinas carajurina, 3′-hidroxicarajurona e carajurona. Outras duas antocianidinas (A2 e A3) cujas estruturas foram sugeridas, mas ainda não confirmadas, também estavam presentes no extrato[29]. Também foi possível isolar as flavonas luteolina e apigenina a partir da ACFF. A análise quantitativa mostrou a luteolina como a flavona mais representativa dessa fração (Figura 9).

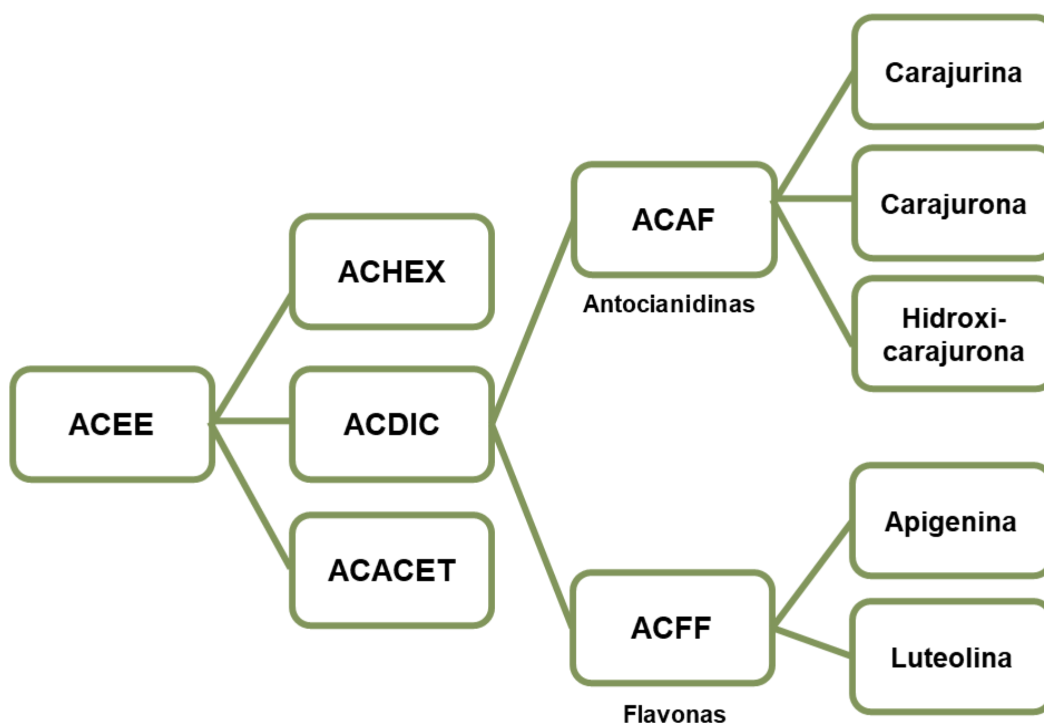

Figura 9 Fluxograma de obtenção das frações enriquecidas com flavonas e antocianidinas e substancias isoladas do extrato hidroalcóolico de *A. chica*.

Resultados anteriores do nosso grupo indicaram que a atividade leishmanicida de *A. chica* está associada ao seu perfil antocianidínico, especialmente o conteúdo de carajurina[29]. No entanto, como a *A. chica* exibe um grande conjunto de compostos polifenólicos - principalmente flavonoides, e os métodos de extração e fracionamento podem auxiliar na atividade biológica, foi realizado um estudo bioguiado a partir do extrato bruto (ACCE) para avaliar se as antocianidinas e/ou a flavonas estão envolvidas na atividade leishmanicida.

A atividade antiparasitária do extrato hidroalcoólico de *A. chica* foi relatada anteriormente contra *Trypanosoma cruzi*[27,28], e *Leishmania*[14,26]. Esses estudos anteriores também mostraram que o processo de fracionamento bioguiado de extratos pode promover um aumento progressivo da atividade contra *T. cruzi* e *Leishmania*, demonstrando o potencial desta planta como fonte de compostos biologicamente ativos. A avaliação do efeito do extrato, fração rica em antocianidinas e flavonas, e dos compostos isolados de *A. chica* contra as formas promastigotas de *L. amazonensis* mostrou que o fracionamento também contribuiu para a melhora da atividade antipromastigota. O aumento da atividade leishmanicida das frações pode estar relacionado ao aumento da concentração desses compostos bioativos em relação ao ACCE ou a um possível aumento do efeito sinérgico entre eles[117].

Assim, na tentativa de identificar o componente responsável pela atividade antipromastigota das frações, foram realizados testes biológicos com as antocianidinas e flavonas isoladas. A 3′-hidroxicarajurona e a carajurona purificadas mostraram atividade antipromastigota menos promissora do que a fração enriquecida em antocianidinas, mas a carajurina exibiu uma capacidade inibitória 6 vezes superior às outras antocianidinas testadas. A diferença de atividade leishmanicida observada está possivelmente relacionada aos diferentes padrões de hidroxilação e metoxilação no cátion flavílio (fenil-2-benzopirílio) dessas substâncias[118,119] (Figura 10). Entre as flavonas, a luteolina isolada apresentou melhor atividade do que o ACFF. A apigenina, no entanto, mostrou atividade semelhante à ACFF. Entretanto, quando se compara os resultados entre os tipos de flavonoides, pode-se observar que as antocianidias apresentam melhor atividade leishmanicida.

A carajurina destacou-se como a antocianidina mais ativa, com IC_{50} menos de 4 µg/mL após 72 h de tratamento. Contudo, utilizando um tratamento de 24 h, observou-se menor efeito sobre os macrófagos peritoneais, aumentando a

seletividade em 7 vezes (IS = 32,4), em relação ao tratamento de 72 horas, resultando em efeito dependente do tempo. Os compostos com índice de seletividade ≥ 10 são considerados compostos anti-*Leishmania* eficazes, em comparação com a citotoxicidad*in vitro*[120]. Assim, a carajurina merece ser considerada como um bom candidato para mais estudos experimentais de quimioterapia contra *Leishmania*.

A alta citotoxicidade contra células L6 (derivadas de mioblastos esqueléticos de rato) também foi relatada, em valores de IC_{50} de 18,1 µg/mL para apigenina e 9,44 µg/mL para luteolina[121] - dados que corroboram a toxicidade observada em nosso estudo. Esse efeito citotóxico talvez possa ser explicado pela presença de grupos hidroxila que possui atividades contra células tumorais, como a apigenina (4′,5,7-triidroxiflavona) que induz apoptose em linhagens celulares de hepatoma humano, Hep G2[122].

Apesar da descrição dos flavonoides mostrarem que eles possuem um amplo espectro de atividade anti-*Leishmania*[123-125], e alguns estudos terem mostrado que extratos e frações de *A. chica* possuem atividade anti-*Leishmania* contra diferentes espécies de *Leishmania*[14,26], nosso estudo é o primeiro a mostrar uma avaliação da atividade anti-*Leishmania* bioguiada de ACCE, ACAF e compostos isolados (carajurina, 3′-hidroxicarajurona e carajurona) contra *L. amazonensis*. Ensaios de atividade anti-*Leishmania* contra as formas promastigotas de *L. amazonensis* mostraram que a apigenina inibe o crescimento dos parasitos com valores de IC_{50} de 23,7 µM[126] e 22,77 µM contra cepas de *Leishmania donovani*[127]. Esta inibição também foi observada com o uso da luteolina no valor IC_{50} de 12,5 µM em atividade contra *L. donovani*[128]. Outro estudo mostra que a apigenina e a luteolina têm atividade inibitória contra amastigotas axênicas de *L. donovani* (IC_{50} 1,9 e 0,8 µg/mL, respectivamente)[121].

Entre os flavonoides ativos contra diferentes espécies de *Leishmania*, aqueles que contêm grupos metoxila em um dos anéis são referidos como moléculas muito promissoras[118,119]. Assim, os resultados antipromastigota sugerem que a presença de um grupo metoxila no anel B favorece a atividade expressa pela carajurina, uma vez que tanto carajurona quanto 3′-hidroxicarajurona, que possuem um e dois grupos hidroxila, respectivamente, e nenhum metoxila no anel B, mostrou menor atividade leishmanicida. A 3′-hidroxicarajurona foi a mais citotóxica entre os compostos avaliados, sugerindo que a presença do grupo hidroxila em 3′ aumenta o

efeito deletério sobre o macrófago. Esta observação é confirmada pela baixa citotoxicidade apresentada pela carajurona (sem grupo hidroxila em 3′), mas neste caso a ausência do grupo metoxila também leva a menor atividade leishmanicida. Além disso, foi observado que a presença da estrutura 5,7-diidroxibenzocromona aumenta muito a atividade leishmanicida, ocorrendo o mesmo com a presença da dupla ligação entre as posições 2 e 3 (C -2,3). Tais características são observadas na apigenina e na luteolina. Assim, é possível inferir que a diferente atividade leishmanicida dessas duas flavonas pode ser devido ao padrão de substituição no anel B, uma vez que a luteolina possui uma porção catecol (3',4'-diidroxifenil) enquanto a apigenina possui apenas uma hidroxila em 4'[121] (Figura 10).

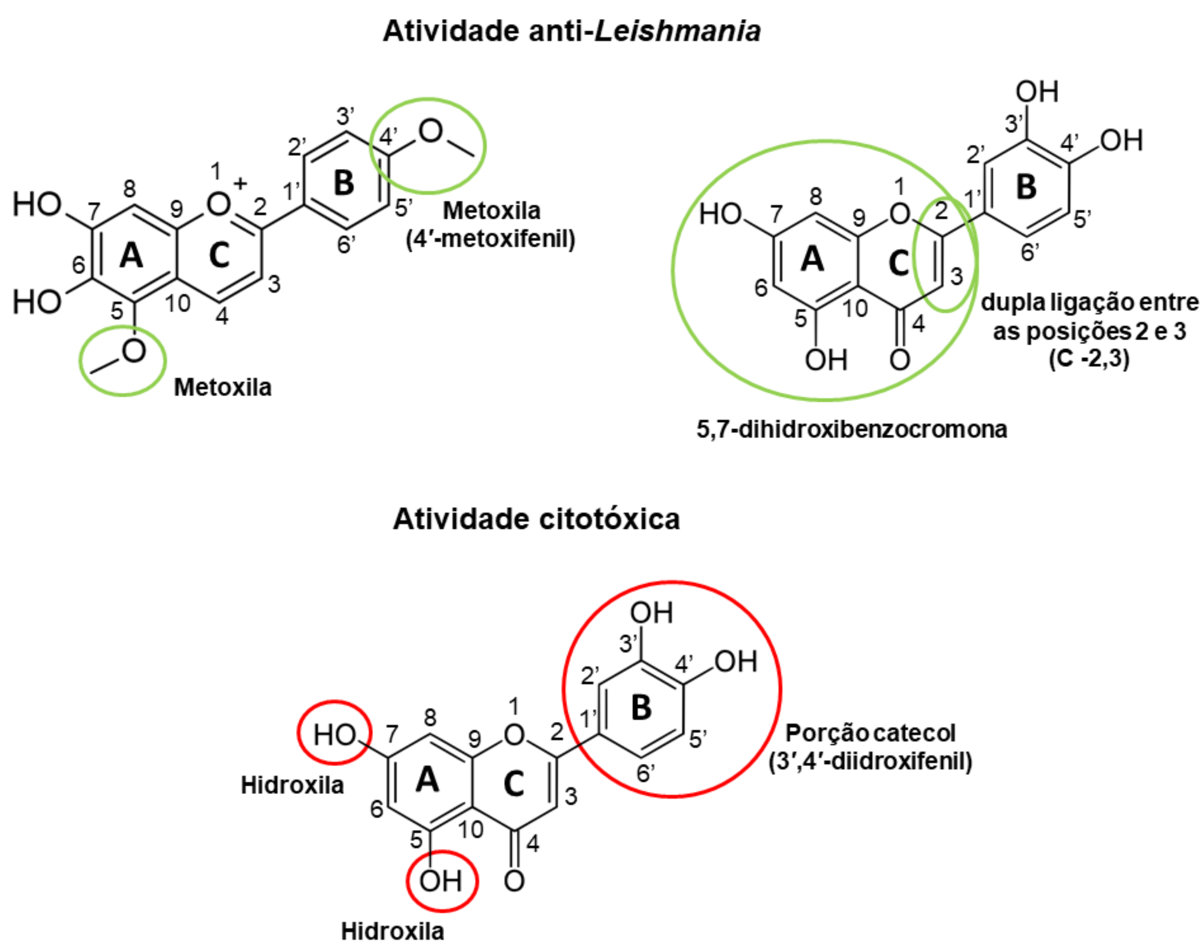

Figura 10 Requisitos estruturais observados para atividade anti-*Leishmania* e citotóxica dos flavonoides de *A. chica*.

Buscando compreender o efeito leishmanicida dos compostos, observações de alterações ultraestruturais e morfológicas são utilizadas para elucidar os mecanismos de ação de novos compostos e para investigar o mecanismo de morte celular envolvido[129]. Consequentemente, para investigar e identificar quais organelas são alvos dos compostos de *A. chica* e os danos dentro do parasito, análises ultraestruturais de promastigotas de *L. amazonensis* foram realizadas por meio de microscopia eletrônica de transmissão.

Nossos resultados mostraram que o tratamento com ACFF promoveu alterações ultraestruturais como processo de vacuolização do citoplasma, corpos lipídicos e multivesiculares, edema do cinetoplasto e mitocôndrias com quebra das cristas mitocondriais. No tratamento com luteolina, o metabólito com maior concentração na composição de ACFF, foi possível observar vacuolização do citoplasma, alteração da cromatina nuclear, corpos lipídicos, com cinetoplasto e mitocôndrias totalmente degeneradas, e vacúolos semelhantes a autofagossomos. Nas formas promastigotas tratadas com carajurina, observamos vesículas com conteúdo elétron-denso; corpúsculos elétron-densos; e edema pronunciado das mitocôndrias.

Poucas informações estão disponíveis na literatura sobre a atividade leishmanicida das antocianidinas para comparação com os resultados aqui apresentados. Contudo, alterações importantes na ultraestrutura de promastigotas de *L. infantum* também foram observadas por Rodrigues et al.[14] em parasitos tratados com a fração obtida com gradiente crescente de polaridade (hexano: acetato de etila) do extrato de hexano de *A. chica*. Nesse estudo, os parasitos expostos à fração ativa (18,6 μg/mL, 24h) apresentaram formas anormais do corpo celular. Também foi observada dilatação mitocondrial com perda do conteúdo da matriz e alterações do complexo de Golgi, seguida de processo de vacuolização do citoplasma e intenso processo exocítico de conteúdo citoplasmático para a bolsa flagelar[14]. Os experimentos com flavonoides diméricos (braquidina 2) obtidos da espécie *Arrabidaea brachypoda* também demonstraram alterações no complexo de Golgi e acúmulo de vesículas dentro da bolsa flagelar em amastigotas de *L. amazonensis*[125].

Além disso, outras drogas interferem diretamente na fisiologia mitocondrial em parasitos como a *Leishmania*[130,131]. As mitocôndrias do protozoário são consideradas um alvo ideal para fármacos, minimizando a toxicidade para as células

do hospedeiro[132,133]. Compostos antitripanossomais como feoforbídeo A[28] obtidos de folhas de *A. chica*, também afetaram a mitocôndria do parasito. As alterações ultraestruturais induzidas pelo feoforbídeo A em tripomastigotas de *T. cruzi* foram semelhantes às observadas no presente estudo para carajurina em *L. amazonensis*.

Estudos realizados por Mittra et al.[128] indicam que os flavonoides podem ter como alvo a enzima topoisomerase II no cinetoplasto dos parasitos de *Leishmania*, uma vez que esses parasitos contêm uma estrutura única de DNA dentro de suas mitocôndrias, o DNA do cinetoplasto (kDNA), que é uma rede de minicírculos interligados e envolve a manipulação mediada por topoisomerase II. Assim, um dos mecanismos de ação que possa envolver os flavonoides luteolina e a quercetina é a capacidade de induzir clivagem significativa do minicírculo de kDNA mediado por essa enzima em *Leishmania*, pois uma inibição semelhante é observada com o uso da droga pentamidina que promove a formação de um "complexo clivável" entre o kDNA e a topoisomerase II. Outro estudo elucida o mecanismo de ação da luteolina analisando as alterações mitocondriais e citosólicas associadas à morte semelhantes à apoptose das células de *L. donovani*[134]. Neste trabalho, Sen et al.[134] relatam que a inibição da produção de ATP glicolítico pela luteolina foi um evento essencial responsável pela despolarização da membrana mitocondrial em células depletadas de mt-DNA para propagar morte semelhante a apoptose em células de *Leishmania*. Esses resultados sugerem o colapso mitocondrial como parte do mecanismo de ação dos compostos de *A. chica* e demonstram seu efeito leishmanicida.

Após a observação do dano à mitocôndria do parasito causado pela carajurina, avaliamos o potencial de membrana mitocondrial por citometria de fluxo com tetrametilrodamina, éster etílico (TMRE), para confirmação dos resultados observados na microscopia eletrônica de transmissão. Observamos que a carajurina induziu a despolarização da membrana mitocondrial das formas promastigotas, mostrando que este composto é capaz de atravessar a membrana plasmática e causar o colapso da membrana mitocondrial do parasito. Vários compostos vegetais que causam dano mitocondrial e morte do parasito têm seus mecanismos de ação atribuídos principalmente à disfunção do potencial da membrana mitocondrial[14,135].

Sabendo que a produção de espécies reativas de oxigênio (ROS) em promastigotas é um dos possíveis eventos desencadeados pela perda da integridade mitocondrial[136,137], investigamos se a carajurina poderia atuar por meio

desse processo. O tratamento de promastigotas de *Leishmania* com carajurina resultou em aumento significativo dos níveis de ROS e demonstrou que a N-acetilcisteína (NAC) protegeu os parasitos da inibição por carajurina, além de reduzir os níveis de ROS em promastigotas tratadas com carajurina. NAC é um composto de tiol que aumenta os níveis de glutationa[138]; é uma molécula importante para proteger os cinetoplastos de ROS ou compostos tóxicos, atuando como um antioxidante[139]. Esses resultados indicam que a inibição do crescimento promovido pela carajurina em *L. amazonensis* é mediada pela produção de ROS, o que talvez pode explicar a despolarização induzida da membrana mitocondrial para este estágio do parasito. Estudos realizados por Fonseca-Silva et al.[133] relataram que a disfunção mitocondrial observada no promastigota de *L. amazonensis* tratado com quercetina é promovida pela produção de ROS, da mesma forma que a apigenina[126], para os mesmos parasitos. Além disso, os resultados desses compostos sugerem o envolvimento de ROS levando a uma alteração do potencial da membrana mitocondrial como parte do mecanismo de ação.

A produção de ROS mitocondrial seguida pela despolarização da membrana mitocondrial pode desencadear a morte do parasito por meio de um mecanismo semelhante à apoptose[132,136,140,141]. Promastigotas de *L. amazonensis* tratadas com carajurina foram duplamente coradas com Anexina V e iodeto de propídio para avaliar a indução da morte celular, observou-se que a carajurina induziu apoptose tardia nos parasitos (Figura 11).

Semelhante aos nossos achados, estudos anteriores relataram a indução de morte celular em promastigotas de *L. amazonensis* induzida por compostos isolados de produtos naturais[142,143]. Além disso, a luteolina e a quercetina inibiram a síntese de DNA em promastigotas de *L. donovani* e promoveram a linearização mediada por topoisomerase-II de minicírculos de kDNA, levando à apoptose[128]. Outros estudos mostraram que os flavonoides fisetina, quercetina e luteolina inibem a enzima arginase de *L. amazonensis*[144,145]. A privação de L-arginina promove uma externalização de fosfolipídios que se ligam à anexina V, sinalizando a morte celular semelhante a apoptose em promastigotas de *L. donovani*[146].

Para analisar o mecanismo de ação da carajurina, o comportamento eletroquímico do composto foi analisado por voltametria cíclica (VC) em um ambiente orgânico aprótico (diclorometano), a fim de mimetizar o ambiente celular apolar[147,148]. VC é um método simples para rastrear compostos redox ativos e

estimar a atividade eletroquímica em diferentes amostras, como plantas medicinais[149]. Em condições aeróbias, predomina o mecanismo de redução do composto, resultando no radical ânion intermediário, que, ao sofrer um processo de retro-oxidação na presença de oxigênio, libera ROS, semelhante ao descrito para outros compostos com ação contra parasitos[147,148,150,151]. Ao analisar a eletroatividade da carajurina, pode-se observar que na ausência de oxigênio molecular o composto apresenta um processo de redução, o que pode levar à geração do radical intermediário. Também foi observado presença de oxigênio molecular, o voltamograma mostra uma mudança no perfil, indicando uma possível interação de produtos de eletrorredução de carajurina com oxigênio dissolvido na célula eletroquímica[147,148], esses efeitos incluem uma mudança da posição do pico de redução da carajurina para potenciais mais positivos. Além disso, os valores do orbital molecular mais alto ocupado (HOMO, do inglês *Highest Occupied Molecular Orbital*) e do mais baixo orbital molecular desocupado (LUMO, do inglês *Lowest Unoccupied Molecular Orbital*) obtidos em voltametria e estudos quânticos sugerem que a carajurina pode adquirir um elétron mais facilmente do que doá-lo, favorecendo sua ação na geração de ROS do que uma ação antioxidante, de acordo com os resultados obtidos em estudos computacionais[152].

Na busca por novos fármacos anti-*Leishmania*, a forma amastigota intracelular é o estágio do parasito considerado o alvo mais relevante para a triagem primária de novos compostos[153], e o indicador mais consistente de atividade *in vivo*[154], adicionalmente considerado "o padrão ouro" de estudos *in vitro*[155]. Portanto, carajurina, ACAF, ACFF e Luteolina foram selecionados para avaliar a atividade contra formas amastigotas intracelulares, por apresentarem a melhor atividade contra formas promastigotas de *L. amazonensis*. Além disso, mesmo com a indicação de possível ação sinérgica dos compostos apresentados na fração, é importante avaliar a atividade do composto majoritário isolado tanto para ACAF quanto para ACFF, na tentativa de caracterizar o possível marcador biológico da espécie *A. chica*.

Foi possível observar melhora na atividade leishmanicida para ACFF e luteolina, com 11,3 e 2,67 vezes mais ativos contra as formas amastigotas intracelulares do que contra as formas promastigotas, respectivamente. Entre carajurina e ACAF, não houve diferença significativa em relação às formas amastigotas intracelulares, apenas uma diferença na análise dos dados de

citotoxicidade, resultando em maior seletividade da carajurina, inclusive com índice de seletividade semelhante quando comparados ao medicamento utilizado como referência, anfotericina B. Os parâmetros de infecção apresentaram redução estatisticamente significativa para o tratamento com todas as amostras avaliadas.

Um estudo realizado por Wong et al.[124] relatou que a luteolina exibiu atividade promissora apenas contra o amastigota intracelular, mas não para promastigotas extracelulares, sugerindo que seus alvos específicos estão presentes apenas na fase intracelular. Essa atividade também foi observada em outro estudo contra *L. donovani*, segundo o qual a luteolina reduziu a carga de amastigotas intracelulares em 70% a uma concentração final tão baixa quanto 12,5 µM[128]. Portanto, a alta concentração de luteolina no ACFF pode ser responsável pelo aumento da inibição contra as formas intracelulares do parasito.

Ao comparar os resultados da atividade antipromastigota contra a fase intracelular, o ACFF foi 3,2 vezes mais ativo do que a luteolina. Vale ressaltar que outra flavona presente na composição do ACFF foi a apigenina, mas esse composto não foi testado contra a forma amastigota intracelular devido ao seu baixo rendimento no processo de extração. No entanto, dados da literatura mostram o efeito inibitório significativo da apigenina contra as formas amastigotas intracelulares de *L. donovani*, em valores de IC_{50} de 45,66 ± 0,01 µM (12,34 µg/mL). Além disso, quando os macrófagos infectados foram tratados com concentrações crescentes de apigenina, houve uma diminuição no número de células infectadas[127]. Portanto, isso nos leva a inferir que a presença de alguns compostos no ACFF pode contribuir para a atividade leishmanicida e diminuir o efeito citotóxico, favorecendo a maior seletividade ao parasito observada ao ACFF quando comparado à luteolina. Essas descobertas são frequentemente consideradas como resultado de um efeito sinérgico ou aditivo dos constituintes do extrato[156].

Apenas algumas espécies do gênero *Arrabidaea* foram investigadas quanto à sua atividade antiprotozoária. Flavonoides diméricos purificados de *Arrabidaea brachypoda* apresentaram atividade anti-*Leishmania*[125]. Ao quantificar os macrófagos infectados *in vitro*, constatou-se que o composto braquidina B foi o mais ativo contra amastigotas intracelulares de *L. amazonensis*, sem apresentar toxicidade celular do hospedeiro. Possivelmente, a braquidina B era mais ativa devido à presença do grupo metoxila, necessário para melhorar a penetração na membrana[125]. Portanto, isso nos leva a inferir que a presença de dois grupos

metoxila na estrutura da carajurina possivelmente seja responsável por sua maior atividade contra as formas amastigotas intracelulares de *L. amazonensis* (Figura 10).

As diferenças observadas na atividade leishmanicida das amostras contra promastigotas de *Leishmania* e amastigotas intracelulares podem ser devidas a características bioquímicas ou metabólicas diferentes dos dois estágios do parasito[157]. O efeito sobre amastigotas intracelulares pode ser indicativo de ativação das funções leishmanicidas dos macrófagos, especialmente induzindo óxido nítrico (NO)[158,159]. Em nossos estudos foram quantificados o nitrito como forma indireta de determinar os níveis de NO na tentativa de compreender a atividade leishmanicida de ACAF, ACFF, luteolina e carajurina contra formas amastigotas intracelulares, e os resultados mostraram que não houve mudanças significativas na quantificação de nitrito para ACFF e luteolina. Em um estudo foi reportado que a inibição da produção de NO pela apigenina e luteolina pode estar relacionado à presença da dupla ligação C-2,3 e que os padrões de substituição de moléculas de flavonoides podem determinar a potência de inibição na produção de NO[160]. Além disso, também foi observada supressão na produção de NO e prostaglandina E2 (PGE2), sem apresentar citotoxicidade em células de macrófagos de camundongo RAW 264.7 ativadas por lipopolissacarídeo bacteriano quando expostas a flavonas, luteolina e sua luteolina-7-O-glicosídeo. A supressão da enzima óxido nítrico sintase induzível (iNOS) e a expressão da proteína ciclooxigenase-2 (COX-2) são responsáveis pelos efeitos inibitórios, e não pela redução da atividade enzimática[161]. Diferentes condições de cultura e tipos de células utilizadas nos experimentos também podem ser responsáveis por algumas diferenças entre os dados descritos na literatura e os resultados obtidos no presente estudo. Portanto, outros mecanismos podem estar envolvidos na atividade leishmanicida de ACFF e luteolina contra amastigotas intracelulares.

Resultados diferentes foram observados para a carajurina. Estudos com *A. chica* descritos por Rodrigues et al.[14] e Da Silva et al.[9] relacionados ao NO e seus efeitos na *Leishmania* confirmam nossos resultados. Após o tratamento com uma fração hexano: acetato de etila, obtido do extrato de *A. chica* hexano, detectou-se os níveis de nitrito no sobrenadante de macrófagos infectados por *L. amazonensis* e *L. infantum* e observou-se que estes níveis de nitrito foram maiores do que aqueles encontrados em culturas de células infectadas e não tratadas[14]. Esses resultados ajudam a contribuir para um melhor entendimento da atividade leishmanicida do

ACAF e da carajurina, pois essa ação estaria associada à capacidade de induzir a ativação da resposta microbicida em macrófagos e promover a produção de NO, os quais levam à morte de amastigotas (Figura 11). Assim, e considerando que todas as isoenzimas da NOS são hemodiméricas, investigamos se o aumento dos níveis de NO investigado pelo método de Griess, que corresponde à atividade leishmanicida relatada em nosso estudo, pode corresponder a um mecanismo de ativação de uma das isoformas desta enzima. Nesse sentido, estudos de docking molecular foram direcionados à análise de possíveis interações da carajurina com sítios de ativação enzimática, principalmente na região importante no processo de dimerização, conforme relatado na literatura.

Uma análise mais precisa da interação mostra que carajurina ocupa o sítio de ligação de 2-amino-6-(1,2-diidroxipropil) octaidropteridin-4 (1 H)-ona (ligante co-cristalizado), para o qual os estudos de redocking mostraram um excelente padrão de alinhamento. No modo de ligação previsto na estrutura 1DF1, carajurina interage com o local de ativação do NOS, especialmente por meio de duas interações do tipo ligação de hidrogênio não covalentes entre os hidrogênios dos grupos hidroxila da carajurina e os oxigênios dos grupos hidroxila e carbonila, presentes no resíduo de serina (112). Além disso, há interação com o resíduo 375 e proximidade espacial com o grupo heme (901), o que é de grande importância no processo de dimerização e ativação dessa enzima. A iNOS ou isoforma II não é expressa constitutivamente, ou seja, não está normalmente presente, sendo induzida em macrófagos e outras células por lipopolissacarídeos bacterianos e/ou citocinas. Esta isoenzima também pode ser chamada de macNOS (macrófago NO sintase). Uma vez induzida, a iNOS é capaz de produzir NO por muito tempo, o que caracteriza seu envolvimento em diversos processos patológicos.

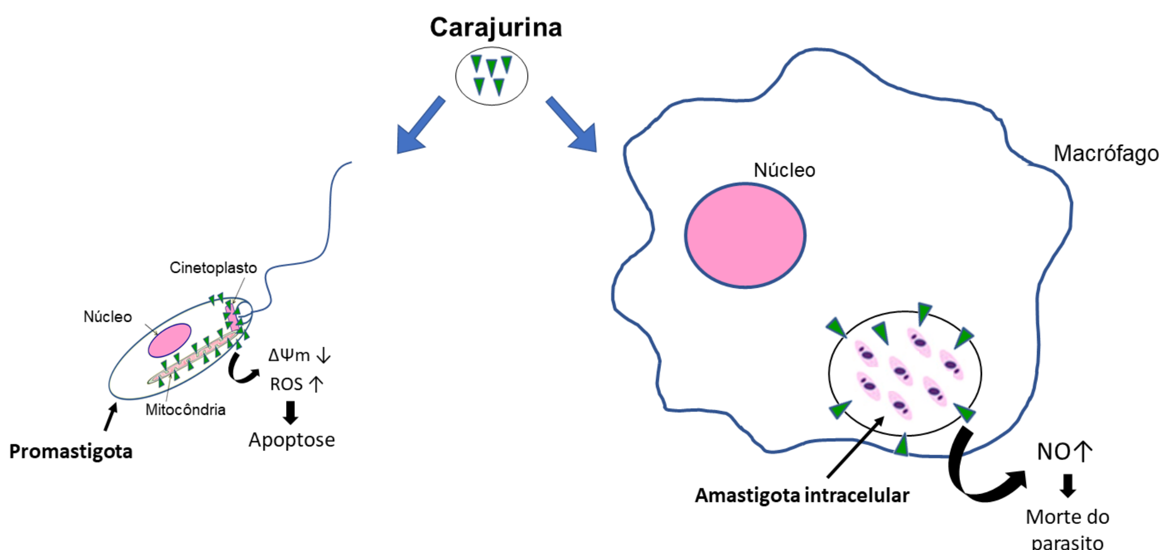

Figura 11 Mecanismo de ação da carajurina contra formas promastigotas e amastigote intracelular de *L. amazonensis*.

Avaliação de propriedades físico-químicas (peso molecular, ligações rotacionais, aceitadores de H-bond, doadores de H-bond, área de superfície TPSA-Å2, e lipofilicidade (log Po/w)) também foram realizadas, e os resultados para a carajurina foram de acordo com o que é esperado para um candidato a fármaco, uma vez que não violam nenhuma das regras estabelecidas por Lipinski, Ghose, Veber, Egan e Mueggue. Além disso, a carajurina não apresentava compostos de interferência de ensaio pan (PAINS, do inglês *Pan Assay INterference compoundS*). Estes resultados fazem avançar os nossos conhecimentos sobre os mecanismos envolvidos no efeito leishmanicida da carajurina, construindo bases sólidas para a descoberta de medicamentos e abrindo novas oportunidades para a investigação nesta área significativa da saúde humana.

Encorajados por esses resultados, direcionamos o processo de fracionamento para uma fração com a carajurina como principal componente. O fracionamento levou a uma fração muito mais concentrada de carajurina (ACCF), quando comparada a fração rica em antocianidinas (ACAF) obtida anteriormente[162]. Assim, investigamos a atividade anti-*Leishmania* da fração rica em carajurina de *Arrabidaea chica* em camundongos BALB/c infectados por via subcutânea com *L. amazonensis*.

Nossos resultados mostraram que a maior dose utilizada de ACCF (200 $mg.kg^{-1}.dia^{-1}$) exibiu uma carga parasitária significativamente mais baixa quando

comparada com os animais infectados e não tratados. No entanto, não encontramos correlação entre o tamanho da lesão e a carga parasitária. Animais tratados com ACCF, na dose de 50 $mg.kg^{-1}.dia^{-1}$, por exemplo, apesar de apresentarem lesões menores quando comparados aos animais tratados com as demais doses, ou com animais não tratados, apresentavam carga parasitária muito semelhante a dos grupos infectados e não tratados. No entanto, o tratamento realizado com a dose de 200 $mg.kg^{-1}.dia^{-1}$ de ACCF não apresentou diferença no tamanho das lesões quando comparado aos animais não tratados, mas com redução significativa da carga parasitária. Foi descrito na literatura que animais infectados com *L. amazonensis* e tratados com extrato hidroalcoólico bruto de *Chenopodium ambrosioides* apresentaram aumento da espessura do coxim plantar, apesar da baixa carga parasitária[163]. Dados da literatura correlaciona o aumento do tamanho das lesões com a presença de edema e o influxo de células inflamatórias ao local da infecção primária. Além disso, a formação de edema no coxim plantar do animal pode ser mantida mesmo sem a presença da amastigota, o que justifica o tamanho da lesão[164].

A baixa carga parasitária em animais tratados com ACCF possivelmente pode ser explicada pela capacidade da carajurina aumentar a produção de óxido nítrico e expressão de iNOS em macrófagos peritoneais infectados com *L. amazonensis*, como foi descrito por nossos estudos *in vitro* e *in sílico*[162]. Além disso, também foram observados altos níveis de IFN-γ e iNOS no tratamento com ACCF. O mecanismo mais eficiente de morte do parasito envolve a produção de IFN-γ e TNF-α, que estimulam a síntese de óxido nítrico sintase induzível (iNOS), gerando a produção de óxido nítrico (NO), uma potente citotoxina envolvida na inibição de *Leishmania*[165-167].

Também foi avaliada a quantificação das citocinas no soro dos animais tratados com ACCF. Nossos resultados revelaram diminuição nos níveis de IL-4 e IL-10, o que contribui para a manutenção de uma resposta Th1 nos grupos tratados. Nenhuma mudança foi observada em IL-2, IL-12 ou TGF-β. No entanto, níveis aumentados de IFN-γ e TNF-α foram observados devido ao tratamento com ACCF. A resistência à leishmaniose está relacionada ao desenvolvimento Th1 e à produção de citocinas pró-inflamatórias como IL-2, IFN-γ e TNF-α que levam à ativação de macrófagos e morte do parasito[168,169]. Portanto, o aumento dessas citocinas contribui para a eficácia dos macrófagos por induzir a iNOS e, consequentemente,

diminuir a carga parasitária. Somado a isso, animais tratados com ACFF não apresentaram alterações nos parâmetros de toxicidade analisados, mostrando que a fração rica em carajurina não irrita o aparelho digestivo, bem como não modifica peso dos animais e não altera os parâmetros bioquímicos séricos da função hepática e renal. Portanto, o tratamento com ACCF não tem efeito tóxico aparente em camundongos.

Em conjunto, nossos resultados fornecem evidências adicionais sobre a atividade anti-*Leishmania* induzida por *A. chica*, principalmente no conteúdo de carajurina. Dessa forma, podemos sugerir que nossos resultados podem ajudar a elucidar os possíveis mecanismos de ação pelos quais a carajurina atua, promovendo um efeito leishmanicida, bem como nossos dados comprovaram a eficácia do ACCF na redução da carga parasitária, demonstrando seus efeitos modulatórios na expressão de citocinas, sem apresentar toxicidade.

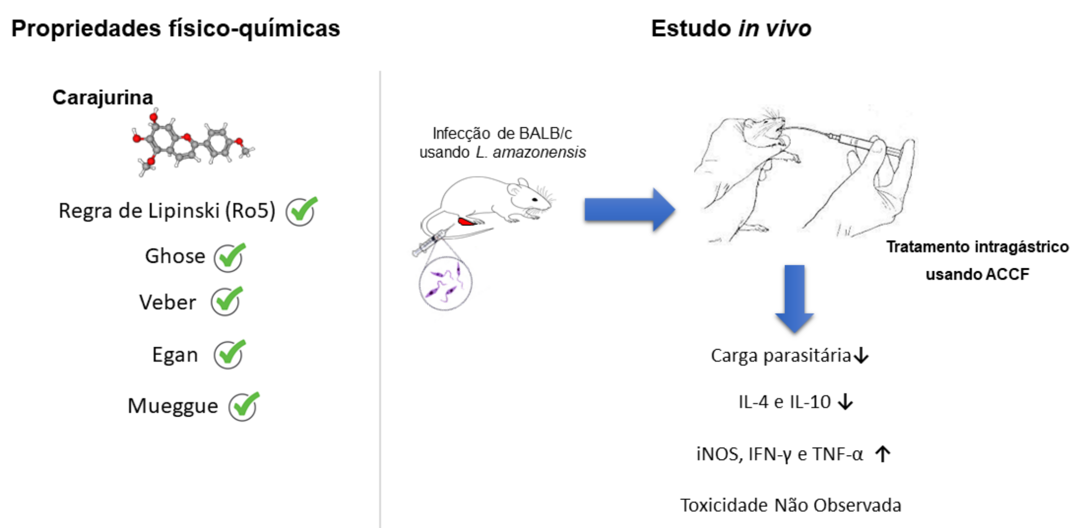

Figura 12 Propriedades físico-químicas da cajurina e avaliação *in vivo* da fração rica em carajurina (ACCF).

5 CONCLUSÕES

Extrato bruto obtido de *A. chica* Verlot (ACCE), fração rica em flavonas (ACFF), fração rica em antocianidinas (ACAF), flavonas e antocianidinas apresentaram atividade leishmanicida contra promastigotas de *L. amazonensis*.

O efeito antiparasitário do ACAF, ACFF, luteolina e da carajurina foi confirmado pelas alterações ultraestruturais com indução de dano mitocondrial observadas.

O efeito letal da carajurina nas formas promastigotas de *L. amazonensis* é resultado de alterações ultraestruturais, diminuição do potencial de membrana mitocondrial e aumento da produção de ROS, que juntos induziram a morte celular por apoptose tardia.

As frações ACAF e ACFF apresentaram baixa citotoxicidade nas células hospedeiras se comparadas às flavonas e antocianidinas isoladas.

ACAF, ACFF, luteolina e carajurina apresentaram atividade leishmanicida contra os amastigotas intracelulares, entretanto, somente a carajurina foi capaz de aumentar os níveis de nitrito no macrófago estimulado ou não com *L. amazonensis*, cujos estudos de docking mostraram uma possível interação da carajurina no sítio de ativação da enzima NOS, hipótese que corrobora os resultados *in vitro* obtidos.

As propriedades físico-químicas da carajurina não violaram nenhuma das regras estabelecidas por Lipinski, Ghose, Veber, Egan e Mueggue.

Fração rica em carajurina (ACCF) diminuiu a carga parasitária na pata de camundongos BALB/c infectados com *L. amazonensis*, além de mostrar efeito modulatórios na expressão de citocinas, sem causar toxicidade.

6 PERSPECTIVAS

Avaliar atividade enzimática *in silico* e *in vitro* da carajurina com o objetivo de melhorar a compreensão do mecanismo pelo qual a carajurina atua na promoção de um efeito leishmanicida.

Investigar os parâmetros farmacocinéticos (ADME) e de toxicidade da carajurina, utilizando uma combinação de estratégias *in silico* e *in vitro*.

Desenvolver formas farmacêuticas contendo a fração rica em carajurina com a finalidade de melhorar sua solubilidade e consequentemente sua biodisponibilidade.

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8 ANEXOS

8.1 ANEXO A - Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado - SisGen

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A670412

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A670412**
Usuário:	**Fiocruz**
CPF/CNPJ:	**33.781.055/0001-35**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	

☑ **Pesquisa Científica** ☐ **Bioprospecção** ☐ **Desenvolvimento Tecnológico**

Espécie
Arrabidaea chica
Phyllanthus sp
Phyllanthus sp
Phyllanthus tenellus
Phyllanthus amarus
Phyllanthus tenellus
Phyllanthus amarus
Phyllanthus niruri
Phyllanthus tenellus
Phyllanthus tenellus
Phyllanthus tenellus
Phyllanthus tenellus

Phyllanthus tenellus

Phyllanthus caroliniensis

Phyllanthus urinaria

Phyllanthus tenellus

Phyllanthus tenellus

Phyllanthus tenellus

Phyllanthus tenellus

Phyllanthus urinaria

Phyllanthus amarus

Phyllanthus amarus

Phyllanthus amarus

Phyllanthus amarus

Phyllanthus urinaria

Phyllanthus niruri

Phyllanthus tenellus

Phyllanthus tenellus

Phyllanthus amarus

Phyllanthus stipulatus

Phyllanthus niruri

Phyllanthus tenellus

Phyllanthus tenellus

Phyllanthus tenellus

Phyllanthus tenellus

Phyllanthus niruri

Phyllanthus tenellus

Phyllanthus niruri

Phyllanthus tenellus

Phyllanthus tenellus

Alpinia zerumbet

Arrabidaea chica

Arrabidaea chica

Varronia curassavica

Mikania laevigata

Mikania glomerata

Título da Atividade: **Estudo químico e biológico de espécies vegetais**

Equipe

Maria das Dores Dutra Behrens	**Fiocruz**
Antonio Carlos Siani	**Fiocruz**
Ana Lucia Abreu Silva	**UFMA**
Carla Junqueira Moragas Tellis	**Fiocruz**
Cintia Ribeiro Manhães Vieira	**IVB**
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Dijalma Barbosa da Silva	**Embrapa**
Daniel Gibaldi	**Fiocruz**
Edmir Fernandez Ferreira	**Fiocruz**
Elisabete Pereira dos Santos	**UFRJ**
Fernando Almeida Souza	**Fiocruz**
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Marcelo Torres Bozza	**Fiocruz**
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Marcos Jun Nakamura	**Fiocruz**

Paulo Victor Ramos de Souza	**Fiocruz**
Ramon Gredilha Paschoal	**Fiocruz**
Renata Oliveira Almeida Soares	**Fiocruz**
Roberto Fontes Vieira	**Embrapa**
Rosa de Belém das Neves Alves	**Embrapa**
Sérgio da Silva Monteiro	**Fiocruz**
Virginia Garcia Correia	**Fiocruz**
Humberto Ribeiro Bizzo	**Embrapa**

Parceiras Nacionais

00.348.003/0038-02 / Embrapa Recursos Genéticos e Biotecnologia

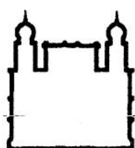
Data do Cadastro: **05/11/2018 13:13:04**

Situação do Cadastro: **Concluído**

Conselho de Gestão do Patrimônio Genético

Situação cadastral conforme consulta ao SisGen em **13:16** de **05/11/2018**.

8.2 ANEXO B - Comissão de Ética no Uso de Animais do Instituto Oswaldo Cruz (CEUA-IOC)



Instituto Oswaldo Cruz

Comissão de Ética no Uso de Animais - CEUA/ IOC

LICENÇA **L-053/2016**

Certificamos que o protocolo (CEUA/IOC-041/2016), intitulado "Desenvolvimento de tratamento contra as leishmanioses: ação leishmanicida in vitro e in vivo de extratos, frações e óleos de origem vegetal", sob a responsabilidade de **KATIA DA SILVA CALABRESE** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais, inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.

Esta licença tem validade até 31/12/2020 e inclui o uso total de:

Camundongo, cepa:

Mus – 3708 animais: 1008 animais machos – 2700 animais fêmeas – 15-18g

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 28 de dezembro de 2016.

Flávio Alves Lara

Coordenador da CEUA/Instituto Oswaldo Cruz

Fundação Oswaldo Cruz

FIOCRUZ-Fundação Oswaldo Cruz/IOC-Instituto Oswaldo Cruz
Av. Brasil, 4365 - Manguinhos - Rio de Janeiro - RJ - Brasil
CEP: 21040-360 Tel: (21) 2562-1056

8.3 ANEXO C – Produção Acadêmica

ARTIGOS PUBLICADOS

1. **da Silva E Silva JV**, Cordovil Brigido HP, Oliveira de Albuquerque KC, Miranda Carvalho J, Ferreira Reis J, Vinhal Faria L, Coelho-Ferreira M, Silveira FT, da Silva Carneiro A, Percário S, do Rosário Marinho AM, Dolabela MF**. Flavopereirine-An Alkaloid Derived from *Geissospermum vellosii*-Presents Leishmanicidal Activity *In Vitro*.** Molecules. 2019; 24(4):785. doi: 10.3390/molecules24040785**.**
2. Silva CMd, Eiró LG, **Silva-Silva JV**, Corrêa-Barbosa J, Coelho-Ferreira MR, Silveira FT, Marinho AMdR, Almeida MdGC, Brigido HPC, Dolabela MF. ***In vitro* antileishmanial activity and phytochemical analysis of *Casearia javitensis* Kunth (Salicaceae)**. Revista Fitos. 2020; 14(4):528-537. doi: 10.32712/2446-4775.2020.950.
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7. **Silva-Silva JV**, Moragas-Tellis CJ, Chagas MDSDS, de Souza PVR, de Souza CDSF, Hardoim DJ, Taniwaki NN, Moreira DL, Dutra Behrens M, Calabrese KDS, Almeida-Souza F. **Antileishmanial Activity of Flavones-Rich Fraction From**

Arrabidaea chica **Verlot (Bignoniaceae)**. Front Pharmacol. 2021; 12:703985. doi: 10.3389/fphar.2021.703985.

8. **Silva-Silva JV**, Moragas-Tellis CJ, Chagas MSS, Souza PVR, Moreira DL, de Souza CSF, Teixeira KF, Cenci AR, de Oliveira AS, Almeida-Souza F, Behrens MD, Calabrese KS. **Carajurin: a anthocyanidin from *Arrabidaea chica* as a potential biological marker of antileishmanial activity**. Biomed Pharmacother. 2021; 141:111910. doi: 10.1016/j.biopha.2021.111910.

9. Pina JRS, **Silva-Silva JV**, Carvalho JM, Bitencourt HR, Watanabe LA, Fernandes JMP, Souza GE, Aguiar ACC, Guido RVC, Almeida-Souza F, Calabrese KDS, Marinho PSB, Marinho AMDR. **Antiprotozoal and Antibacterial Activity of Ravenelin, a Xanthone Isolated from the Endophytic Fungus *Exserohilum rostratum***. Molecules. 2021; 26(11):3339. doi: 10.3390/molecules26113339.

10. Faria LV, Brígido HPC, Bentaberry-Rosa AA, Correa-Barbosa J, **Silva-Silva JV**, Bastos MLC, Costa EVS, Coelho-Ferreira M, Silveira FT, Dolabela MF. **Anti-leishmania activity of extract and fractions from the stem and leaf of *Montrichardia linifera* (Arruda) schott (Araceae) against *Leishmania amazonensis***. Research, Society and Development. 2021; 10(2):e9310212312. doi: 10.33448/rsd-v10i2.12312.

11. Teles AM, **Silva-Silva JV**, Fernandes JMP, Calabrese KDS, Abreu-Silva AL, Marinho SC, Mouchrek AN, Filho VEM, Almeida-Souza F. ***Aniba rosaeodora*** **(Var. amazonica Ducke) Essential Oil: Chemical Composition, Antibacterial, Antioxidant and Antitrypanosomal Activity. Antibiotics (Basel)**. 2021; 10(1):24. doi: 10.3390/antibiotics10010024.

12. **Silva-Silva JV**, Moragas-Tellis CJ, Chagas MSS, Souza PVR, Moreira DL, Hardoim DJ, Taniwaki NN, Costa VFA, Bertho AL, Brondani D, Zapp E, Oliveira AS, Calabrese KS, Behrens MD, Almeida-Souza F. **Carajurin Induces Apoptosis in *Leishmania amazonensis* Promastigotes through Reactive Oxygen Species Production and Mitochondrial Dysfunction**. Pharmaceuticals. 2022.

PREMIAÇÕES

1. This is to certify that the abstract Antileishmanial Activity of *Geissospermum Allemão* (Apocynaceae). **João Victor da Silva e Silva**; Andrey Moacir do Rosário Marinho; Fernando Tobias Silveira; Sandro Percário; Maria Fâni Dolabela. Was presented as pôster in the 4th International Symposium on Challenges and New Technologies in Drug Discovery & Pharmaceutical Production held in Rio de Janeiro, Brasil, 7th-9th November, 2017. Sendo premiado como **Melhor Pôster na categoria Pesquisa científica**.

2. Certificamos que o trabalho 17.012 - *In silico* studies and antileishmanial activity of ravenelin B isolated from fungus *Exserohilum rostratum* - **Silva-Silva JV**1, Pina JRS2, Souza CSF1, Fernandes JMP, Lima AHL3, Almeida-Souza F4, Marinho AMR2, Marinho PSB2, Calabrese KS, 1 Laboratório de Imunomodulação e Protozoologia - FIOCRUZ 2 Laboratório de Química e Pesquisa - UFPA 3 Laboratório de Planejamento e Desenvolvimento de Fármacos LPD - UFPA 4 Pós-Graduação em Ciência Animal - UEMA foi contemplado com **MENÇÃO HONROSA** durante a **XXXIV Reunião Anual da FeSBE**, realizado de 09 de setembro de 2019 de setembro de 2019 no Campos do Jordão Convention Center.

3. Declaro para os devidos fins que o egresso do Programa de Pós-Graduação em Medicina Tropical, **João Victor da Silva e Silva** recebeu **bolsa de doutorado FAPERJ Nota 10** no período de 08/2019 a 09/2021. O Programa de Pós-graduação *Stricto sensu* em Medicina Tropical (Mestrado e Doutorado) está credenciado pela CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, de acordo com Portaria no. 1740, de 20.12.94, do Ministro de Estado da Educação e do Desporto e obteve o conceito 6 (seis) na última avaliação quadrienal da CAPES.

PARTICIPAÇÃO EM BANCA

1. Certificamos que **João Victor da Silva e Silva** participou da comissão científica para a revisão de trabalhos encaminhados ao **II CONGRESSO DE TECNOLOGIAS E DESENVOLVIMENTO NA AMAZÔNIA e III CONGRESSO DE EDUCAÇÃO E SAÚDE DO SUDESTE DO PARÁ. 2018. Universidade Federal do Pará**, realizado nos dias 7, 8 e 9 de novembro de 2018 em Tucuruí (PA).

2. Certificamos que, **João Victor da Silva e Silva** Participou da **banca de julgamento** do trabalho intitulado "Avaliação da eficácia do pré-natal e dos cuidados da atenção primária à saúde para a redução da mortalidade infantil com enfoque em puérperas de situação socioeconômica vulnerável no município de Santa Inês-MA", durante as **defesas dos projetos de pesquisa do módulo de Métodos de Estudo e Pesquisa I (MEP I)**, realizada no dia 15 de janeiro de 2021, no Curso de Medicina da Faculdade ITPAC, na cidade de Santa Inês - MA.

3. Certificamos que, **João Victor da Silva e Silva** Participou da **banca de julgamento** do trabalho intitulado "Parâmetros de biossegurança adotados pelos profissionais da área da saúde após o surgimento da COVID-19 em Santa Inês", durante as defesas dos projetos de pesquisa do módulo de Métodos de Estudo e Pesquisa I (MEP I), realizada no dia 15 de janeiro de 2021, no Curso de Medicina da Faculdade ITPAC, na cidade de Santa Inês - MA.

4. Certificamos que, **João Victor da Silva e Silva** Participou da **banca de julgamento** do trabalho intitulado "Estudo clínico vinculado a não utilização da técnica de mastectomia por efeito da quimioterapia neoadjuvante", durante as **defesas dos projetos de pesquisa do módulo de Métodos de Estudo e Pesquisa I (MEP I)**, realizada no dia 15 de janeiro de 2021, no Curso de Medicina da Faculdade ITPAC, na cidade de Santa Inês - MA.

5. Certifico, para fins de comprovação curricular, que o Prof **João Victor da Silva e Silva** participou **da Banca Examinadora do Trabalho de Conclusão de Curso (TCC II)**, que se constitui como requisito final para obtenção do grau de Bacharel em Biomedicina, da estudante Jênifer Johnes Gonçalves Fiuza, cujo título "Aplicabilidade da técnica de sequenciamento de nova geração com enfoque na aclaração de investigações na perícia forense."

PARTICIPAÇÃO EM CURSOS E EVENTOS

1. Certificamos que Juliana Correa Barbosa, Lara Vinhal Faria, Alexandre Augusto Rosa, Marcio Luiz Costa Amaro, Milena Cristina Martins Da Silva, **João Victor da Silva e Silva**, Heliton Patrick Cordovil Brígido, Andreza Do Socorro Silva Veiga and Maria Fâni Dolabela participaram do **XXV Congresso Brasileiro de Parasitologia**, realizado em Búzios, de 03 a 06 de setembro de 2017, no Rio de Janeiro, com o

trabalho Phytochemical studies and evaluation of leishmanicide activity of Montrichardia linifera (Arruda) Schott.

2. This is to certify that **João Victor da Silva e** Silva attended the **4th International Symposium on Challenges and New Technologies in Drug Discovery & Pharmaceutical Production** held in Rio de Janeiro, Brasil, 7th-9th November, 2017.

3. This is to certify that the abstract Antileishmanial Activity of *Geissospermum Allemão* (Apocynaceae). **João Victor da Silva e Silva**; Andrey Moacir do Rosário Marinho; Fernando Tobias Silveira; Sandro Percário; Maria Fâni Dolabela. Was presented as pôster in the **4th International Symposium on Challenges and New Technologies in Drug Discovery & Pharmaceutical Production** held in Rio de Janeiro, Brasil, 7th-9th November, 2017.

4. This is to certify that the abstract Phytochemical Study and Leishmanicidal Activity of Montrichardia Linifera (Arruda) Schott. **João Victor da Silva e Silva**; Lara Vinhal Faria; Heliton Patrick Cordovil Brigido; Kelly Cristina Oliveira de Albuquerque; Josiwander Miranda Carvalho; Maria Fâni Dolabela. Was presented as pôster in the **4th International Symposium on Challenges and New Technologies in Drug Discovery & Pharmaceutical Production** held in Rio de Janeiro, Brasil, 7th-9th November, 2017.

5. Certificamos que **João Victor da Silva e Silva** participou do **VI Ciclo Carlos Chagas de Palestras**, nos dias 05 e 06 de abril de 2018, totalizando 10 horas, realizado na Fiocruz – Rio de Janeiro, RJ.

6. Certificamos que o trabalho intitulado "Molecular docking studies and antitrypanosomal activity of monomethylsulochrin and pseutotin A isolated from *Aspergillus* ssp", de autoria de **João Victor Silva-Silva**; Jordano Ferreira Reis; Luciano Almeida Watanabe; Rosiane Fernandes Moreira; Agnaldo da Silva Carneiro; Andrey Moacir do Rosario Marinho; Patrícia Santana Barbosa Marinho; Daiana de Jesus Hardoim; Fernando Almeida-Souza; Kátia da Silva Calabrese, foi publicado no **Livro de resumo do VI Ciclo Carlos Chagas de Palestras**.

7. Certificamos que o trabalho intitulado "*In silico* studies and antitrypanosomal activity of ravenelin B, a new xanthone, as potential inhibitors of cruzain", de autoria de **João Victor Silva-Silva**; Jeferson Rodrido S. Pina; Jordano Ferreira Reis3; Celeste da Silva Freitas de Souza; Juan Matheus Pereira Fernandes; Flávia de Oliveira Cardoso; Agnaldo da Silva Carneiro; Fernando Almeida-Souza1; Kátia da Silva Calabrese; Andrey Moacir do Rosario Marinho; Patrícia Santana Barbosa

Marinho, foi publicado no **Livro de resumo do VII Ciclo Carlos Chagas de Palestras**.

8. Certificamos que **João Victor da Silva e Silva** participou do **I Simpósio de Medicina Tropical e Doenças Negligenciadas da Universidade Federal do Rio de Janeiro**, realizado em 25 e 26 de Julho de 2018, no Centro de Ciências da Saúde, totalizando 18h de atividades. Rio de Janeiro, 15 de agosto de 2018.

9. Certificamos que o trabalho intitulado "Estudos de docking molecular e atividade antiprotozoária de substâncias obtidas por síntese de derivados da 4-hidróxi-acetofenona", dos autores **João Victor Silva-Silva**; André de Oliveira Feitosa; Jordano Ferreira Reis; Andrey Moacir do Rosario Marinho; Agnaldo da Silva Carneiro; Heriberto Rodrigues Bitencourt; Antônio Pedro da Silva Souza Filho; Fernando Almeida-Souza; Kátia da Silva Calabrese foi apresentado no **I Simpósio de Medicina Tropical e Doenças Negligenciadas da UFRJ**, realizado em 25 e 26 de Julho de 2018, no Centro de Ciências da Saúde.

10. Certificamos que **João Victor da Silva e Silva**, concluiu 22 de Jan de 2018 sua participação no **módulo O que os profissionais e gestores da área da saúde precisam saber sobre a febre amarela?**, com carga horária de 20 horas/aula produzido por Centro de Telessaúde do Hospital das Clínicas da UFMG (CTHCUFMG), Rede de Teleassistência de Minas Gerais (RTMG), Ministério da Saúde (MS) e ofertado por Universidade Federal do Rio Grande do Norte (UFRN). No período de 20 de Jan de 2018 à 22 de Jan de 2018 Brasília/DF, 22 de Jan de 2018.

11. A Universidade Federal de Pernambuco (UFPE), por meio da Universidade Aberta do Sistema Único de Saúde (UNA-SUS UFPE), do Hospital das Clínicas e do Grupo SABER Tecnologias Educacionais e Sociais declara que **João Victor da Silva e Silva** concluiu o **curso "Delineando um projeto de pesquisa"**, na categoria de curso livre, com carga horária de horas.

12. A Universidade Federal do Mato Grosso do Sul (UFMS), por meio da Universidade Aberta do SUS (UNA-SUS), certifica que **João Victor da Silva e Silva** concluiu o **curso "Manejo Clínico de Chikungunya",** na categoria de **curso de extensão profissional,** com carga horária de 30 horas**.**

13. Atestamos que o trabalho "Leshimanicidal activity of extracts of endophytic fungi from *Apidosperma excelsum*", autoria de Carvalho, J. M.; Santos, L. A.; **Silva, J. V. S.**; Dolabela, M. F.; Marinho, P. S. B.; Marinho, A. M. R. foi apresentado na forma de

pôster durante a **41ª Reunião Anual da Sociedade Brasileira de Química**. Foz do Iguaçu, 24 de maio de 2018.

14. Certificamos que **João Victor da Silva e Silva** participou do **"12º Congresso Brasileiro de Saúde Coletiva"**, realizado de 26 a 29 de julho de 2018 na Fiocruz em RIO DE JANEIRO/RJ.

15. Certificamos que o trabalho Avaliação dos Benefícios e dos Riscos do Tratamento Medicamentoso da Esquizofrenia dos autores: Selma Siqueira Franco; **João Victor da Silva e Silva**; Maria Fâni Dolabela, foi aprovado na modalidade **Comunicação Oral Curta**, no **12º Congresso Brasileiro de Saúde Coletiva** ocorrido de 26 a 29 de julho de 2018 na Fiocruz em RIO DE JANEIRO/RJ.

16. Certificamos que o trabalho Atividade Leishmanicida *in vitro* e Estudos *in silico* Flavopereirina cujos autores são: **João Victor da Silva e Silva**, Yasmin Hanna Couto Brandão, Jordano Ferreira Reis, Andrey Moacir do Rosário Marinho, Agnaldo da Silva Carneiro, Maria Fâni Dolabela foi apresentado no **54° Congresso da Sociedade Brasileira de Medicina Tropical – Medtrop 2018**, realizado no período de 02 a 05 de setembro de 2018, no Centro de Convenções de Pernambuco, Olinda – PE, na modalidade E-POSTER.

17. Certificamos que o trabalho 17.012 - *In silico* studies and antileishmanial activity of ravenelin B isolated from fungus *Exserohilum rostratum* - **Silva-Silva JV**1, Pina JRS2, Souza CSF1, Fernandes JMP, Lima AHL3, Almeida-Souza F4, Marinho AMR2, Marinho PSB2, Calabrese KS, 1 Laboratório de Imunomodulação e Protozoologia - FIOCRUZ 2 Laboratório de Química e Pesquisa - UFPA 3 Laboratório de Planejamento e Desenvolvimento de Fármacos LPD - UFPA 4 Pós-Graduação em Ciência Animal - UEMA foi apresentado na forma de painel durante a **XXXIV Reunião Anual da FeSBE**, realizado de 09 de setembro de 2019 a 13 de setembro de 2019 no Campos do Jordão Convention Center.

18. Certificamos que o trabalho intitulado "Caracterização fitoquímica e avaliação da atividade leishmanicida de flavonoides das folhas de *Arrabidaea chica* Verlot.", de autoria de Carla J. M. Tellis; Paulo Victor R. Souza; Maria S. S. Chagas; **João Victor S. Silva**; Fernando A. Souza; Kátia S. Calabrese; Davyson L. Moreira; Maria D. Behrens, foi apresentado na forma de pôster no **12º Simpósio Brasileiro de Farmacognosia e 17º Simpósio Latinoamericano de Farmacobotânica**, realizados na FMP/FASE em Petrópolis-RJ entre os dias 07 a 10 de maio de 2019.

19. This is to certify that **João Victor da Silva e** Silva attended the **IV International Symposium on Immunobiologicals** held in Rio de Janeiro, Brasil, 7th-9th May, 2019.
20. A Fundação Oswaldo Cruz, por meio da Unidade Instituto Oswaldo Cruz certifica que o(a) aluno(a), **João Victor Da Silva E Silva**, concluiu o evento educacional **I Simpósio de Promoção à Saúde**: Chega de Chagas! (1º Oferta) com duração de 6 hora(s), no dia 03/12/2019. Rio de Janeiro, 17/12/2019.
21. Certificamos que **João Victor da Silva e Silva** participou do **55º Congresso da Sociedade Brasileira de Medicina Tropical e XXVI Congresso Brasileiro de Parasitologia**, realizados de 28 a 31 de Julho de 2019, em Belo Horizonte, Minas Gerais.
22. Certificamos que o trabalho Avaliação antileishmania e modelagem molecular de uma nova neolignana como potencial inibidor da tripanotiona redutase, de autoria de **João Victor da Silva e Silva**, foi apresentado na sessão de e-pôster do eixo Eixo 8 - Leishmanioses, durante o **55º Congresso da Sociedade Brasileira de Medicina Tropical e XXVI Congresso Brasileiro de Parasitologia**, realizados de 28 a 31 de Julho de 2019, em Belo Horizonte, Minas Gerais.
23. Certificamos que **João Victor da Silva e Silva** participou do minicurso Redação e publicação científica, que ocorreu durante o **55º Congresso da Sociedade Brasileira de Medicina Tropical e XXVI Congresso Brasileiro de Parasitologia**, realizados de 28 a 31 de Julho de 2019, em Belo Horizonte, Minas Gerais.
24. We hereby certify that the poster entitled Quantitative analysis by HPLC/DAD of luteolin and apigenin in flavonoid-enriched bioactive fraction of Arrabidaea chica (Bonpl.) Verlot from the authors Paulo Victor Souza, Carla Tellis, Maria do Socorro Chagas, **João Victor Silva-Silva**, Fernando Almeida-Souza, Katia Calabrese, Davyson Moreira, Maria Behrens was presented during the **7th Brazilian Conference on Natural Product/ XXXIII RESEM** helded between November 10-13, 2019.
25. O Diretor do Instituto de Ciências Biomédicas, nos termos do artigo 74, parágrafo único, inciso 5, alínea "b", do Estatuto da Universidade de São Paulo, certifica que **João Victor da Silva e Silva**, concluiu **o Curso de Extensão Universitária na modalidade de Difusão: Capacitação no Uso e Manejo de Animais de Laboratório** e, para que possa gozar de todos os direitos e prerrogativas legais, outorga-lhe o presente Certificado.

26. Researcher Academy On Campus Certificate of Attendance This certifies that **João Victor Silva-Silva** has attended the following **ScienceDirect para a Fiocruz** at Zoom - lasting 1 hour, on Monday 25 May, 2020 Presented by Aline Bastos Training Analyst, Sergio Vidal Customer Consultant.

27. Certificamos que o trabalho intitulado “Investigation of the antioxidant and antichagasic potential of luteolin: analysis of structure-activity relationships”, de autoria de **João Victor Silva-Silva**; Paulo Victor Ramos de Souza; Carla Junqueira Moragas Tellis, Maria do Socorro dos Santos Chagas; Maria Dutra Behrens; Fernando Almeida-Souza; Aldo Sena de Oliveira; Adriano Defini Andricopulo e Kátia da Silva Calabrese, foi publicado no Livro de resumo do **VIII Ciclo Carlos Chagas de Palestras**.

28. Certificamos que o trabalho intitulado Atividade Antitumoral e Citotoxicidade de *Geissospermum sericeum* Benth. and Hook. f. ex Miers de autoria de Mírian Letícia Carmo Bastos, **João Victor Silva-Silva**, Josiwander Miranda Carvalho, Márlia Regina Coelho-Ferreira, Patricia Santana Barbosa Marinho, Andrey Moacir do Rosario Marinho, Marcelo de Oliveira Bahia, Maria Fâni Dolabela foi apresentado no **Congresso Virtual Em Ciências Farmacêuticas**, realizado no período de 03/12/2020 à 05/12/2020, na modalidade Comunicação oral.

29. Certificamos que o trabalho intitulado Relação Entre o Teor de Antocianidinas e Atividade Leishmanicida dos Extratos de *Arrabidaea chica* (Bonpl.) Verlot (Bignoniaceae): Importância de um Desenho Experimental de autoria de Carla Junqueira Moragas Tellis, Paulo Victor Ramos de Souza, Ygor Jessé Ramos dos Santos, Maria do Socorro dos Santos Chagas, **João Victor Silva-Silva**, Fernando Almeida-Souza, Kátia da Silva Calabrese, Davyson de Lima Moreira e Maria Behrens, foi apresentado no evento **XIII Simpósio Brasileiro de Farmacognosia**, realizado em 12/10/2021 a 15/10/2021, na cidade de São Cristóvão, contabilizando carga horária total de 30 horas.

30. Acceptance letter Dear Paulo Victor Souza On behalf of the Scientific Committee, we are pleased to inform you that the abstract Optimization of ultrasound-assisted extraction for obtention of bioactive compounds of Arrabidaea chica (Bonpl.) Verlot by the authors Paulo Victor Souza, Ygor Ramos, Carla MoragasTellis, Maria do Socorro Chagas, **João Victor Silva-Silva**, Fernando Almeida-Souza, Katia Calabrese, Maria Behrens, Davyson Moreira was accepted for publication in the **8th BCNP/XXXIV RESEM** Proceedings.

31. Certificamos que o trabalho intitulado Carajurina: uma antocianidina da *Arrabidaea chica* como um potencial marcador biológico com atividade antileishmanicida de autoria de Katia Calabrese, Celeste S.f. de Souza, Kerolain Faoro Teixeira, **João Victor Silva-Silva**, Carla Tellis, Maria do Socorro Chagas, Paulo Victor R. Souza, Davyson L. Moreira, Arthur Ribeiro Cenci, Aldo Oliveira, Fernando Almeida-Souza, Maria D. Behrens foi submetido, aprovado e apresentado na divisão de Química Medicinal - MED como e-Pôster na **44ª Reunião Anual da Sociedade Brasileira de Química - Virtual**, realizada no período de 15 a 26 de novembro de 2021.