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DANIELA RIBEIRO ALVES

METABOLITOS DE FUNGOS E VEGETAIS DA CAATINGA COM ATIVIDADE ANTILEISHMANIAL

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2019
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Area de concentração: Reprodução e Sanidade Animal.

Orientador: Profa. Dra. Selene Maia de Morais
Co-orientador: Dr. Francisco das Chagas de Oliveira Freire

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BANCA EXAMINADORA:

Selene Maia de Morais
Profa. Dra. Selene Maia de Morais (Orientadora)

Prof. Dr. Francisco das Chagas de Oliveira Freire (Co-Orientador)
Empresa Brasileira de Pesquisa Agropecuária – CNPAT

Prof. Dr. Fabio Roger Vasconcelos (Avaliador)
Instituto Federal do Ceará - Campus Boa Viagem.

Prof. Dra. Maria Teresa Salles Tevisan (Avaliadora)
Universidade Federal do Ceará – UFC

Prof. Dr. Wesley Eyeverton Correia Ribeiro (Avaliador)
Universidade Federal do Ceará - UFC

FORTALEZA - CE
2019
À minha amada esposa Karine e meu filho querido Rúben Davi (in memoriam),
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À Deus.
RESUMO

A leishmaniose tornou-se uma das doenças mais importantes da atualidade, dada a sua incidência e alta letalidade em indivíduos não tratados, idosos e crianças desnutridas. Dessa forma, na tentativa de descoberta de novos agentes mais eficazes e menos tóxicos para o combate à leishmaniose, os produtos naturais foram uma opção considerada no presente trabalho. Então foi realizada avaliação dos metabolitos primários e secundários de fungos e vegetais oriundos da Caatinga quanto às suas características químicas e às atividades biológicas, incluindo toxicidade às formas promastigotas e amastigotas de Leishmania sp., bem como o comparativo dessa atividade à de outros fitoterápicos. Foi selecionada a planta Caryocar coriaceum, bem como fungos endofíticos da planta Jatropha curcas a serem utilizados para a extração de enzimas hidrolíticas. De fungos do gênero Dichothomophtora e endofíticos dos gêneros Vermisporium-like e Emericella nidulans isolados de J. curcas, foram obtidos extratos proteicos ricos em lipases que foram testados contra fungos dermatófitos e Leishmania amazonensis. Os metabolitos secundários produzidos por C. coriaceum demonstraram possuir substâncias potencialmente bioativas com atividade antioxidante que atuaram como antifúngicas e antileishmaniais, principalmente pela eliminação de radicais livres dentro do vacúulo parasitário e mecanismos anticolinesterásicos de membrana. A caracterização química por cromatografia líquida de alta eficiência da presença de queracética, rutina e isoqueracétina e indicativa dessas atividades. Extratos da folha de C. coriaceum exercem efeito leishmanicida atuando em formas primitivas por mecanismo semelhante a apoptose e amastigotas intracelulares pela resposta dependente da ferrugina Nr2 / HO-1 / ferritina e depleção de ferro. Já extratos de frutos de C. coriaceum causam ação leishmanicida de forma direta em promastigotas e indireta em amastigotas intracelulares por modulação do metabolismo de ferro. Foi determinada a atividade antileishmanial de extratos proteicos de fungos endofíticos na caatinga, sem que essas substâncias causassem danos a células do hospedeiro, evidenciando nível de seletividade superior à droga padrão. Os dados obtidos nesse estudo comprovam a importância do estudo de metabolitos secundários obtidos de vegetais e de fungos endofíticos da caatinga, abrindo perspectivas ao uso desses extratos naturais, e substâncias isoladas, como agentes terapêuticos capazes de interromper o curso de infecções por Leishmania sp.

ABSTRACT

Leishmaniasis has become one of the most important diseases today, given its incidence and high lethality in untreated individuals, the elderly and malnourished children. Thus, to find new, more effective and less toxic agents to combat leishmaniasis, natural products were an option considered in the present work. Then, the primary and secondary metabolites of Caatinga fungi and vegetables were evaluated for their chemical characteristics and biological activities, including toxicity to the promastigote and amastigote forms of *Leishmania* sp., as well as the comparison of this activity to that of other phytotherapeutics. The *Caryocar coriaceum* plant was selected, as well as endophytic fungi of the *Jatropha curcas* plant to be used for the extraction of hydrolytic enzymes. From fungi of the genus Dichothomosphorata and endophytic genera of *Vernisporium*-like and *Emericella nidulans* isolated from *J. curcas*, lipase-rich protein extracts were tested against dermatophytes fungi and *Leishmania amazonensis*. Secondary metabolites produced by *C. coriaceum* were shown to have potentially bioactive substances with antioxidant activity that acted as antifungal and antileishmanial, mainly by the elimination of free radicals within the parasitic vacuole and anticholinesterase membrane mechanisms.

The chemical characterization by high performance liquid chromatography of the presence of quercetin, rutin and isoquercitrin is indicative of these activities. Extracts of *C. coriaceum* leaf exert leishmanicidal effect acting in primitive forms by apoptosis-like mechanism and intracellular amastigotes by Nrf2 / HO-1 / ferritin-dependent response and iron depletion. Already fruit extracts of *C. coriaceum* cause leishmanicidal action directly in promastigotes and indirectly in intracellular amastigotes by modulation of iron metabolism. The antileishmanial activity of protein extracts of endophytic fungi in the caatinga was determined, without these substances causing damage to host cells, showing a level of selectivity superior to the standard drug. The data obtained in this study confirm the importance of studying secondary metabolites obtained from caatinga endophytic plants and fungi, opening perspectives for the use of these natural extracts and isolated substances as therapeutic agents capable of interrupting the course of infections by *Leishmania* sp.

**Keywords:** Antileishmanial, Antioxidant, Anticholinergic, Lipases. *Caryocar coriaceum*
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1 INTRODUÇÃO

Leishmaniose é uma doença negligenciada cujos patógenos causam a morte de 20-30 milhões de pessoas por ano em 98 países (WHO, 2014). Considerada emergente a leishmaniose tornou-se uma das doenças mais importantes da atualidade, dada a sua incidência e alta letalidade em indivíduos não tratados, idosos e crianças desnutridas (ZANET et al., 2014). Embora apresente opções terapêuticas, como os azoderivados e drogas antimoniais pentavalentes para quimioterapia, essas drogas apresentam efeitos colaterais graves, incluindo alta toxicidade e baixa eficácia (DEPS et al., 2000; BRENZAN et al., 2007; BORBOREMA et al., 2011).

Esta tese versa sobre a atuação de metabolitos de fungos e vegetais oriundos da Caatinga quanto à sua toxicidade às formas promastigotas e amastigotas de Leishmania sp. bem como sua alta seletividade ao parasito, não sendo tóxico ao hospedeiro, e avaliados alguns mecanismos de ação para as atividades destes compostos.

Protozoários do gênero Leishmania são patógenos intracelulares infectam principalmente macrófagos, mas também neutrófilos e células dendríticas (SALUD, 2013; HERMIDA et al., 2014; WHO, 2014, 2016). O parasito precisa vencer diversas barreiras naturais no hospedeiro, intermediário ou não, para completar seu desenvolvimento. Inserir-se e permanecer em um hospedeiro implica que o patógeno possui meios de evitar ou destruir o sistema imune do hospedeiro.

Ocupar um nicho intracelular é um dos mecanismos utilizados para evitar que o sistema imune reconheça o invasor, além de requerer que o patógeno seja resistente aos mecanismos microbicidas das células hospedeiras. O uso de plantas medicinais como tratamento ou prevenção de enfermidades é tão antigo quanto o surgimento do homem. A preocupação com a cura de doenças se fez presente ao longo da história, sendo repassado ao longo das gerações. Estudos comprovam que fontes naturais apresentam muitos compostos com atividade farmacológica podendo ser usados no desenvolvimento de novas drogas (MAGALHÃES et al., 2010).

A exploração destes recursos pode levar a identificação de metabolitos inestimáveis que podem servir como drogas ou conduzir ao desenvolvimento de novas substâncias terapêuticas (ALVES et al., 2018; RODRIGUES et al., 2018).

Dentre os metabolitos primários destacam-se as substâncias proteicas e no trabalho de Hasan et al. (2006), os autores relatam as enzimas hidrolíticas, caracterizando-as como o grupo mais importante de biocatalizadores em aplicações biotecnológicas,
sendo esse um importante metabolito primário. Quanto a atuação da leishmaniose no indivíduo, essa se multiplica pois tem acesso a diversas fontes de carbono dentro do macrófago. Se as enzimas hidrolíticas atuarem sobre estas fontes diminuindo o acesso dos alimentos para o parasito, estas enzimas constituiriam novas opções para o desenvolvimento de agentes quimioterápicos que tenham como alvo a absorção de nutrientes ou o metabolismo intracelular do parasita causador da leishmaniose.

Metabólitos secundários como terpenóides, flavonóides, alcalóides, são ferramentas importantes na terapêutica e busca clínica do tratamento da leishmaniose (figura 01). Os flavonóides se destacam como a classe que tem maior número de compostos leishmanicidas, dentre outros como os acetogeninas, alcalóides e componentes de óleos essenciais (VILA-NOVA et al., 2012, 2013).

Figura 01 – Metabólitos secundários como potenciais alvos para uso no tratamento da leishmaniose (FARIAS-JUNIOR et al., 2012; SILVA et al., 2014; RODRIGUES et al., 2018; CATANEO et al., 2019; MORAIS et al., 2019).

Caryocar é um gênero botânico pertencente à família Caryocaraceae, distribuído na América do Sul e Central no bioma Cerrado. As frutas são usadas em alimentos, cosméticos e na medicina popular. Rico em compostos fenólicos, esses extratos têm sido descritos como potentes antioxidantes, além de apresentar efeitos anti-inflamatórios, antineoplásicos, antimicrobianos e leishmanicida (BATISTA et al., 2010; DE OLIVEIRA
et al., 2015; DE FIGUEIREDO et al., 2016; TOMIOTTO-PELLISSIER et al., 2018).

Desta forma se faz necessária a realização de estudo sobre a atuação destes metabolitos de fungos e vegetais oriundos da Caatinga relativo às características químicas, às atividades e à toxicidade às formas promastigotas e amastigotas de Leishmania sp., bem como comparar esta atividade com a de outros candidatos a fitoterápicos.

2 REVISÃO BIBLIOGRÁFICA

2.1 A PROBLEMÁTICA DA LEISHMANIOSE

Infecções por leishmaniose podem causar grande espectro de sintomas, sendo divididas em três tipos clínicos de apresentação da doença: cutânea (LC), mucocutânea (LMC) e visceral (LV). Cerca de 95% dos casos ocorrem nas Américas, na bacia do Mediterrâneo e no Oriente Médio e Ásia Central. Cerca de 0,7 a 1,3 milhões de novos casos ocorrem em todo o mundo anualmente. Dos casos que ocorrem nas Américas, quase 90% dos casos ocorrem na Bolívia, Brasil e Peru. Estima-se que 200 a 400 mil novos casos de LV ocorrem no mundo a cada ano e, desses, acima de 90% dos novos casos ocorrem no sul da Ásia e no Brasil, onde é registrada em 19 das 27 Unidades da Federação, principalmente na região Nordeste, sendo considerada uma zoonose (SALUD, 2013; HERMIDA et al., 2014; WHO, 2014, 2016).

A leishmaniose é transmitida pela picada da fêmea flebotomínea em mamíferos, onde o ser humano é hospedeiro eventual e não obrigatório, podendo este contrair a doença (SAÚDE; CEARÁ, 2015). O diagnóstico clínico é precário e complexo, devido a sintomatologia variável e inespecífica. A imunossupressão causada por Leishmania spp. pode gerar infecções oportunistas, dificultando ainda mais o diagnóstico (VAN DEN BOGAART et al., 2014). LC é a forma mais comum da doença e provoca úlceras, deixando cicatrizas e estigmas. LMC produz granulomas de leucócitos infectados, conduzindo à destruição parcial ou total das membranas mucosas do nariz, boca e garganta. LV é fatal se não tratada. No ambiente doméstico, o cão (Canis familiaris) é o principal reservatório envolvido na manutenção do ciclo zoonótico predominante em várias regiões do país.

A quimioterapia utilizada no tratamento da leishmaniose é baseada em medicamentos que possuem metais pesados tóxicos, conhecidos como antimoniatos,
entre eles os mais usados são o antimoniatô de meglumine (Glucantime®) e o estibogluconato de sódio (Pentostan®)(SILVA et al., 2014).

Quando este tipo de tratamento não é eficaz, outros medicamentos como pentamidina e antofericina B também são utilizados. Todos estes são de administração injetável e requerem supervisão clínica ou hospitalização devido a severidade dos efeitos colaterais.(DEPS et al., 2000; TIUMAN et al., 2011; TESTASICCA et al., 2014). Assim o tratamento da leishmaniose atual não é satisfatório em termos de efetividade e toxicidade, visto que a resistência de diferentes cepas às drogas existentes dificulta o tratamento (OSÓRIO et al., 2007).

A ausência de uma vacina eficaz contra leishmaniose leva a necessidade urgente de drogas efetivas para substituir ou suplementar aquelas de uso corrente. A busca por novas drogas anti-Leishmania tem proporcionado a origem de protótipos para o desenvolvimento de novos compostos quimioterápicos com melhor atividade e menos efeitos colaterais (TESTASICCA et al., 2014).

A obtenção de novos princípios ativos se torna uma alternativa rentável, assim pesquisas sobre diferentes produtos de origem vegetal têm sido realizadas. Além disso até meados dos anos 90 de 65 a 80% da população dos países em desenvolvimento recorreram plantas medicinais como única forma de acesso aos cuidados básicos de saúde (PENIDO et al., 2017)

2.2 COMPOSTOS NATURAIS BIOATIVOS ORIUNDOS DE VEGETAIS

O Brasil é um país com uma vasta biodiversidade. As propriedades terapêuticas de muitos metabolitos de fungos e plantas conduzem à pesquisa de princípios ativos de várias espécies. A exploração desses recursos pode ajudar na identificação de produtos naturais que podem levar ao desenvolvimento de novas substâncias terapêuticas (ALVES et al., 2017a; PENIDO et al., 2017; MARTINS et al., 2018).

A Caatinga (do tupi: ka'a [mata] + tinga [branca] = mata branca), dos biomas brasileiros, é um dos mais frágeis e é único, o que significa que grande parte do seu patrimônio biológico não pode ser encontrado em nenhum outro lugar do planeta (FREIRE; GONÇALVES, 2012; ALVES et al., 2018). No entanto, muitas plantas de outros países são muito bem adaptadas a esse bioma.

Por apresentarem efeitos protetores, os compostos naturais despertaram interesse crescente na comunidade científica e na indústria farmacêutica (PENIDO et al., 2017). A
diversidade de compostos naturais de diferentes origens tem sido descrita prevenir ou atenuar várias patologias. Vários estudos in vitro e in vivo comprovaram o potencial terapêutico de compostos naturais (ANDRADE et al., 2019).

Fármacos oriundos de produtos naturais incluem ciclosporina, digoxina, doxorrubicina, morfina, paclitaxel, penicilina G, quinina, teofilina, vincristina, vitamina A, entre muitos outros exemplos (CHEUKA; AL, 2016). Ainda, podemos observar a presença de compostos bioativos do grupamento fenólico, estes possuem um ou mais anéis aromáticos com um ou mais grupos hidroxila em formas livres, combinadas ou conjugadas que são consideradas parte dos mecanismos de defesa em plantas (TELLES; KUPSKI; FURLONG, 2017). O grupamento fenólico se divide em ácidos fenólicos, ácidos hidroxibenzôicos, ácidos hidroxicinâmicos, estilbenos, flavonóides, lignanas, polifenóis e taninos (SÁNCHEZ, 2017).

Compostos fenólicos têm sido investigados conforme seu potencial como sequestradores de radicais livres. O estresse oxidativo (SO) pode levar a complicações em condições patológicas bem como gerar novas condições patológicas. É um fenômeno associado a mecanismos patogênicos de várias doenças, incluindo asma, aterosclerose, câncer, diabetes mellitus, Doença de Alzheimer, doença de Parkinson, doença inflamatória, doença ocular degenerativa, doenças causadas por bactérias, fungos e protozoários, doenças psicológicas, doenças renais, outras doenças neurodegenerativas, estão altamente correlacionadas com o desequilíbrio celular redox e os radicais livres (LI et al., 2012; MATHEW; SUBRAMANIAN, 2014; EGBUNA, 2017; TOMIOTTO-PELLISSIER et al., 2018). Podemos caracterizar o SO como o desequilíbrio entre oxidantes e antioxidantes em um sistema biológico (SINGH et al., 2019) e a capacidade das células de desintoxicá-las e negar seus efeitos prejudiciais sobre proteínas, lipídios e DNA é determinante na manutenção a vida (AHMADINEJAD et al., 2017).

O desequilíbrio, que gera doenças e contribui para a deterioração celular, resulta do excesso de espécies reativas de oxigênio (EROs), espécies reativas de nitrogênio (ERNs) ou funcionamento ineficaz do sistema antioxidante, advém da modulação da função de vários sistemas de biomoléculas (SINGH et al., 2019).

Os flavonóides desempenham uma cadeia de reações de supressão da peroxidação lipídica, inibindo mediadores inflamatórios, modulando e expressão génica e ativando de enzimas antioxidantes, ajudando na manutenção do estado antioxidante endógeno celular (MONTEIRO et al., 2018). Considerando que essas substâncias são capazes de atravessar até mesmo a barreira hemato-encefálica, considerada estrutura de permeabilidade
altamente seletiva, os flavonoides são considerados uma ótima classe de substâncias com possíveis atividades terapêuticas (BRAGA et al., 2018).

![Quercetina](image1.png)
![Rutina](image2.png)
![Isoquercitrina](image3.png)

**Figura 02 – Quercetina (3,3',4',5,7-pentahidroxiflavona) e seus derivados Rutina e Isoquercitrina.**

Como exemplo de flavonoide ubíquo, versátil e abundante, tem-se a quercetina (3,3',4',5,7-pentahidroxiflavona) e seus derivados (figura 02). Conhecida por seus efeitos clínicos, como inibição carcinogênica, diminuição de doenças cardiovasculares, efeito antioxidante, dentre outros (KAZEMIPOUR et al., 2018), a quercetina têm maior ocorrência como glicosídeo hidrossolúvel, e não em sua forma de aglicona, sendo a posição 3 da molécula o local de glicosilação preferido.

A diferença entre os derivados da quercetina deve-se a modificações nos resíduos de açúcar e locais de glicosilação (ZHANG; CHEN, 2018), o que faz alterar também os seus efeitos farmacológicos, incluindo efeitos antialérgicos, anticancerígenos, antiinflamatórios, anti-malogênico, antiviral e atividade antioxidante (KIM; NEVITT; THIELE, 2008; KIM et al., 2013).

Isoquercitrina, quercetina e rutina são amplamente reconhecidos na literatura como compostos naturais com propriedades antioxidantes (WANG; LI; BI, 2018). Esses compostos apresentam estrutura o-dihidroxi no anel B e grupos 3- e 5-OH em conjugação com a função 4-oxo, conforme figura 02 (KAURINOVIC; VASTAG, 2019).
2.3 PLANTAS COM ATIVIDADE ANTILEISHMANIAL

_Cariocar coriaceum_ (pequi-zeiro), _Dimorphandra gardneriana_ (Fava’anta) _Myracrodruon urundeuva_ (Aroeira), _Astronium fraxinifolium_ (Gonçalo Alves), _Spondias mombin_ (Cajazeira) dentre outras plantas com compostos flavonóides bioativos, possuem potencial atividade antileishmanial (VILA-NOVA et al., 2013, 2011, 2012; DE LIMA et al., 2014; CALIXTO JÚNIOR et al., 2015; SILVA et al., 2016; PENIDO et al., 2017; BRAGA et al., 2018; PEREIRA et al., 2018; RODRIGUES et al., 2018; MORAIS et al., 2019).

Nos estudos anteriormente citados foi avaliado o potencial de extratos vegetais. As atividades _in vitro_ desses produtos se correlacionam de forma positiva com sua capacidade de modular a integridade mitocondrial e alterar a membrana plasmática de células de patógenos intracelulares, sem causar danos às células hospedeiras (TOMIOTTO-PELLISSIER et al., 2018), determinar atividades antifúngicas, antibacterianas, antileishmaniais, citotóxicas (SOARES et al., 2015; SILVA et al., 2016; ALVES et al., 2017b; MARTINS et al., 2018; PEREIRA et al., 2018), antiprotozoários (TEMPONE et al., 2005; VILA-NOVA et al., 2012, 2013), antioxidante (MORAIS et al., 2017), dentre outros.

O flavonóide epigallocatequina 3-O-galato, uma substância fenólica abundante em _Camellia sinensis_ (Theaceae), foi avaliado contra camundongos BALB/C infectados por _L. amazonensis_ (MHOM/BR/75/LTB0016) com resultados que reduzem significativamente tamanho de lesão e carga parasitária (NEAGU et al., 2018).

Resultados semelhantes ao da quercetina (3,3’,4’,5,7-pentahidroxiflavona), também classificada dentro do grupo dos compostos químicos flavonóides e comumente encontrada em plantas da caatinga, quando utilizada promove o efeito antipromastigota, aumentando a produção de espécies reativas de oxigênio (EROs) e anti-amastigota, regulando positivamente a expressão de NRF2/OH⁻¹, afetando a disponibilidade de ferro (CATANEÓ et al., 2019).

O ácido caurenóico, extraído do vegetal _Sphagneticola trilobata_, possui atividade leishmanicida, desencadeando uma via NLRP12/IL-1β/espécies reativas de nitrogênio (ERNs) / ON (MIRANDA et al., 2015). O ácido cafêico, ácido fenólico comumente encontrado em plantas do gênero _Coffea_, possui atividade antipromastigota por processo semelhante à apoptose; e anti-amastigota pela produção de TNF-α / EROs / ON e diminuição da disponibilidade de ferro (BORTOLETI et al., 2019), demonstrando o potencial efeito antileishmanial de compostos naturais oriundos de vegetais.
2.4 FUNGOS ENDOFÍTICOS COMO FONTES DE AGENTES BIOATIVOS

Quanto à biodiversidade mundial, os fungos representam o segundo maior grupo de organismos no planeta, atrás apenas dos insetos. Eles são elementos chave nos ecossistemas tropicais, ocorrendo nos mais diversos habitats (psicrófilos a termófilos). São organismos heterotróficos, podendo se comportar como saprófitas e/ou parasitas (FREIRE; GONÇALVES, 2012).

Durante a evolução, quando as plantas colonizaram a terra, os fungos desenvolveram diferentes tipos de relações com elas. Um grupo, denominado de “endofíticos”, formou um tipo de associação o qual tem sido confirmado por meio de registros fósseis, sugerindo que a associação endofítico-planta evoluiu desde o estabelecimento das primeiras plantas sobre a terra (KRINGS; TAYLOR; DOTZLER, 2012; SURYANARAYANAN et al., 2012; FIELD; PRESSEL, 2018).

Os estudos sobre a proporção do número de espécies de fungos para cada espécie vegetal indicam que o número estimado de espécies fúngicas é de 1,5 milhões, entretanto o número de espécies descritas até hoje é de aproximadamente 100 mil. Postulados sobre o número de espécies fúngicas existentes chegam até mesmo a estimar 8,25 milhões de espécies fúngicas, considerando que a diversidade é maior em regiões tropicais (FREIRE; GONÇALVES, 2012). Acredita-se que todas as espécies de plantas são potenciais hospedeiros para diversos fungos. Esses dados revelam o grande potencial de exploração da diversidade genética, materiais biotecnológicos fúngicos, além biodiversidade e potenciais fitopatógenos no ambiente agrícola (FREIRE; VASCONCELOS; COUTINHO, 2014a).

Os fungos normalmente exercem uma associação mutualística, antagônica ou neutra com uma infinidade de organismos heterotróficos e autotróficos. Essas relações apresentam diversos graus de associação e interdependência nutricional. O termo endofíticos foi criado com o intuito de diferenciá-los dos fungos epífiticos, que ocorrem apenas na superfície das plantas. Vários outros autores têm sugerido definições, entretanto, a mais aceita é a definição onde os fungos endofíticos são descritos como “organismos que durante algum estágio de seu desenvolvimento sobrevivem no interior dos tecidos vegetais, sem causar qualquer dano aparente ou sintoma de doença no hospedeiro” (FREIRE; VASCONCELOS; COUTINHO, 2014; ALVES et al., 2018).

Apesar da importância dos microrganismos, muito pouco da diversidade existente
é conhecida, tornando imperativa a exploração de microrganismos de ecossistemas como a caatinga. A versatilidade bioquímica e diversidade biológica de fungos endofíticos representam uma enorme variedade de genes que ainda são desconhecidos, os quais podem apresentar importantes aplicações biotecnológicas e agrícolas (FREIRE; VASCONCELOS; COUTINHO, 2014a).

O potencial biotecnológico dos fungos endofíticos tem sido muito estudado por vários autores (KUMAR et al., 2004; SESSITSCH; REITER; BERG, 2004; THINES; ANKE; WEBER, 2004; DAVIS et al., 2005; SURYANARAYANAN et al., 2009; ANYAOGU; MORTENSEN, 2015; PUSZTAHELYI; HOLB; PÁCSI, 2015; MACHELEIDT et al., 2016).

Com o número crescente de trabalhos sobre a interação entre planta e endófitos, juntamente com os resultados promissores observados, têm sido importantes os estudos de fungos endofíticos como agentes de controle biológico de inúmeros patógenos (VIDAL; JABER, 2015; JABER; OWNLEY, 2018), promotores de crescimento vegetal (BUSBY; RIDOUT; NEWCOMBE, 2016), produtores de enzimas (ALVES et al., 2018) e ainda o uso de fungos endofíticos em plantas como fitoremediadores de áreas poluídas (DENG; CAO, 2017; FENG et al., 2017).

É importante ressaltar que produtos de valor biotecnológico, como antibióticos e outros princípios ativos, com inúmeras propriedades farmacológicas, têm sido sintetizados por fungos endofíticos, tornando esses um importante alvo em estudos aplicados na área médica e agronômica. O taxol, um potente agente antitumoral, sintetizado pelo fungo endofítico Taxomyces andreanae da planta hospedeira Taxus brevifolia, vem sendo estudado para a sua produção em escala industrial (JI et al., 2006). Os fungos têm sido uma fonte de antibióticos muito utilizados na prática clínica como a penicilina produzida pelo fungo Penicillium notatum, e a cefalexina sintetizada pelo fungo Cefalosporium acremonium (OWEN; HUNDLEY, 2004).

Como mencionado anteriormente, microrganismos endofíticos são bactérias ou fungos que colonizam o interior dos tecidos de plantas, sem apresentar efeito patogênico no hospedeiro. Dessa maneira, ocorre uma associação simbiótica entre a planta hospedeira, que o protege e alimenta, e o microrganismo endofítico, produtor de metabólicos bioativos que auxiliam o crescimento e a proteção da planta contra o ataque de fitopatógenos (VIDAL; JABER, 2015; BUSBY; RIDOUT; NEWCOMBE, 2016; MACHELEIDT et al., 2016; DENG; CAO, 2017; FENG et al., 2017; JABER; OWNLEY, 2018).
Cada uma das 300 mil espécies de plantas existentes é hospedeira de uma ou mais espécies de microrganismos endofíticos, mas poucas delas têm sido completamente estudadas em relação à sua biologia endofítica. Estima-se que menos de 7% das espécies de fungos sejam conhecidas (DENG; CAO, 2017), sugerindo que milhões de espécies microbianas poderão fornecer importantes biomoléculas.

Devido ao fato que somente parte da diversidade biológica dos fungos tenha sido descritas e estudadas, existe uma enorme e inexplorada reserva de compostos naturais de grande variedade estrutural, como fonte potencial de novas substâncias bioativas (FREIRE; VASCONCELOS; COUTINHO, 2014a; ALVES et al., 2018).

A facilidade de isolamento dessas substâncias de microrganismos em meio de cultura torna esse reino interessante para a produção de produtos biotecnológicos. Consequentemente, torna-se grande a oportunidade de se descobrir novos microrganismos endofíticos de plantas hospedeiras de diferentes ecossistemas (FREIRE; KOZAKIEWICZ; PATERSON, 1999, 2000; ROCHA et al., 2014; FREIRE; ROCHA, 2016).

Historicamente, de todos os microrganismos estudados, os fungos têm sido os maiores produtores de metabólitos secundários. Suger-se ainda que os fungos são fundamentais para a sustentabilidade, biodiversidade e manutenção de muitos ecossistemas terrestres (KUMAR et al., 2004; OWEN; HUNDLEY, 2004; THINES; ANKE; WEBER, 2004; BUSBY; RIDOUT; NEWCOMBE, 2016; MACHELEIDT et al., 2016), produtores de metabolitos e princípio de fármacos em uso na medicina popular e clinica (FREIRE; KOZAKIEWICZ; PATERSON, 1999; FREIRE; VASCONCELOS; COUTINHO, 2014a).

Dentre todos os produtores de compostos bioativos naturais, os fungos representam uma fonte rica de metabólitos biologicamente ativos que apresentam ampla aplicação como antibióticos, antiparasitários, antifúngicos, antitumorais, entre muitos outros compostos. Ao produzirem compostos alcaloides e terpenos, por exemplo, microrganismos endófitos protegem os vegetais ao produzirem o efeito inseticida ou repelente, tornando a planta menos susceptível ao ataque de artrópodes (ABRAHAM et al., 2016; SHYMANOVICH et al., 2019; ZHANG et al., 2019).

Várias classes de metabólitos secundários como alcaloides, esteroides, fenilpropanoides, fenóis, flavonoides, cumarinas, lactonas, pirolizidinas, policetídeos, quinonas, terpenos, terpenóides, dentre outros, são produzidos por fungos endofíticos, o que faz com que esses sejam considerados uma origem encorajadora para estudos de
exploração do potencial biotecnológico de produtos farmacológicos na prática humana (FREIRE; VASCONCELOS; COUTINHO, 2014a; FREIRE; ROCHA, 2016) e veterinária (ROCHA et al., 2014; ALVES et al., 2018).

Figura 03 – Substâncias bioativas isoladas de fungos com atividade antileishmanial descritas na literatura.

Podemos tomar por exemplo de substancias bioativas isoladas de fungos (Figura 03) a hipocrellina A, obtida do fungo *Hypocrella bambusae*, que mostrou atividade antileishmanial significativa contra *L. donovani*, enquanto a hipocrellina B foi moderadamente ativo (MA et al., 2004). O metabolito policetídeo conhecido como citrinina, isolado de extrato metanólico de um fungo endofítico *Penicillium janthineillum* (DO ROSÁRIO MARINHO et al., 2005) também foi ativo contra *Leishmania* sp., bem como um derivado de bifenil, altenusina isolado de *Alternaria* sp. (COTA et al., 2008).

Também foi estudado outro fungo *Cochliobolus* sp., onde dois compostos isolados, coclocioquinona A e a isococlocioquinona A apresentaram atividade antileishmanial contra *L. amazonensis* (CAMPOS et al., 2008; ROSA et al., 2010). A investigação fitoquímica do fungo *Chaetomium* sp. levou ao isolamento de três novos compostos de xantona. Esses chaetoxantanonas A-C exibiram atividades significativas contra *L. donovani* (BORBOREMA et al., 2011). De *Edenia* sp. endofítica, isolada de *Petrea volubilis*, cinco compostos antileishmaniais potentes foram separados. Estes compostos eram
preussomerina EG1, palmarumicina CP2, palmarumicina CP17, palmarumicina CP18 e CJ-12, 37. Metabólitos secundários de *Eurotium repens* que foram examinados quanto às atividades antibacteriana, antifúngica, antimalárica e antileishmanial *in vitro* (MA et al., 2004).

Rodrigues e colaboradores (2014) estudaram o efeito antileishmanial *in vitro e in vivo* de ácido kojico em *L. amazonensis*. Fungos endofíticos podem então ser considerados também como fonte alternativa de metabólitos que podem combater os danos advindos da EROs e provocar SO em tecidos e órgãos, devido à liberação de radicais livres gerados nos processos sistêmicos bioquímicos e metabólicos promotores ou agravantes de diversos processos patológicos (OWEN; HUNDLEY, 2004; ANYAOGU; MORTENSEN, 2015; ABRAHAM et al., 2016; MACHELEIDT et al., 2016; ALVES et al., 2018), como descrito anteriormente.

### 2.5 LIPASES COMO BIOAGENTES CONTRA MICRORGANISMOS

Em relação à enzima lipase, na Medicina Veterinária a sua importância começa com a interação entre o estudo da manutenção da homeostase e a patogênese. Historicamente, a determinação da atividade sérica da lipase, amilase e imunorreatividade à tripsina têm sido utilizadas para o diagnóstico de pancreatite (HULSEBOSCH et al., 2016).

O teste de concentração lipolítica é relatado como o biomarcador não invasivo mais sensível (65-94%) e específico (66-100%) disponível para o diagnóstico de pancreatite em animais (STEINER; WILSON; WILLIAMS, 2004; TRIVEDI et al., 2011; XENOULIS et al., 2014). Assim, a enzimologia clínica é de fundamental importância para identificar déficits hepáticos e pancreatite em animais através da análise do perfil metabólico do sangue (XENOULIS; STEINER, 2012).

A engenharia lipolítica começou há mais de 150 anos, mas foi somente após meados da década de 1980 que a maioria das enzimas produzidas veio de fontes microbianas. Somente quando se aceitou que as enzimas lipase permaneceram ativas, mesmo em solventes orgânicos, que várias investigações com essas enzimas começaram como objetos de estudo, otimizando para torná-las ferramentas para a indústria (MOBARAK-QAMSARI; KASRA-KERMANSHAHI; MOOSAVI-NEJAD, 2011).

Ao longo dos anos, foi demonstrado que, para obter a produção de enzimas com alta qualidade e especialização, é necessário priorizar e observar as propriedades de
produção dos microrganismos estudados, além da purificação e caracterização dessa produção para obter uma enzima estável e eficaz. Apesar do conhecimento expressivo da ampla possibilidade de produção enzimática por microrganismos, apenas um pequeno número de lipases é comercialmente explorado (JAEGER; REETZ, 1998).

O desenvolvimento de aplicações lipolíticas na produção e uso nas indústrias apenas aumentou o interesse por seus genes codificadores nos diferentes microrganismos, pois essas enzimas são altamente variáveis em composição, tamanho e estrutura.

O interesse recente na produção lipolítica é justificado pela descoberta de suas mais variadas aplicações (SALIHU et al., 2012). Como aditivos alimentares, as lipases atuam na síntese de ésteres como agentes aromatizantes, na hidrólise de triaçilgliceróis (óleos ou gorduras), como detergentes ou agentes de limpeza (WANG et al., 2012) e na composição de medicamentos em produtos farmacêuticos (RODRIGUES et al., 2014).

2.6 ATIVIDADES BIOLÓGICAS

2.6.1 Atividade antioxidante

Figura 04 – Transformação celular por meio de radicais livres. É demonstrada uma célula normal sendo atacada por radicais livres e sofrendo estresse oxidativo (SO), levando a dano tecidual, inflamação e apoptose.

Radicais livres são moléculas ou fragmento moleculares altamente reativos, podendo reagir por várias interações, podendo iniciar uma reação em cadeia, conforme podemos visualizar na figura 04. Essas moléculas contêm um ou mais elétrons desemparelhados em seu orbital atômico (SAILAJA RAO et al., 2011) sendo geradas a
partir de fontes endógenas, como por ativação de células imunes, câncer, estresse mental e/ou envelhecimento, exercício excessivo, infecção, inflamação, isquemia, ou de fontes exógena como resultado pelo uso de álcool, certos medicamentos (ciclosporina, tacrolimus, gentamicina e bleomicina), má alimentação (alimentos processados e ultraprocessados, altos níveis de gorduras, corantes e conservantes), exposição a radiações, gases provenientes de cigarro ou escapamento de carros, metais pesados, exposição a poluentes ambientais, solventes químicos, dentre outros (PIZZINO et al., 2017).

Organismos vivos possuem sistemas antioxidantes que são capazes de regenerar ou prevenir todo ou parte dos danos oxidativos. Entretanto, substâncias sequestradoras de radicais livres também podem ser obtidas de fontes externas, como alimentos e bebidas. Diversos estudos acerca dos radicais livres e o desenvolvimento de novos métodos para avaliação de atividade antioxidante (AOX) têm aumentado consideravelmente, onde as descobertas dos efeitos deletérios dos radicais livres e sua relação com certas doenças impulsionam a busca por novas substâncias capazes de prevenir ou minimizar os danos oxidativos às células vivas (SILVA et al., 2014; CALIXTO JÚNIOR et al., 2015; SOARES et al., 2015; MORAIS et al., 2017; PENIDO et al., 2017; ALVES et al., 2018; PEREIRA et al., 2018) demonstrando produtos naturais como boa alternativa como AOX.

A avaliação do potencial de remoção de radicais livres DPPH* (2,2-difenil-1-picril-hidrazila) (Figura 05) de uma molécula antioxidante é considerada como um dos métodos colorimétricos padrão e de rápida avaliação de propriedades antioxidantes de compostos puros (MISHRA; OJHA; CHAUDHURY, 2012). As substâncias antioxidantes reagem com o DPPH, que é um radical livre estável e é reduzido ao DPPH·H, bem como é observada a redução de sua absorbância no espectro de espectroscopia eletrônica (KARRAR, 2014).

Figura 05 – Evidencia-se o método de determinação de atividade antioxidante pela capacidade de substâncias sequestrarem o radical livre DPPH*.
2.6.2 Atividade antiacetilcolinesterase

A acetilcolinesterase (AChE) provou ser o alvo terapêutico mais viável para a melhora sintomática de doenças voltadas a degradação celular, porque o déficit colinérgico é um achado consistente e precoce, sendo importante alvo com correlação positiva com substâncias antioxidantes (MORAIS et al., 2017), determinante em doenças neurodegenerativas (PENIDO et al., 2017), bem como em doenças causadas por protozoários, como leishmanioses (ALVES et al., 2017b, 2018; MARTINS et al., 2018; TOMIOTTO-PELLISSIER et al., 2018), doenças causadas por microrganismos (PEREIRA et al., 2018), dentre outras.

A sinalização da acetilcolina é terminada dentro da fenda sináptica por clivagem pela acetilcolinesterase (AChE). Assim, fármacos que mimetizam a atividade da acetilcolina (ACh) (colinomiméticos) ou fármacos que limitam a degradação da acetilcolina (inibidores da AChE) fornecem uma estratégia terapêutica para aumentar a sinalização colinérgica (CARTER et al., 2017). Evidências crescentes mostraram redução da atividade da AChE em vários distúrbios cerebrais, incluindo distúrbios neurodegenerativos (PAUL; BORAH, 2017).
Figura 06 – Descrição de atividade da enzima acetilcolinesterase. É evidenciado o mecanismo enzimático (U) na fenda sináptica, realizando a lise da acetilcolina (●) em ácido acético (¶) e colina (¶). Demonstam-se ainda os receptores nicotínicos (N) e muscarínicos (M) como determinantes na formação de sinalização desse neurotransmissor.

A acetilcolinesterase desempenha um papel importante em muitas condições...
patológicas, diminuindo a disponibilidade de acetilcolina na fenda sináptica, como demonstrado na Figura 06. Assim, a inibição da AChE emergiu como o principal alvo terapêutico baseado nesta "hipótese colinérgica", considerada uma estratégia terapêutica promissora para vários tipos de doenças neurodegenerativas como: demência, miastenia gravis, glaucoma e doença de Parkinson, além da Doença de Alzheimer (DA) (MATHEW; SUBRAMANIAN, 2014). Galantamina (nivalina, razadyne, reminil), que estimula a neurotransmissão colinérgica com apenas efeitos adversos leves em doses baixas, é usada para melhorar o desempenho cognitivo em pessoas com esse tipo de doenças (EJSMOND; PROVENZA, 2018)

Figura 07 – Hidrólise promovida pela enzima AChE.

Os inibidores da acetilcolinesterase (AChE) são, até o momento, os únicos fármacos que demonstram eficácia clínica no tratamento da DA. Reduzindo a hidrólise da acetilcolina (ACh), em ácido acético e colina (Figura 07), a inibição da AChE aumenta a função dos neurônios colinérgicos centrais, permitindo que a ACh permaneça mais tempo na fenda sináptica estimulando receptores N e M no cérebro. Atualmente, apenas a abordagem de inibição da AChE parece produzir melhorias sintomáticas encorajadoras em ensaios clínicos (LOVERRE et al., 2008).

Atualmente, quatro inibidores de AChE são licenciados pelo Ministério da Saúde brasileiro para o tratamento sintomático da DA: memantina, donepezil, rivastigmina e galantamina (ABU MOHSEN et al., 2016). De fato, esses agentes prolongam a duração da ação da acetilcolina e proporcionam alívio sintomático nessa doença (ALIABADI, 2013). A relevância dos fármacos baseados em produtos naturais está evidenciada pelo fato de três dos cinco fármacos utilizados para fins medicinais serem produtos naturais (galantamina e tacrina) ou semi-sintéticos (rivastigmina) (TZVETKOV; ATANASOV, 2018), onde somente a galantamina e a rivastigmina são permitidos no Brasil.
2.6.3 Atividade antileishmanial

Como anteriormente citado, infecções por *Leishmania* sp. podem causar grande espectro de sintomas, LC é a forma mais comum da doença e provoca úlceras, deixando cicatrizes e estigmas (GRANO et al., 2014; VAN DEN BOGAART et al., 2014). Lesões cutâneas localizadas, lesões cutâneas difusas, destruição da membrana mucocutânea, doenças viscerais de órgãos do sistema hematopoético, dentre outros. Para tanto, o parasito precisa vencer diversas barreiras naturais no hospedeiro, intermediário ou não, para completar seu desenvolvimento.

Os órgãos alvo para infecções por *Leishmania* sp. são aqueles que contêm as células do sistema fagócito mononuclear, tais como o fígado, o baço, nódulos linfáticos, a medula óssea, e também a pele, a difusão para o sistema nervoso central pode ocorrer para dar suporte à proliferação, quando da proteção do parasito contra as drogas antileishmaniais (CHATTERJEE et al., 2014). A co-infeção de um hospedeiro por mais de uma espécie de parasitas é uma condição recorrente e a suscetibilidade individual do hospedeiro, bem como o grau de infectividade e patogenicidade do parasito, são sensibilizados em diferentes níveis no sistema de interações parasito-hospedeiro (HERMIDA et al., 2014).

O padrão de transmissão da doença tem apresentado mudanças importantes, inicialmente predominado pelas características de ambientes rurais e, mais recentemente, ocorrendo em centros urbanos. A LV é também considerada emergente em indivíduos portadores do vírus da imunodeficiência adquirida (HIV), tornando-se uma das doenças mais importantes da atualidade, dada a sua incidência e alta letalidade em indivíduos não tratados e crianças desnutridas (BRASIL, 2011, 2013a,b).

3 JUSTIFICATIVA

Em destaque por sua larga aplicação, a enzima lipolítica representa um possível papel como leishmanicida por apresentar efeitos adversos em outros organismos. Foi demonstrada a ação nematicida, bactericida, fungicida das soluções de lipase, protease e enzimas hidrolíticas (BUTTLE et al., 2011; HU et al., 2012; PARAMESWARAN et al., 2014).

Como efeito, a presença de vários compostos bioativos em *C. coriaceum*, são determinantes na sua utilização na medicina tradicional (BEZERRA et al., 2002; AGRA;
FREITAS; BARBOSA-FILHO, 2007; ALVES et al., 2017; TOMIOTTO-PELLISSIER et al., 2018). Plantas medicinais constituem uma fonte importante para o desenvolvimento de fármacos, de modo que o estudo dos metabólitos secundários das mesmas torna-se faz necessário e relevante para a pesquisa em questão.

Dessa forma, se faz necessário primeiramente reconhecer a importância da identificação e caracterização destes metabolitos primários e secundários para estudos posteriores e, em segunda instância, analisar a interação entre os metabólitos e o patógeno com exames de toxicidade frente a espécies de *Leishmania* descritos amplamente na literatura, comparando-os com a metodologia apresentada por Vila-Nova et al. (2011) e Silva et al. (2014a,b), com a possibilidade da avaliação da biodiversidade de compostos.

A biodiversidade existente na região nordeste detém um potencial latente mediante o patrimônio gênico que este tem a oferecer como fonte promissora de metabolitos bioativos de importância farmacológica. As propriedades terapêuticas de muitos metabólitos de plantas conduzem à pesquisa de ingredientes ativos de várias espécies. A exploração desses recursos pode levar à identificação de produtos naturais que podem servir como fármacos ou levar ao desenvolvimento de novas substâncias terapêuticas. Podemos afirmar ainda que a Caatinga detém grande potencial biotecnológico, pois seu potencial de produção metabólico ainda não foi plenamente investigado e grande parte do seu patrimônio biológico não ser encontrado em outro lugar do planeta.

Ao identificar os mecanismos de ação sobre os patógenos a partir de produtos naturais enzimáticos de fungos, bem como por metabólitos secundários vegetais como fenóis, flavonoides, taninos, alcaloides, tripterínóides, etc., há a possibilidade de contribuir para a determinação de metodologias de intervenção, alterando o metabolismo do organismo e impedindo assim a infecção pelo protozoário e/ou interrompendo o curso de infecção de *Leishmania*.

4 HIPÓTESE CIENTÍFICA

Metabólitos de vegetais da Caatinga, e de seus fungos endofíticos, possuem ação antileishmanial.
5 OBJETIVOS

5.1 OBJETIVO GERAL

Obter extratos proteicos de fungos endofíticos de *Jatropha curcas* e extratos orgânicos de *Caryocar coriaceum* para avaliação das atividades antioxidante, anticolinesterasica e antileishmanial

5.2 OBJETIVOS ESPECÍFICOS

- Coletar e preparar extratos com solventes orgânicos de folhas, cascas e frutos de *Caryocar coriaceum*
- Isolar e identificar fungos endofíticos associados a *Jatropha curcas*;
- Obter extratos proteicos dos fungos isolados;
- Caracterizar as lipases dos fungos endofíticos
- Caracterizar os metabólitos secundários presentes nos extratos vegetais;
- Testar os produtos selecionados contra *Leishmania* sp. em formas promastigotas e amastigotas;
- Determinar a eficiência, a seletividade e a concentração média letal dos produtos selecionados.
Lipolytic enzymes and their use in production of human and animal biotechnology

(Enzimas lipolíticas e seu uso na produção de biotecnologia humana e animal)

Daniela R. Alves, Selene M. Morais, Fábio R. Vasconcelos, Francisco C. O. Freire

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Lipolytic enzymes from fungus and their use for human and animal biotechnology
Daniela R. Alves¹⁺, Selene M. Morais¹*, Fábio R. Vasconcelos², Francisco C. O. Freire³

ABSTRACT

Lipases are omnipresent on the nature and they act as catalysts for hydrolysis reactions of triglycerides, or synthesis of esters from fatty acids and glycerol. Although they are differentiated by their origins and properties, these enzymes have been highlighted in several industrial sectors, from food products, textiles, cosmetics and the formation of diagnostic tools. The lipases need a "key" that gives access to its active site, as well as something that stabilizes the molecule when it undergoes activation. Nevertheless, few studies are available to designate and classify the genetic sequence of lipases obtained from producing microorganisms. In this context, this literature review aims to search for the molecular determination, through gene expression, and registering of eukaryotic lipases in silico to make the enzyme employment as an economic alternative to produce specific and feasible alternatives for industrial needs. The production and thermostability's importance of some microbial enzymes are also approached.


INTRODUCTION

Microorganisms, as bacteria and fungi, are in general ease of nutrition and cultivation, present high rates of growth and production, as well as synthetize a variety of bioactive compounds including enzyme molecules, which allow the bioengineering for the production of new bioproducts. When comparing microbial enzymes with animal and

¹ Veterinarian Sciences Post Graduation Program, Ceará State University, Av. Dr. Silas Munguba, 1700, CEP 60740913, Campus Itaerê, Fortaleza, Ceará, Brazil
² Embrapa Agroindustria Tropical, Rua Sara Mesquita n 2270 Planalto do Pici, Fortaleza, Ceará, Brazil Author to whom correspondence should be addressed: alves.danielaribeiro@gmail.com; selenemaiaademorais@gmail.com*; Tel.: +55-85-988938523
vegetable enzymes, the former exhibit properties that determine their preference in the
most diverse applications (1–8).

Among the microorganisms, fungi are distinguished by the production of
extracellular enzymes, which contribute for the synthesis or hydrolysis of the substances
produced in the medium. In each environment the determinant for their performance on
metabolic processes are the sequence of nucleic acids expressed in each microorganism,
which translated into proteins for environmental need (2,3,9–16).

Each factor, such as carbon and nitrogen sources, pH and temperature, is variable
and specific for each microorganism and determines the activity of the proteins produced.
In general, the preparation of optically active compounds as biodiesel synthesis has been
a major challenge for organic chemists and biochemists due to the increasing need for
thermostable substances (17,18) for use in the various industrial sectors as Automotive,
Chemistry, Cooked food and drinks, Cosmetics, Dairy Products, Detergent Industry, Fats
and oils, Leather, Paper, Pharmaceutical, etc.

Among these substances the lipase enzyme stands out in the industries of its most
varied sectors, from food products, textiles, cosmetics and the formation of diagnostic
resources, acting as enzymatic markers (1). Lipases are classified in the superfamily of α
/ β hydrolase and have as an example of sister enzymes esterases, proteases, peroxidases,
lyases, among others (19).

Lipases are ubiquitous in industrial sectors and constitute the most important
group of biocatalysts in biotechnological applications (1). In researches of mutagenic
lipolytic determination there is a search for ways to improve the protein sequence to
determine functions different from those previously expressed (8,20–23). Thus, the effect
of pH, temperature, metal ions and substrate are specific in the bioproduction of any
substance of high reactivity, as lipases.

It is possible to produce high amounts of optically active and improved enzymes
for industrial use (24). The choice of the microorganisms to produce a specific enzyme
will vary according to a characteristic gene sequence in the active site of the enzymes
from chosen microorganisms. As example, Fusarium heterosporum produces a solvent-
tolerant lipase, which cDNA was cloned, and was expressed using Saccharomyces
cerevisiae.

Most lipases require a "key" that gives access to their active site, consisting of one
of two α-helices polypeptide chains attached to the protein body by flexible structural
elements, as lids that protect their active site (25). It is necessary the determination of the
molecules of the enzyme's active site, to measure its qualitative and quantitative action in the industrial production. The studies available in the literature that address lipases do not explore them extensively, designating their characteristics and differentiating them by their origins and properties.

In this context, the present review proposes the determination of the enzymatic characterization, through the search of gene expression, molecular, structural and functional characteristics, and the cataloging of inelastic eukaryote lipases with data from the last 20 (twenty) years, addressing the production, the importance of thermostability of some microbial enzymes as well as their biotechnological applications including in the veterinary medicine.

**LIPASES (TRIACYLGLYCEROL ACYLHYDROLASE, EC 3.1.1.3)**

Lipases are enzymes capable of catalyzing the synthesis (development) and / or hydrolysis (breaking) of a broad spectrum of carboxylic esters, as well as the use or production of organic acids and glycerol (9,26), even in a disadvantaged environment of water molecules, according to the need of the microorganism (19,27).

During the catalysis the enzyme is produced in extracellular medium, facilitating its recovery from it (28,29). This exoenzyme is susceptible to change in its structural conformations by changes in temperature, pH, nitrogen and carbon sources, as well as inorganic salts and oxygen concentration. Each characteristic, expressed by the enzyme, will be determined by the genetic sequence that transcribes it and is regulated by its affinity with the substrate.

Several studies demonstrate the production of this type of enzyme by fungi, either naturally or by molecular bioengineering (14–16,30,31). Mobarak-Qamsari et al. (32) performed the genetic sequencing and verified the increase in lipolytic activity through the improvement of production conditions through differentiated carbon and nitrogen concentrations for the selected bacteria.

Sometimes the use of the effluent to produce lipases (Roveda et al., 2010; Mobarak-Qamsari et al., 2011) is a viable and low-cost process. Effluents present high nutrient load still available for microbiological growth and subsequent enzyme production (Ferreira et al., 2005; Santos et al., 2007; Bandeira et al., 2014).

Lipase-producing fungi have already been isolated from greasy industrial waste (Mobarak-Qamsari et al., 2011), of soil contaminated with oil (Bandeira et al., 2014), factory processing of vegetable oils and dairy products (Santos et al., 2007; Mahanta et
lipases for industrial applications

In order to characterize the alkaline lipase enzyme for industrial applications, such as the use of lipolytic enzymes in detergents, animal leather processing industries and high-quality chemicals, the authors used *Pseudomonas aeruginosa* strain KM110 from the (Figure 1).

![Pseudomonas aeruginosa lipase structure](image)

Figure 1: *Pseudomonas aeruginosa* lipase structure β-strips are represented as arrows (brownish red) and α-propellers as roll (blue and yellow); The yellow helix may form a lid on the active site. Residues of the active site triad Ser82, Asp229 and His251 are marked, and the potential position of the Ca²⁺ ion is indicated by the green ball (13).

Nagao et al. (24) also verified the influence of carbon and nitrogen concentration on biological development and lipase production. For this a transfection of the amino acid code present in *Saccharomyces cerevisiae* was carried out in a strain of *Fusarium heterosporum*. However, the authors observed that although the peptide expressions are very similar, the production of this enzyme is strongly influenced by the medium. This fact was also observed in cultivation of strains with similar gene loads, even though from different genera, such as *Pseudomonas* sp. and *Burkholderia* sp. (41).

There are certain species of fungi that produce and degrade esters, using more stable lipases and better quality, being more active and stable in extreme environments, in the presence of detergents, alkaline pH and temperatures above 60°C (42,43). And it is these enzymes that the industry employs to dissolve solids coming from treatment plants,
clearing and/or preventing oleic accumulation on wastewater surfaces (32,44–46).

**Molecular characterization of lipases**

In general lipases have different amino acid sequences, since they catalyze different types of hydrolysis reactions then: i) They do not have any similarity between amino acids (AA) sequences; ii) do not operate with identical substrates and iii) do not have the same nucleophile (negative ion or neutral molecule acting as a Lewis base). Structural and spatial similarities are limited to its catalytic region, thus, although they do not have the same sequence of AA, after the packaging it is observed a conserved region for active site, showing its common ancestry (20,47–50).

Lipases are classified into eight families (I to VIII), described in Table 01, according to their properties, structures and protein sequences, all of which are considered α/β hydrolases produced in extracellular medium, and have similar enzymes as esterases, proteases, peroxidases, lyases, among others (51). After protein packaging these enzymes demonstrate the characteristic α/β structure, so they are considered as possessing a common ancestor only when active.

On the conserved sites, these enzymes become homologous only when activated after packaging, with α/β packaging structure, when finally the catalytic triad is shown (25). Where the hydrophobic surface can be centralized, in the zone of lipid contact, there are protein residues such as PHE, ILE, TRP, LEU and TYR, where the first two probably have the function of coupling and penetrating the lipid surface due the apolar R group linked to α-carbon (19).

Family I is the largest and most branched, covering seven other subfamilies (I.1 to I.7). In relation to these subfamilies, the first three are derived from gram-negative bacteria and thus considered true lipases. Families I.1 and I.2 have about 30% similarity in their genetic sequences, being secreted by the type 2 secretion system. Family I.3 have less than 20% similarity to the previous two, being produced by the type 01 secretory pathway.

The family II does not have the conventional catalytic triad (a group of three amino acids that are found in the active sites), but an association between GLY, ASP, SER and LEU within the catalytic residue of the SER, so the same is closer to the amine terminal of other lipase. The family III consists of monomers of acetylhydrolases that also function as a factor for activation of platelets (tiny blood cells that help body form clots to stop bleeding) and is constituted of the typical catalytic triad that plays the versatility
of α/β hydrolases.

The variations of family IV lipases have a great similarity with mammalian hormone-sensitive lipase enzymes, demonstrating their origin in mesophilic bacteria. Those of the V family may also originate from mesophilic families, but are thermophilic in general, and can be adapted to cold or heat. The VI family has the smallest enzymes known as esterases, thus not having activity in long chains of triglycerides, its active form is that of a dimer.

These latter enzymes are 40% like the eukaryotic lysophospholipases. The VII family has great similarity with acetylcholinesterases in the intestines of mammals and is particularly active even in the presence of the herbicide phenylecarbamate (acetylcholinesterase inhibitor), in general the protein family has a length around 55kDa (52). Finally, the family VIII has the most active site β-lactamases, which suggests that its SER residue also has LYS, a hydrophilic amino acid that assists in the formation of the oxyanion hole (51, 53, 54). This process of stabilization occurs only by the formation of two hydrogen bonds between amide bonds in the forming residues of this electrophilic region (55).

For Pleiss et al. (25) the lipase’s classification is not based on numbers but on the name of each "superfamily," which support individuals of different species, but with the same characterization based on their properties, structures, and protein sequences. With integrates information on sequence and structure of lipases and related proteins sharing the same α/β hydrolase fold to facilitate protein engineering. The classification from Arpigny e Jaeger (51), is the most used among the searches found in this review.
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<td>D13253</td>
<td></td>
<td>15</td>
<td>51 True Lipases</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td><em>Bacillus subtilis</em></td>
<td>M74010</td>
<td></td>
<td>16</td>
<td>100 True Lipases</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bacillus pumilus</em></td>
<td>A34992</td>
<td></td>
<td>13</td>
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<td></td>
<td></td>
<td><em>Bacillus stearothermophilus</em></td>
<td>U78785</td>
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<td>15</td>
<td>100 True Lipases</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td><em>Bacillus thermocatenulatus</em></td>
<td>X95309</td>
<td></td>
<td>14</td>
<td>94 True Lipases</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Staphylococcus hyicus</em></td>
<td>X02844</td>
<td></td>
<td>15</td>
<td>29 Phospholipase</td>
</tr>
<tr>
<td>Species</td>
<td>Accession</td>
<td>Activity Type</td>
<td>Description</td>
<td></td>
<td></td>
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<tr>
<td>Staphylococcus aureus</td>
<td>M12715</td>
<td>14</td>
<td>Phospholipase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
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<tr>
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<tr>
<td>Aeromonas hydrophila</td>
<td>P10480</td>
<td>100</td>
<td>Acyltransferase secreted</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Streptomyces scabies*</td>
<td>M57297</td>
<td>36</td>
<td>Esterase secreted</td>
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<tr>
<td>Pseudomonas aeruginosa</td>
<td>AF005091</td>
<td>35</td>
<td>Binding membrane esterase</td>
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<td>Salmonella typhimurium</td>
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<td>Photorhabdus luminescens</td>
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<td>28</td>
<td>Esterase secreted</td>
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<td>Streptomyces exfoliatius*</td>
<td>M86351</td>
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<td>Streptomyces albus</td>
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<td>Moraxella sp.</td>
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<td>Extracellular Esterase 1</td>
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<td>Alicyclobacillus acidocaldarius</td>
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<td>Archaeoglobus fulgidus</td>
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<td>48</td>
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<td>Alcaligenes eutrophus</td>
<td>L36817</td>
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<td>Escherichia coli</td>
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<td>Moraxella sp.</td>
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<td>Pseudomonas oleovorans</td>
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<td>Polyhydroxyalkanoate Depolymerase</td>
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<td>Haemophilus influenzae</td>
<td>U32704</td>
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<td>Moraxella sp.</td>
<td>Sulfobacter pasteurianus</td>
<td>Acinetobacter sp.</td>
<td>Synechocystis sp.</td>
<td>Pseudomonas fluorescens</td>
<td>Rickettsia prowazekii</td>
<td>Chlamydia trachomatis</td>
</tr>
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<td>X53869</td>
<td>AF071233</td>
<td>AB013096</td>
<td>S70419</td>
<td>S70600</td>
<td>Y11778</td>
<td>AE001287</td>
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<td>34</td>
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<th>Extracellular Esterase 3</th>
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<th>Esterase</th>
<th>Carboxylesterases</th>
<th>Carboxylesterases</th>
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<th>Carboxylesterases</th>
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<td>20</td>
<td>100</td>
<td>50</td>
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<td>20</td>
<td>16</td>
<td>100</td>
<td>48</td>
<td>45</td>
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</tbody>
</table>

VI

VII

VIII
Active site mechanisms – the role of the oxyanion hole

It is called "oxyanion" the internal hydropathic structure which, through hydrophilic forces, causes hydrophobic residues, generally: MET, CYS, PHE, LEU, VAL and/or ILE, to localize externally to the protein. Figure 2 shows two types of oxyanion holes from enzymes GX e GGGX.

![Diagram of GX and GGGX](image)

**Figure 2. Illustration of two types of oxyanion holes. Modified from (25).**

In figure 2 (a), the GX type enzyme in *Rhizomucor miehei* lipase (PDB register 4TGL) the stabilization of the substrate is made through the diethylphosphate DEP inhibitor by hydrogen bonds in the first residue of the oxyanion hole (S82); Stabilization of S82 by hydrogen bonds anchored to residue D91. In b) In the enzyme type GGGX in *Candida rugosa* lipase (PDB 1LPM register), stabilization of the substrate is made through the analogous 1R-methyl hexyl phosphonate inhibitor where the first oxyanion hole contains a residue G (G124); stabilization of the interlaced oxyanion the A210 side chain occurs between G124 and the side chain of X (F125).

As more hydrophilic the nucleophiles, more they express their characteristics: i) the polar amino acids will be inside the protein; ii) a better equilibrium of the electric charges of the free carbonyl, which also increases its thermostability and iii) maintains the protein spatial arrangement in its active form (25,56–58).

**Biotechnological Applications in Veterinary Medicine**

Regarding the lipase enzyme in veterinary medicine, its importance starts with the
interaction between the study of the maintenance of homeostasis and the pathogenesis. Historically, the determination of lipase serum activity, amylase, and trypsin immunoreactivity have been used for diagnosis. (Hulshof et al., 2016)

The lipolytic concentration test is reported to be the most sensitive (65-94%) and specific (66-100%) non-invasive biomarker available for the diagnosis of pancreatitis in animals (Steiner et al., 2004; Trivedi et al., 2011; Xenoulis et al., 2014). Thus, clinical enzymology is of fundamental importance to identify hepatic deficits and pancreatitis in animals through the analysis of the metabolic profile of the blood (Xenoulis et Steiner, 2012; Scheffer et González, 2016).

Lipolytic engineering began more than 150 years ago, but it was only after the mid-1980s that most of the enzymes produced came from microbial sources. Only when it became accepted that lipase enzymes remained active, even in organic solvents, that several investigations with these enzymes began as objects of study, rising to make them tools for the industry (Wong et Schotz, 2002; Jaeger et Eggert, 2002).

Over the years, it has been demonstrated that in order to obtain the production of enzymes with high quality and specialization it is necessary to prioritize and observe the production properties of the studied microorganisms, purification and characterization of this production to achieve a stable and effective enzyme. Despite the expressive knowledge of the wide possibility of enzymatic production by microorganisms only a small number of lipases are commercially exploited (Jaeger et Reetz, 1998; Ramani et al., 2010).

The development of lipolytic applications in the production and use in industries only increased the interest for their coding genes in the different microorganisms, because these enzymes are highly variable in composition, size and structure.

The recent interest in lipolytic production is justified by the discovery of its most varied applications (Salihu et al., 2012). As food additives, lipases act in the synthesis of esters to be flavoring agents, in the hydrolysis of triacylglycerols (oils or fats), to act as detergents or cleaning agents (Liu et al., 2009) and in the composition of medicines in pharmaceutical industry (Gurung et al., 2013). See table 2.

Lipolytic enzymes can also be used in the treatment of wastewater by performing the decomposition and removal of oily substances (32,45,46), developed as an alternative to conventional treatment(33), on anaerobic biodigestion of swine manure (69,70), in the degreasing of skin and animal coatings and in cosmetics (71) in the removal of lipids.
Table 2 - Industrial applications of microbial lipases. Modified from (32,33,70,72,73).

<table>
<thead>
<tr>
<th>Industry</th>
<th>Action</th>
<th>Product application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergent</td>
<td>Fat Hydrolysis</td>
<td>Oil stain removal from factories</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anaerobic biodigestion of swine manure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment of wastewater</td>
</tr>
<tr>
<td>Dairy Products</td>
<td>Milk and fat hydrolysis, cheese ripening, butter fat modification</td>
<td>Development of causative / flavor modifying agent, milk, cheese and butter</td>
</tr>
<tr>
<td>Cooked food</td>
<td>Flavor Development</td>
<td>Storage time extension</td>
</tr>
<tr>
<td>Drinks</td>
<td>Flavor development</td>
<td>Drinks</td>
</tr>
<tr>
<td>Food Adornment</td>
<td>Quality development</td>
<td>Mayonnaise, ornamentation and fiber breaking (meat)</td>
</tr>
<tr>
<td>Healthy food</td>
<td>Transesterification</td>
<td>Healthy food</td>
</tr>
<tr>
<td>Meat and fish</td>
<td>Flavor Development</td>
<td>Meat and fish products; fat removal</td>
</tr>
<tr>
<td>Fats and oils</td>
<td>Transesterification, hydrolysis</td>
<td>Cocoa Butter, Margarine, Fatty Acids, Glycerols, Mono-, and Diglycerides</td>
</tr>
<tr>
<td>Chemistry</td>
<td>Enantioselectivity, synthesis</td>
<td>Construction of chiral blocks, chemical compounds</td>
</tr>
<tr>
<td>Pharmaceutical</td>
<td>Transesterification, hydrolysis</td>
<td>Special lipids and digestion aids</td>
</tr>
<tr>
<td>Cosmetics</td>
<td>Synthesis</td>
<td>Emulsifiers, Moisture Controlling Agents</td>
</tr>
<tr>
<td>Leather</td>
<td>Hydrolysis</td>
<td>Leather products</td>
</tr>
<tr>
<td></td>
<td>Skin animal coating</td>
<td></td>
</tr>
<tr>
<td>Paper</td>
<td>Hydrolysis</td>
<td>Better fiber quality papers</td>
</tr>
<tr>
<td>Cleaning</td>
<td>Hydrolysis</td>
<td>Fat removal</td>
</tr>
<tr>
<td>Automotive</td>
<td>Biodiesel Synthesis</td>
<td>Transesterification of vegetable oils</td>
</tr>
</tbody>
</table>

Gomes et al. (2007) states that the same microorganisms that produce lipase with hydrolytic activity can be used in papermaking for processing of starch and food (74). Lipases inhibitors can also be applied as an additive for animal nutrition, causing the previous breakdown of fats and oils from the mixture to be inserted into the feed (Jensen et al., 1997; Meena et al., 2014) and/or preventing fats from being absorbed in the intestine (Fouad e El-Senousey, 2014).

With lipolytic enzymes addition in food, absorption of fats by the intestine is reduced and the resulting composition of fats in foods is presumed to facilitate the development of lean and qualitatively high meat (Jensen et al., 1997; Ghosh, 2006; Fouad e El-Senousey, 2014; Meena et al., 2014; Polycarpo et al., 2014; Suiryanrayna e Ramana, 2015).

Over the past years lipases have been used in the synthesis of many biologically
active compounds. For example, lipases to catalyze the acylation of substances, forming novel optically active compounds (Itoh et al., 1993, 1997). As well as it is used in the regioselective esterification of diacetates, since this is impossible by chemical reactions like the alkaline hydrolysis (Itoh et al., 1993; Uza et al., 1996).

As an example, pancreatic lipase has the ability to catalyze the ester emulsion hydrolysis in glycerol and long chains of derivative fatty acids (Angajala et al., 2016). These reactions of transesterification and enzymatic hydrolysis are complementary methods for the resolution of secondary alcohols in the synthesis of chiral drugs (Kazlauskas et al., 1991).

In the animal production some products containing fats of dairy products are normally used and lipase can be added, which provides the transformation of long chains into short chains in order to facilitate absorption and accelerated growth of goat, camel, cow, buffalo and pig pups (Jensen et al., 1997; Meena et al., 2014).

Lipases have also been used in flavor enhancement (Jaeger e Eggert, 2002; Sabat et al., 2012), making changes in food coloring and creaminess characteristics (Hasan et al., 2006), according to the size and degree of unsaturation of the carbon chain (Schmitt et al., 2002; Macedo et al., 2003), as shown in table 3.

Table 3. Some of the commercially available lipases of microbial origin produced by different companies. Modified from (20,66,88)

<table>
<thead>
<tr>
<th>Fonts</th>
<th>Trade</th>
<th>Name</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alcaligenes ssp.</em></td>
<td>LipasePL</td>
<td>MeitoSangyo, Co.</td>
<td>modifications in oils and fats / food additives</td>
</tr>
<tr>
<td><em>Pseudomonas cepacia</em></td>
<td>LipaseSL</td>
<td>Amano</td>
<td>synthesis of chiral compounds</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>LipaseDS</td>
<td>Amano</td>
<td>food supplements</td>
</tr>
<tr>
<td></td>
<td>Lipase</td>
<td>Sigma</td>
<td>organic and analytical synthesis</td>
</tr>
<tr>
<td></td>
<td>Lipopan® F³</td>
<td>Novozyme</td>
<td>dough / paste (hardness)</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>Lipomod™62</td>
<td>Biocatalysts</td>
<td>flour / pasta dough (texture and shelf life)</td>
</tr>
<tr>
<td></td>
<td>7P-L627P</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Lipomod™36</td>
<td></td>
<td>Dietetics</td>
</tr>
<tr>
<td></td>
<td>P-L036P</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Yeast</strong></td>
<td>Palatase²a</td>
<td>Novozyme</td>
<td>development of dairy products flavors (Cheese)</td>
</tr>
</tbody>
</table>

²a
<table>
<thead>
<tr>
<th></th>
<th>LipaseMY</th>
<th>MeitoSangyo, Co.</th>
<th>Dietetics</th>
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<tbody>
<tr>
<td><em>Candida antarctica</em></td>
<td>Novozym®</td>
<td>Novozym</td>
<td>olive oil specialties</td>
</tr>
<tr>
<td></td>
<td>435a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Noopazyme®</td>
<td></td>
<td>pasta / noodles</td>
</tr>
<tr>
<td><em>Candida cylindracea/ porcine páncreas</em></td>
<td>LipomodTM 29 P-L029P</td>
<td>BiocatalystsLt d.</td>
<td>development of dairy products flavors (Cheddar Cheese)</td>
</tr>
</tbody>
</table>

*a trade names may change.

The most important element for lipolytic expression is the carbon source, i.e. from alternative carbon sources such as sugars and polysaccharides, and triacylglycerols (Panaiotov et al., 1997; Romdhane et al., 2011). Triacylglycerols, fatty acids, bile salts, glycerol are common sources of carbon, although olive oil is usually the most used for lipolytic production in scientific studies (Sileo, 2001; Diaz et al., 2006; Rodriguez et al., 2006; Gonçalves, 2007; Stoytcheva et al., 2011).

**CONCLUSION**

There are few studies available in the literature that address lipases in a broad way, designating the gene sequence that produces this enzyme in the microorganisms studied, their molecular, structural and general classification characteristics.

Although they have a catalytic triad composed of conserved residues of SER-ASP/GLU-HIS/THR, lipolytic enzymes have a wide variety of characteristics and similarities with other protein groups, which makes the α / β family hydrolase so diverse, such as *Candida antarctica* lipase, which has two regulatory protein layers and *Fusarium solani* cutinase.

Metal ions such as Ca$^{2+}$ or Mg$^{2+}$, among others, are determinant components in lipolytic activation, as well as the oxyanion hole becomes indispensable through its stabilizing function of electrons in the molecular protein structure. The more hydrophilic, the more amino acids will be close to the protein core and, therefore, the better the equilibrium of the electric charges, which maintains the protein arrangement in its active form and increases the molecular thermostability.

In the search for assisting veterinarians, microbiologists and biochemists in the complete understanding of the functioning of these enzymes, this review aims to elucidate and even confront the existing characteristics and knowledge. Therefore, further studies
are needed to characterize and elucidate this enzymatic group. In industrial processes with
the synthesis of new products, biocatalysis is a remarkable tool, and without any doubt
the lipases constitute one of the important current biocatalysts. The limitations found in
the synthetic application of enzymes in their native form are currently being circumvented
by altering stereospecificity, thermostability and activity involving molecular biology
techniques of site-directed or random mutations.

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Flavonoid composition and biological activities of ethanol extracts of *Caryocar coriaceum* Wittm, a native plant from Caatinga Biome

(Composição flavonóide e atividades biológicas de extratos etanólicos de *Caryocar coriaceum* Wittm, planta nativa do bioma Caatinga)


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Flavonoid composition and biological activities of ethanol extracts of *Caryocar coriaceum* Witum, a native plant from Caatinga Biome

Daniela Ribeiro Alves¹, Selene Maia de Morais¹*, Fernanda Tomiotto-Pellissier³, Milena Menegazzio Miranda-Sapla³, Fábio Roger Vasconcelos², Isaac Neto Goes da Silva¹, Halisson Araujo de Sousa³, João Paulo Assolini³, Gustavo Adolfo Saavedra Pinto², Ivete Conchon-Costa³, Wander Rogério Pavanelli³, Francisco das Chagas Oliveira Freire².

¹Veterinarian Sciences Post Graduation Program, Ceará State University, Av. Dr. Silas Munguba, 1700, CEP 60740913, Campus Itaperi, Fortaleza, Ceará, Brazil
²Embrapa Tropical Agroindustry, Rua Sara Mesquita n 2270 Planalto do Pici, Fortaleza, Ceará, Brazil
³Pathological Sciences Center, Londrina State University; Rodovia Celso Garcia Cid, PR: 445, Km 380, Campus Universitário, Londrina, Paraná, Brazil.
⁴Chemical Course, Ceará State University; Av. Dr. Silas Munguba, 1700 Campus Itaperi, Fortaleza, Ceará, Brazil

*Author to whom correspondence should be addressed: selenemaiademorais@gmail.com;
Tel.: +55-85-988938523
Abstract

Caryocar coriaceum fruit is largely used in culinary and in this work ethanol extracts of pulp and peel of C. coriaceum fruits were evaluated in relation to phenols and flavonoids content and biological activities such as antifungal, antileishmanial, anticholinesterase and antioxidant. Peel extracts contain higher content of total phenols and flavonoids. HPLC analysis of flavonoids revealed Isoquercitrin as the main flavonoid in the extracts. Fruit peel extract also showed the best antioxidant activity, compared to standard. In the inhibition of the acetylcholinesterase assay, pulp and peel extracts demonstrate action comparable to physostigmine. The antimicrobial activity of extracts was evaluated against strains of Malassezia sp. and Microsporum canis, using the broth microdilution technique, in which the extracts showed similar MIC and MFC. Moreover, the extracts present antileishmanial activity and low toxicity on murine macrophages and erythrocytes. Therefore, these results suggest a potential for the application of C. coriaceum fruit ethanol extracts in the treatment against dermatophyte fungi and leishmaniasis, probably due to the presence of quercetin and its glucosides. Further studies are recommended for in vivo tests, aiming the development of possible new pharmaceutical compounds.

Keywords: fungicidal; antileishmanial; crude extract; antiacetylcholinesterase; antioxidant; Isoquercitrin

Introduction

In veterinary care some diseases are common in Brazil as fungal dermatophytosis and cutaneous/mucocutaneous leishmaniasis. Regarding to fungal diseases, Malassezia, Microsporum and Trichophyton genera are the main infectious agents of human and animal cutaneous mycoses [1,2].

Leishmaniasis are zoonosis caused by protozoa of the Leishmania genus with a wide range of clinical symptoms: cutaneous, mucocutaneous and visceral [4]. In these parasitic diseases, humans and wild, synanthropic and domestic mammals act as hosts and/or reservoirs of several Leishmania spp [5].

The cutaneous/mucocutaneous leishmaniasis is usually characterized by chronic skin lesions and permanent scars of arms with deformation of the infected area [6]. This disease presented more than 1 million cases reported in the last five years and over 431
million people living in endemic areas at risk of infection [4]. *Leishmania* spp. are pathogens that infect mainly macrophages, but also neutrophils and dendritic cells. The parasites are able to evade of microbicidal mechanism of this cells resulting in the different forms of disease, according to the *Leishmania* specie [7].

Although both diseases present therapeutic options such as the azoderivatives for fungal treatment, especially ketoconazole and itraconazole and pentavalent antimonial drugs for leishmaniasis chemotherapy, these drugs present severe side effects, including high toxicity and low efficacy [2,8–10]. Thus, many researchers have been trying to find safer plant-derived natural products to treat these diseases [7,9,11,12].

The *Caryocar* genus, known popularly as Pequi, has a wide distribution and is represented in several Brazilian biomes such as the Cerrado, the Atlantic Forest, Amazon and the Caatinga [13,14]. It is used in the regional cooking and as promising drug of several diseases due to its bactericidal, fungicidal, leishmanicidal, nematicidal and wound healing effects [15–17].

Thus, the aim of this study is evaluate the role of flavonoid constituents, from peel and pulp extracts of *C. coriaceum* fruits, on the toxicity to dermatophyte fungi and *Leishmania amazonensis* parasites. Therefore, this trying the discovery of new antifungal and antileishmanial agents. In addition, this work investigated the antioxidant and antiacetylcholinesterase activities, which support their antileishmanial and antifungal properties.

**Material and Methods**

**Chemicals**

Quercetin, isoquercitrin, rutin, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 5,59-Dithiobis-(2-nitrobenzoic acid) (DTNB), Acetylthiocholine iodide (ATCI) and other reagents were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

**Preparation of plant part extracts**

Peel and pulp of *C. coriaceum* mature fruits were obtained at the Campus of the Universidade Estadual do Ceará (UECE) lat: -3.792222 long: -38.556111, Fortaleza, Brazil. These *C. coriaceum* plants were submitted and identified by Prisco Bezerra Herbarium under the code EAC57060.

The extracts were obtained by cold maceration [24] with 96% ethanol, at 12h cycle
of light, without agitation for 7 days. Filtration of the supernatant and evaporation of the solvent at reduced pressure in a rotary evaporator led to crude ethanol extracts of Pequi fruit pulp (EEPUCC) and Pequi fruit peel (EEPECC).

**Chemical constituents of plant extracts**

Qualitative tests for the presence of secondary metabolites were based on visual observation of color changes or precipitate formation reactions [9,25]. The reactions were conducted by acidification and alkalinization test for detection of anthocyanins and catechins; Lieberman-Burchard reagent (acetic anhydride plus sulfuric acid) to steroids (green color) or triterpenes (red color) detection. Ferric chloride solution was used to react with phenols and tannins, Shinoda reagent (concentrated HCl and granulated magnesium ribbon) to flavonoids and xanthones (a pink to red color); the pH variation pattern (with sodium hydroxide and sulfuric acid) to flavonols, flavanones and flavanones and distilled water with formation of foam to characterize saponins after shaking.

Total phenol content was quantitatively determined using the Folin–Ciocalteu’s method [26]. The absorbance was measured at 750 nm using a UV/VIS spectrophotometer. The blue color indicated the presence of phenol content. The results are expressed in mg of gallic acid equivalent per gram of extract (mg GAE/g) based on a linear equation for a standard curve prepared with gallic acid.

The flavonoid content was determined using Funari and Ferro’s method [27]. The absorbance was measured at 425 nm. Yellow color indicated the presence of flavonoids. The flavonoid content are expressed in mg of quercetin equivalent per gram of extract (mg EQ/g), on a linear equation for a standard curve prepared with quercetin.

**Characterization of flavonoids by HPLC**

The identification and quantification of flavonoids on EEPUCC and EEPECC were performed by high-performance liquid chromatography (HPLC) with SHIMADZU liquid chromatograph coupled to a SCL-10AVP controller system, UV-VIS detector SPD-10AVP and isocratic pump LC-10ATVP. The software LC Solution software was used to record the chromatograms and measure of peak areas. The column used was a Shimadzu analytical CLC-ODS M (C-18) of 25 cm.

The calibration curve was constructed using the standards rutin, isoquercetin and quercetin, injected at different concentrations (0.25; 0.05; 0.025 and 0.005 mg/mL) into
the liquid chromatograph. The flow rate was 1.8 mL per minute for quercetin and 1.25 mL per minute to rutin both in a wavelength of 350 nm and a mobile phase of 20% acetonitrile and 80% solution of H3PO4 pH buffer 2.8. The linear regression equation was obtained by using the Microsoft Office Excel 2010 program.

The chromatographic profile of flavonoids Rutin, Quercetin and Isoquercitrin, the chosen standards, were obtained preparing ethanolic solutions at a concentration of 0.5 mg/mL, and then injecting into the chromatograph. As mobile phase the same solution was used for calibration curve at the same wavelength and flow rate of 1.80 mL per minute.

**Antioxidant activity**

Antioxidant activity of EEPucc and EEPECC via 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical reduction were assessed using a previously described method [23], with some modifications as follows. Dilution series of the samples, and positive control (rutin), in methanol, were prepared, to obtain the concentrations 100-3.125 μg/mL. Methanol was used as a negative control. The absorbances were measured at 515 nm using a UV-VIS spectrophotometer. The percentage inhibition (PI) were calculated according to the equation: PI% = [(Absorbance of DPPH – Absorbance of the extract)/Absorbance of DPPH] × 100. The IC₅₀ values were determined by linear regression of the plotted data followed by Tukey’s test for multiple comparisons.

**Inhibition of acetylcholinesterase (AChE) enzyme**

The AChE inhibitory activity was qualitatively assessed using Ellman's [28] methodology, adapted for thin layer chromatography by Rhee et al. [29]. Solutions of the EEPucc and EEPECC at the concentration of 2.0 mg/mL were applied to an TLC aluminum chromatoplate Silica gel 60 F254 (MERCK®) forming 2 mm spots. The plate was sprayed with 5,59-Dithiobis-(2-nitrobenzoic acid) (DTNB)/Acetylthiocholine iodide (ATCI) reagent (1 mM DTNB and 1 mM ATCI in 50 mM Tris–HCl, pH 8) until the silica was carefully saturated with the solvent. Plates were allowed to dry and then 5 U/mL of AChE enzyme solution was sprayed. A yellow background was observed, with white zones, indicating the presence of AChE enzyme inhibiting compounds. These zones became visible after 5 min. The zones were observed, measured and recorded. Physostigmine was used as standard.
**Fungicidal assay**

The minimum concentration capable of inhibit 100% fungi growth (MIC) was determined by the dilution technique, according to CLSI method [30]. Six strains were tested (3 *Malassezia* sp. - MA239, MA276 and MA355 - and 3 *Microsporum canis* - MC017/15, MC029/15 and MC115/15), isolated from infected domestic animals, identified and stocked at the Microbiology section and kindly donated by VETTINGS®.

The spore suspension solution for initial inoculation was prepared from filamentous fungi cultivated on potato dextrose agar (PDA) and incubated at temperature at 28± 2°C for 7 days. The spore count was performed in a Neubauer chamber to achieve the concentration of 10⁵ to 10⁶ cells.

In laminar flow cabinet, 100.0 μL of RPMI medium were distributed into each well of a 96-well microplate. 100.0 μL of EEPUCC and EEPECC extract were added and serial dilutions performed from 2500.0 to 2.44 μg/mL. Well intervals were prepared to evaluate the sterility control of the medium; to control of fungal growth; by testing the fungicide (TECTO® RC Thiabendazole; Syngenta®), the fungicide concentration was in accordance with manufacturer instructions (4.0 mL/L). Finally, 50.0 μL of the fungal suspension were added to all wells except the lines intended for the control of the sterile medium.

The readings were taken by checking the MIC, with the aid of a stereoscopic checking the lowest concentration of the samples capable of inhibiting 100% the growth of the microorganism, after 5 days of incubation. The plates were also inspected under an inverted microscope to assure growth of the controls and sterile conditions.

The Minimal fungicide concentration (MFC), as considered as minimum concentration capable of kill 100% fungi, were measured by transfer 50.0 μL from wells without fungal growth and inoculate on PDA. MFC was established according to the fungus growth after incubation under the same conditions for 5 days.

**Leishmania parasite**

*Leishmania (Leishmania) amazonensis* (MHOM/BR/1989/166MJO) was used in promastigote forms, in the stationary growth phase (day 5 of culture). The parasites were obtained from popliteal lymph nodes of *L. amazonensis*-infected BALB/c mice and maintained in 199 culture medium (GIBCO) supplemented with 10%-fetal bovine serum
(FBS) (GIBCO), 10mM-HEPES Biological Buffer (AMRESCO), 0.1%-human urine, 0.1%-L-glutamine (SYNTH), penicillin (10 U/mL) and streptomycin (10 μg/mL)(GIBCO) and 10%- sodium bicarbonate (SYNTH). Cell cultures were incubated at 25°C in 25-cm² flasks. All parasites were from culture that was serially passed for less than 5 weeks.

Viability of *L. amazonensis*-promastigote forms

The direct effect of EEPUCC and EEPECC extracts against *L. amazonensis* was performed in 24-well microtiter plates, each well containing 1000 μL of 199 supplemented culture medium with 1 x 10⁶ promastigote forms in stationary phase with or without the extracts of interest at final concentration of 0.1, 0.05 and 0.025 mg/mL. Viable promastigote concentration was determined by Neubauer chamber counting after 24, 48 and 72h of treatment. In the stock solutions of extracts, 0.01% dimethyl sulfoxide (DMSO) (GIBCO) was used as vehicle. Untreated parasites and vehicle only (0.01% DMSO) were included as negative controls. The plates were also inspected under an inverted microscope to assure growth of the controls and sterile conditions. The 50% inhibitory concentration (IC₅₀) were determined by non-linear regression analysis of the obtained data.

Animals

BALB/c mice weighing approximately 25-30 g and aged 6-8 weeks were used according to protocols approved by the ethics committee of Universidade Estadual de Londrina approved the protocol for animal use (13134.2016.62).

Peritoneal macrophages viability assay

The viability of peritoneal macrophages treated with EEPUCC and EEPECC extracts were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described by Mosmann (1983). BALB/c peritoneal macrophages (5 x 10⁵ U/mL) were cultured in 24-well plates with 500 μL of 199 medium for 2 h for adherence at 37°C and 5% CO₂. The cells were washed with PBS and then, adherent cells were incubated with different concentrations of extracts (2.5 - 0.025 mg/mL) or with vehicle (0.0,1% DMSO) and maintained in culture for 24h at 37°C and 5% CO₂. After incubation with extracts, the macrophages were washed with PBS and added MTT at final concentration of 5 μg/mL in each well, followed by incubation for 4h
at 37°C/ 5% CO₂. The MTT formazan product was solubilized with 300 μL of DMSO, plates were read at 570 nm in a spectrophotometer. The 50% cytotoxicity concentration (CC₅₀) was determined by non-linear regression analysis of the obtained data.

**Selectivity index (SI)**

The degree of selectivity of EEPUC and EEPCC extracts were expressed as SI = IC₅₀ of extracts on macrophages/IC₅₀ of the same extract on promastigotes.

**Hemolytic assay**

Blood from healthy subjects was collected with heparinized vacuum tube, and the erythrocytes were washed 3 times with PBS (centrifugation at 1000 rpm for 10 minutes). A 2% red cell suspension was prepared with PBS. Treatments (0.5, 0.25, 0.1 and 0.025 μg/mL) were incubated 1:1 in a total volume of 200 μL, with a suspension of 2% red cells in 96-well plate for 3 hours at 37°C 5% CO₂. PBS was used as negative control, and distilled water to control hemolysis. The plates were centrifuged at 1000 rpm for 10 minutes, and supernatants were collected and analyzed for absorbance reading at 550 nm. This experiment was performed in duplicate and repeated three times.

**Statistical Analysis**

All of the experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (or SEM). Results were contrasted with negative and a positive control. The differences between the values were examined using analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons and a p-value<0.05 were considered to be statistically significant. Data were analyzed in Graphpad Prism 6.01 software for Windows (GraphPad Software, San Diego California, USA)

**Results**

**Chemical constituents of plant extracts**

In phytochemical qualitative screening the extracts of C. coriaceum showed similar constituents for both EEPUC and EEPCC extracts presenting the secondary metabolites as alkaloids, steroids, phenols, flavonoids, saponins and tannins (table 1). Catechins were present only in EEPUC extract as shown on Table 1, corroborating with Batista et al. [31].
Table 1. Phytochemical screening of *Caryocar coriaceum* extracts.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>EEPUCC</th>
<th>EEPECC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catechins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) Presence of the metabolite; (-): Absence of the metabolite. EEPUCC: Pulp extract; EEPECC: Peel extract of *C. coriaceum* fruits

Since many activities of phenols were already determined in literature and as all the extracts exhibit positive results to phenols and flavonoids, was performed the quantification of the target metabolites. The EEPECC extract presented higher amount of phenols, with $55.617 \pm 7.92$ mg of gallic acid equivalents (GAE)/g of extract (mg GAE/g) and flavonoids with $3.881 \pm 0.10$ mg quercetin equivalent (QE)/g of extracts (mg QE/g) when compared with EEPUCC extract which showed $24.539 \pm 3.55$ GAE/g plus $1.334 \pm 0.21$ QE/g, as we can observe at Table 2.

Table 2: Quantifications of total phenols and standard flavonoids by high performance liquid chromatography (HPLC) of *Caryocar coriaceum* extracts.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total Phenols (mg GAE/g)</th>
<th>Total Flavonoids (mg QE/g)</th>
<th>HPLC quantification of flavonoids (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HPLC quantification of flavonoids (µg/mL)</td>
</tr>
<tr>
<td>EEPUCC</td>
<td>$24.539 \pm 3.55$</td>
<td>$1.334 \pm 0.21^b$</td>
<td>$1.965^a$, $5.025^a$, $47.665^b$</td>
</tr>
<tr>
<td>EEPECC</td>
<td>$55.617 \pm 7.92$</td>
<td>$3.881 \pm 0.10^a$</td>
<td>$0^b$, $4.169^b$, $129.198^a$</td>
</tr>
</tbody>
</table>

Similar small letters indicate significant similarities between rows ($p < 0.0001$, according to ANOVA followed by Tukey test). EEPUCC: Pulp extract; EEPECC: Peel extract of *C. coriaceum* fruits
Characterization of flavonoids by HPLC

Further, was conducted the HPLC identification (Fig. 1-2) for identify the composition of the main flavonoids from these extracts. The flavonoids found in the extracts were rutin, with was greater in EEPucc and an expressive quantity of isoquercetin, mainly in EEPECC extract. Quercetin was present only in EEPucc extract. Regarding to Isoquercetin bioavailability it was shown that its results are better than its analogue quercetin [32], also observed at Table 2.

Fig. 1. Chromatogram obtained by injection Pulp extract of C. coriaceum fruits

![Chromatogram obtained by injection Pulp extract of C. coriaceum fruits](image)

(1): Rutin; (2): Isoquercetin; (3): Quercetin.

Fig. 2. Chromatogram obtained by injection Peel extract of C. coriaceum fruits

Fixar os números no cromatograma

![Chromatogram obtained by injection Peel extract of C. coriaceum fruits](image)

(1): Rutin; (2): Isoquercetin.

Antioxidant and AntiAChE activity

We assess the capability of the EEPucc and EEPECC extracts to inhibit free radicals. Radical inhibition occurred at greater power with increasing the concentration
of the extracts when compared to standard (Rutin). Table 3 displays the results obtained for the extracts and the respective standard substances. The IC₅₀ values for EEPUCC and EEPECC extract were 49.4 µg/mL and 25.5 µg/mL, respectively. Compared the extracts, the EEPUCC presented significantly higher antioxidant potential, due lower values are needed from this sample to rescue free radicals.

Table 3. Biological activities of C. coriaceum extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Antioxidant activity (IC₅₀ µg/mL)</th>
<th>AChE inhibition (mm)</th>
<th>Malassezia spp. Values (µg/mL)</th>
<th>M. canis Values (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEPUCC</td>
<td>49.4 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.06 ± 1.79&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.77 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EEPECC</td>
<td>25.5 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.06 ± 0.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.88 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rutin</td>
<td>13.7 ± 0.25&lt;sup&gt;A&lt;/sup&gt;</td>
<td>-</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>-</td>
<td>9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

Antioxidant and AChE assays, similar letters indicate significant similarities between rows (p < 0.05, according to ANOVA followed by Tukey test). MFC/MIC assays, similar letters indicate significant similarities between referred data (p < 0.0001, according to ANOVA followed by Tukey test). Rutin: positive control to antioxidant activity. Physostigmine: positive control to AChE inhibition. EEPUCC: Pulp extract; EEPECC: Peel extract of C. coriaceum fruits; -: Not Realized; NE; No Effect.

We have also tested the extracts effect as anti-cholinesterase which inhibits the acetylcholinesterase enzyme from breaking down acetylcholine, by thin layer chromatography (TLC), comparing with the control (physostigmin). All of the extracts yielded positive results when compared with physostigmin. EEPUCC extract showed inhibition comparable to that presented by physostigmine, standard substance. EEPECC presented significantly lower AChE potential.

**Fungicidal assay**

By the Holetz’s antimicrobial activity index [33], all the extracts can be classified
as good antifungal. Against *M. canis*, the referred extracts obtained better results. EEPECC extract exhibit great results with 4.88 μg/mL MIC and MFC. EEPUCC extract was also considered great obtaining 4.88 μg/mL MIC and 9.77 μg/mL MFC. Both extracts exhibit 39.06 μg/mL MFC against *Malassezia* spp., despite MIC, EEPECC extract demonstrates better results than EEPUCC extract, with 9.77 μg/mL and 19.53 μg/mL, respectively (Table 3).

**L. amazonensis**-promastigote, macrophage viability and SI

The effect of extracts on *L. amazonensis* promastigotes were monitored for 3 days, as demonstrated at Table 4. The concentration of EEPUCC extract which induced 50 percent inhibition (IC₅₀) in parasite proliferation was 30 ± 5.0 μg/mL and EEPECC extract by 38 ± 3.0 μg/mL at 24h. At the time of 48h, the IC₅₀ of EEPUCC extract was 39 ± 8.0 μg/mL and of the EEPECC extract 31 ± 9.0 μg/mL. We also shows dose-dependent reaction due after 72h IC₅₀ results reduces to 17 ± 7.0 μg/mL and 22 ± 3.0 μg/mL, respectively.

**Table 4. Inhibitory concentration of Caryocar coriaceum extracts on promastigotes forms of L. amazonensis (IC₅₀ μg/mL) after 24, 48 and 72h of treatment, toxicity to peritoneal macrophages (CC₅₀ μg/mL) and selectivity index (SI) after 24h of treatment. Values are expressed in μg/mL.**

<table>
<thead>
<tr>
<th></th>
<th>24h</th>
<th></th>
<th>48h</th>
<th></th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀</td>
<td>CC₅₀</td>
<td>SI</td>
<td>IC₅₀</td>
<td>IC₅₀</td>
</tr>
<tr>
<td>EEPUCC</td>
<td>30 (±5.0) A</td>
<td>253b (±42.0)</td>
<td>8.43b</td>
<td>39 (±8.0)</td>
<td>17 (±7.0)</td>
</tr>
<tr>
<td>EEPECC</td>
<td>38 (±13.0) A</td>
<td>454a (±11.0)</td>
<td>11.94c</td>
<td>31 (±9.0)</td>
<td>22 (±3.0)</td>
</tr>
<tr>
<td>Pentamidin [11]</td>
<td>23.71A (18.44–30.50)</td>
<td>17.9c (0.002–0.026)</td>
<td>0.75a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucantine [25]</td>
<td>13.95A (±2.06)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Data represent the mean ± SEM of at least three independent experiments performed in triplicate. CC₅₀: cytotoxic concentration of 50% of macrophages (μg/mL). IC₅₀: inhibitory concentration of 50% of promastigotes forms (μg/mL). SI: selectivity index = CC₅₀ / IC₅₀. Similar letters indicate significant similarities between rows (p ≤
0.05, according to ANOVA followed by Tukey test). EEPUCC: Pulp extract; EEPECC: Peel extract of *C. coriaceum* fruits.

To test the selectivity of EEPUCC and EEPECC extracts, murine macrophages were treated with different concentrations of the extracts and the viability of these cells were assessed by the MTT reduction. EEPUCC extract induced 50 percent citotoxic effect (CC50) in peritoneal macrophages at 253.0 ± 42.0 μg/mL and EEPECC extract by 454.0 ± 11.0 μg/mL at 24h. In addition, EEPUCC extract presented a selectivity index of 8.43 and EEPECC extract 11.94, indicating a good predilection of the extract by the parasites, being greater than the pentamidine that presented SI of 0.75.

**Hemolytic assay**

Another way to evaluate the cytotoxicity of extracts is by the ability to cause hemolysis in human erythrocytes. As shown in Table 5, the EEPUCC and EEPECC extracts showed low toxicity. The lowest concentration determines hemolytic activities at 0.909 ± 0.746 and 0.616 ± 0.224 per cent, respectively, although with very low levels of hemolysis. The Caryocar extracts presented low hemolytic activity, and concentrations of 0.1, 0.05 and 0.025 μg/mL were not able to cause significantly hemolysis.

**Table 5. Hemolytic activity (%) of *Caryocar coriaceum* extracts (mg/mL).**

<table>
<thead>
<tr>
<th></th>
<th>0.025</th>
<th>0.05</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEPUCC</td>
<td>0.909</td>
<td>1.300</td>
<td>1.295</td>
<td>2.406</td>
<td>8.777</td>
</tr>
<tr>
<td>(±0.746)</td>
<td>(±0.341)</td>
<td>(±0.564)</td>
<td>(±1.206)</td>
<td>(±4.102)</td>
<td></td>
</tr>
<tr>
<td>EEPECC</td>
<td>0.616</td>
<td>1.229</td>
<td>0.925</td>
<td>3.068</td>
<td>6.872</td>
</tr>
<tr>
<td>(±0.224)</td>
<td>(±0.740)</td>
<td>(±0.315)</td>
<td>(±1.368)</td>
<td>(±3.056)</td>
<td></td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM of at least three independent experiments performed in triplicate. Data were normalized to the positive control (distilled water). EEPUCC: Pulp extract; EEPECC: Peel extract of *C. coriaceum* fruits

**Discussion**

In the recent decades, development of synthetic drugs caused disaffection toward natural products as an attractive resource for search new chemotherapeutic compounds.
However, the emergence of some limitations in the use of synthetic drugs as high toxicity, side effects, and elevated costs caused a shift in situation and interest in the field of ethnobotanical research [34–36].

Plant-derived natural products are valuable sources in traditional medicine due to having less side-effects, low cost, and high availability [37–39]. Indeed, numerous plant-derived bioactive compounds that display a wide variety of pharmacological effects have been described as quercetin and glucosides rutin and isoquercitrin, common flavonoids present in plants which display several activities, including antifungal and antileishmanial [40–43].

The *C. coriaceum* extracts stand out due to an antioxidant-rich pulp, which contains carotenoids and phenolic compounds, and a good profile of unsaturated fatty acids [44]. In phytochemical tests both extracts showed positive results to phenols and flavonoids, leading to the search of active flavonoids like quercetin, rutin, and isoquercitrin, which are biological targets to identify and quantify in the extracts by HPLC analysis. The presence of the target molecules in both extracts explains the biological activities found in this study which were based on *in vitro* [19] and *in silico* [21] predictions.

Antioxidants can have a pathogen neutralizing action, directly by scavenging ROS or indirectly by activating pathways that promote ROS degradation [37]. Regarding to the capacity of scavenging free radicals of the *C. coriaceum* extracts, the samples proved to be better than obtained in other plant extracts by Moura et al. [45] and Morais et al. [12]. Other studies [46,47] have been demonstrated that compounds as flavonoids, including quercetin, rutin, and isoquercitrin, are important due to their several biological activities.

Studies have revealed the potential of the various antioxidant constituents as fungicidal agents [31,48]. Other plants extracts also presented similar results that connect antioxidant mechanisms of action with *in vitro* fungicide activities of natural compounds [9,10,47].

In this work, the ethanol extracts proved to be effective against six animal pathogenic strains: three of the genus *Malassezia* sp. and three strains of *M. canis*. The MIC and MFC varied between 39.1 and 4.1 in tested microorganisms strains. However, all the extracts have better results, when compared to other extracts or isolated compounds [12,18,49–51].
Regarding leishmaniasis, the interaction between parasites and host immune cells leads to an inflammatory response essential for parasite control. However, an exacerbated proinflammatory response may cause tissue damage, resulting in lesion formation observed in cutaneous leishmaniasis [52,53]. On the other hand, the lack of an effective inflammatory response may promote increased parasite burden [36]. In this scenario, the antioxidant effect of *C. coriaceum* extracts can control the inflammatory response being ideal for an effective control of the disease.

In this study, the *Caryocar* extracts had higher toxicity to *L. amazonensis* promastigotes form and demonstrate lower cytotoxicity on murine macrophage and erythrocytes whereas statistically better results than demonstrated in previous studies for pentamidin and glucantime as standards [11,19,25]. This results lead to the referred degrees of selectivity of 8.43 and 11.94 per cent, respectively, with as statistically better results than demonstrated in previous studies for the standard [11].

These good activities were probably due the presence of the main flavonoids Quercetin, Rutin and Isoquercitrin that are large known in the literature with great biological activities. Therefore Peel extract that present higher yields of Quercetin and glucosides showed better effects, as predicted by previous studies [19,21,40,54,55].

The investigated flavonoids also lead to possible mechanism of action of leishmanicidal agents as inhibiting the action of Acetylcholinesterase. Acetylcholinesterase inactivates acetylcholine by hydrolyzing it, into the acetyl and choline groups. Phospholipids compose biological membranes account for approximately 70% of total cellular lipid in *Leishmania* spp [56]. Phosphatidylcholine (PC) is the major phospholipid species, generally comprising ~50% in mammals [57], ~30-40 in fungi [58] and ~20% in *Leishmania* spp. [19] of the total phospholipid mass.

PC is develop by Kennedy pathway in *Leishmania* spp., equally in many higher eukaryotes. PC synthesis requires recovery of the choline precursor. In general inhibitors of AChE also can present toxicological effects, due to phospholipids metabolism inhibition and affecting stabilization the parasite’s membrane composition, and are important markers for fungicidal [59] and leishmanicidal [25] activities. These extracts may act at phospholipid pathway resulting in a negative effects chain in parasites cell membranes.
Conclusions

The secondary metabolites produced by *C. coriaceum* have potentially bioactive substances acting as antifungal and antileishmanial, mainly by scavenging of free radicals and anticholinesterase mechanisms. Quercetin, rutin and Isoquercitrin, are an important indicative of these activities. The results obtained in this study corroborate the potential of these plant species and may form the basis for new antifungal and antileishmanial agents, mainly due the presence of quercetin and glucosides. Nevertheless further studies are necessary for the isolation and characterization of other substances and perform *in vivo* studies to detect bioavailability of these extracts, aiming the development of possible new pharmaceutical compounds.

Conflict of interest

The authors declare no competing financial interest.

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Caryocar coriaceum extracts exert leishmanicidal effect acting in promastigote forms by apoptosis-like mechanism and intracellular amastigotes by Nrf2/HO-1/ferritin dependent response and iron depletion. Leishmanicidal effect of Caryocar coriaceum leaf extracts

(Os extratos de Caryocar coriaceum exercem efeito leishmanicida atuando em formas promastigotas por mecanismo semelhante à apoptose e amastigotas intracelulares pela resposta dependente de ferro, Nrf2 / HO-1 / ferritina e depleção de ferro)


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Caryocar coriaceum extracts exert leishmanicidal effect acting in promastigote forms by apoptosis-like mechanism and intracellular amastigotes by Nrf2/HO-1/ferritin dependent response and iron depletion Leishmanicidal effect of Caryocar coriaceum leaf extracts

Fernanda Tomiutto-Pellissier\textsuperscript{a,*}, Daniela Ribeiro Alves\textsuperscript{b}, Milena Menegazzo Miranda-Sapla\textsuperscript{a}, Selene Maia de Morais\textsuperscript{b}, João Paulo Assolini\textsuperscript{a}, Bruna Taciane da Silva Bortoleti\textsuperscript{a}, Manoela Daiele Gonçalves\textsuperscript{a}, Allan Henrique Depieri Cataneo\textsuperscript{a}, Danielle Kian\textsuperscript{c}, Tiago Bervelieri Madeira\textsuperscript{d}, Lucy Megumi Yamauchi\textsuperscript{c}, Suzana Lucy Nixdorf\textsuperscript{d}, Idessania Nazareth Costa\textsuperscript{a}, Ivete Conchon-Costa\textsuperscript{a}, Wander Rogério Pavanelli\textsuperscript{a,*}

\textsuperscript{a} Department of Pathological Sciences, Center of Biological Sciences, State University of Londrina, Londrina, Paraná, Brazil
\textsuperscript{b} Department of Veterinarian Sciences, Ceará State University, Fortaleza, Ceará, Brazil
\textsuperscript{c} Department of Microbiology, Center of Biological Sciences, State University of Londrina, Londrina, Paraná, Brazil
\textsuperscript{d} Department of Chemistry, Center of Exact Sciences, State University of Londrina, Londrina, Paraná, Brazil

**ABSTRACT**

Ethnopharmacological relevance: Plants belonging to the Caryocar genus are found in Brazilian cerrado, where the fruits are used as food and in folk medicine, as treatment for respiratory, ophthalmological and hepatic diseases.

Aim of the study: To evaluate the in vitro effect of two different solvents extracts obtained from leaves of Caryocar coriaceum plant on L. amazonensis promastigote and amastigote forms in macrophages of BALB/c mice infected with this parasite.

Materials and methods: It were used ethyl acetate (EAC) and methanol (MET) leaf extracts, that were tested at 25; 50 and 100 \( \mu \text{g/mL} \). The antipromastigote assay was performed through Neubauer chamber counting and scanning electron microscopy, and the death mechanism was performed by techniques using a TMRE and H2DCFDA probe, propidium iodide and annexin V labeling. Cytotoxicity was assessed by MTT assay. The antiamastigote assays were performed by infection of BALB/c mice macrophages with L. amazonensis promastigotes (1:5) and afterwards, verified different parameters: infection index, cytokine production, lipoperoxidation, reactive oxygen species (ROS) and nitric
oxide (NO) measurement, total iron and total iron bound capacity levels, Nrf2, heme oxygenase (HO-1) and ferritin expression.

Results: C. coriacum extracts showed antipromastigote effect after 24, 48 and 72 hours of treatment. The extracts also induced loss of mitochondrial membrane potential, as well reactive oxygen species production on promastigotes, damage on plasma membrane and phosphatidylserine exposure on extracellular parasites, and it was verified that the most parasites were under late apoptosis-like process. The range of concentrations used did not alter the viability of peritoneal macrophages of BALB/c mice, then it was observed that the treatment with extracts was able to reduce the infection on host cells. Next, we verified through CBA that the extracts were able to significantly improve TNFa, IL-6, MCP-1 and IL-10 levels, but reduce the MDA and ROS levels, without interfere in NO levels. In addition, EAC and MET upregulated NRF-2/HO-1/Ferritin expression and modulated the labile iron pool in infected macrophages. Conclusion: Based on the data obtained, It was possible infer that different solvent extracts of the C. coriacum leaves exerts leishmanicidal effect, acting in promastigote forms through apoptosis-like mechanisms and in intracellular amastigote forms involving an antioxidant response, which culminates in a depletion of available iron for L. amazonensis replication.

INTRODUCTION

*Leishmania (Leishmania) amazonensis* is one of the causative agents of American cutaneous leishmaniasis (ACL). The pathogenesis of this zoonosis depending both the virulence factors of the parasite strain and the host immune response. The clinical manifestations may ranging from a single granulomatous skin lesion to diffuse lesions, where it may or may not affect the mucous membranes or even progress to visceral disease (Barral et al., 1991).

One of the challenges in controlling the disease is due to the fact that *Leishmania* parasites have a sophisticated survival mechanism that involves down regulation of microbicidal mechanisms of macrophages (Cecilio et al., 2014; Liu and Uzonna, 2012a; Olivier and Gregory, 2005). Besides that, studies have demonstrated that *Leishmania* parasites can sequester hostis nutrients, as scavenge labile iron pool and uses it to survival and replication inside macrophages (Das et al., 2009; Zaidi et al., 2017).

The currently available chemotherapy for the treatment of ACL is based on
pentavalent antimonials such as sodium stibogluconate (Pentostam®) and antimoniate N-methyl-glucamine (Glucantime®) and in case of lack of response, second-line drugs such as amphotericin B or pentamidines are used. However the available drugs present toxicity, low efficiency and difficulty of administration, prompting a search for alternative treatments (Alvar et al., 2006). In this sense, natural compounds could complement current therapy or even help control the disease in cases of disease recurrence or resistance.

Brazilian cerrado is a biome that occupies vast areas of the country, with large diversity of plants, which have been generated interest for their varied biological properties with potential use in medicine (Bailão et al., 2015; Correia et al., 2016; Justino et al., 2016; KELLNER and KELLNER, 2016).

Plants belonging to the Caryocar genus are found in this vegetation, and their fruits are used as food and in folk medicine. Studies have demonstrated that as anti-inflammatory by reducing interleukin (IL)-6, leukotriene-4 and 5 and tumor necrosis factor (TNF) receptor (Torres et al., 2016), antifungal on A. solani and V. pirina (Breda et al., 2016), antibacterial on E. faecalis, E. coli, P. aeruginosa and S. aureus, antinociceptive (de Oliveira et al., 2015), antineoplastic (Colombo et al., 2015; Suffredini et al., 2007), immunomodulatory by inducing TNF-α synthesis (Gusman et al., 2015), wound healing on skin lesions (Oliveira et al., 2010) and nematicide (Nogueira et al., 2012). In addition, Paula-Ju et al., 2006b described the antioxidant and antipromastigote effect of hydroethanolic extracts of leaves of another specie of Caryocar genus, C. brasiliense. However, there are no studies that verify the potential of Caryocar coriaceum (pequi) extracts on Leishmania spp.

In this way, the aim of this work is investigate the effect of ethyl acetate and methanol C. coriaceum leaves extracts on promastigote forms as well on intracellular amastigote forms of L. amazonensis. We further investigated a putative mechanism of action of this compound as a modulator of molecules such as cytokines, nitric oxide, and labile iron pool in experimental models.

MATERIALS AND METHODS

Leishmania (Leishmania) amazonensis maintenance

Promastigotes forms of L. (L.) amazonensis (MHOM/BR/1989/166MJO) were maintained in culture medium 199 (GIBCO, Invitrogen, New York, USA) pH 7.18-7.22
supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen, New York, USA), 10mM HEPES buffer, 0.1% human urine, 0.1% L-glutamine, 10U/mL penicillin and 10μg/mL streptomycin (Invitrogen-GIBCO) and 10% sodium bicarbonate. The cell culture was maintained in a B.O.D at 25°C in 25 cm² culture flask. In all experiments, promastigote forms in the stationary growth phase were used (5-day culture).

*Caryocar coriaceum* extracts

The leaf extracts of *C. coriaceum* were kindly supplied by Dr. Selene Maia de Morais of the State University of Ceará, and obtained at the Campus of the State University of Ceará (UECE) lat.: -3.792222 long.: -38.556111, in the Fortaleza city (Brazil). Leafs of *C. coriaceum* were presented and identified in the Herbarium Prisco Bezerra (UFC) under the code EAC57060. The leaves were washed in tap water, dried in an oven and cut into pieces with a maximum of 1 cm².

Subsequently, the obtained material was subjected to extraction by soxhlet firstly with ethyl acetate then with methanol for 4 days each solvent until the exhaustion of metabolites extraction of the total mass. Both treatments were performed in 12h light cycle and without shaking. After exhaustion, all crude extracts were filtered and concentrated under vacuum until complete elimination of the organic solvent. Then the crude extracts were kept at room temperature for further use and fractionation.

The extracts were named according to solvent, respectively: (EAC) ethyl acetate and (MET) methanol. For the biological assays, the extracts were diluted in DMSO 0.01%.

**Antipromastigote assay**

*L. amazonensis* promastigote forms (1×10⁶ cells/mL) were treated with *C. coriaceum* extracts 25, 50 and 100 μg/mL. Parasites were counted in a Neubauer chamber after 24, 48 and 72 h of treatment. As a control, was used *L. amazonensis* promastigote maintained in culture medium, DMSO 0.01% or amphotericin B (AMB) 1 μM.

**Scanning electron microscopy of promastigotes**

Scanning electron microscopy (SEM) of promastigotes forms was performed according to (Silva et al., 2013), where the parasites (1×10⁶) were treated with 50 μg/mL of EAC and MET extracts and incubated for 24 hours. The promastigotes were, then, collected, washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 M of Sodium
cacodylate buffer containing 1 mM CaCl$_2$. After fixation, the samples were placed in poly-L-lysine treated coverslips. The samples were then dehydrated with graded ethanol baths, CO$_2$ dry point, gold coated, and observed by SEM (FEI QUANTA 200 scanning electron microscope).

**Determination of parasites cell volume**

Promastigotes (10$^6$ cells/mL) were treated or untreated with were treated with 50 µg/mL of EAC or MET and incubated for 24 hours at 24°C, harvested, and washed with PBS. Subsequently, the parasites were analyzed using a BD Accuri™ C6 Plus personal flow cytometer. Histograms were generated, and FSC-H represented the cell volume. A total of 10,000 events were acquired in the region that corresponded to the parasites.

**Determination of mitochondrial-membrane potential**

To evaluate the inner mitochondrial membrane potential, was conducted a tetramethylrhodamine ethyl ester (TMRE) staining (Sigma, St. Louis, MO, USA). For this purpose, promastigote forms (1×10$^6$ cells/mL) previously treated for 24h with 50µg/mL of extracts were washed and incubated in 25 nM of TMRE for 30 min at 25°C, washed once again in PBS and immediately analyzed in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland). It was used excitation wavelength of 480 nm and emission wavelength of 580 nm.

**Determination of ROS generation on *L. amazonensis***

To evaluate the ROS generation in promastigote forms of *L. amazonensis*, 1x10$^6$ parasites were incubated with 50µg/mL of each treatment for 24 h. Then, they were washed in PBS (pH 7.4) and loaded with 10µM of a permeant probe diacetate 2',7'-dichlorofluorescein (H2DCFDA) (Sigma, St. Louis, MO, USA) diluted in DMSO, incubated in the dark for 45 min, 24°C. Reactive oxygen species (ROS) were measured as an increase in fluorescence caused by the conversion of non-fluorescent dye to highly fluorescent 20,70-dichlorofluorescein, with an excitation wavelength of 488 nm and emission wavelength of 530 nm in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

**Determination of phosphatidylserine exposure in parasite membrane**
Phosphatidylserine (PS) exposure was detected using Annexin-V FITC (Invitrogen, Eugene, USA), a calcium-dependent phospholipid binding protein used as an apoptosis marker. Promastigotes (1×10^6 cells/mL) were treated with 50 μg/mL of the extracts for 24 h at 25°C. Afterward, the parasites were washed and resuspended in 100 μL of binding buffer (140 mM NaCl, 5 mM CaCl₂, and 10 mM HEPES-Na, pH 7.4), followed by the addition of 5 μL of the calcium-dependent phospholipid binding protein Annexin-V FITC for 15 min at room temperature. After the incubation binding buffer (400 μL) was then added. Data acquisition was performed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) at an excitation wavelength of 488 nm and emission wavelength of 520 nm. In order to compare the different treatments, the fluorescent values obtained were normalized to the respective number of cells.

**Determination of promastigotes membrane integrity**

Parasites (1×10^6 cells/mL) treated with 50 μg/mL of the different extracts for 24 h at 25°C were harvested, washed with PBS and directly incubated with propidium iodide (PI) (Sigma, St. Louis, MO, USA) (0.50 μg/mL) for 5 min according to the manufacturer’s instructions. Immediately thereafter, the promastigotes were analyzed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) with an excitation wavelength of 480 nm and emission wavelength of 580 nm. The fluorescent values obtained were normalized to the total number of cells of each treatment.

**Co-determination of annexin V and propidium iodide label**

Promastigotes (10^6 cells/mL) under the same conditions mentioned above were washed and resuspended in 100 μL of assay buffer 1x (Santa cruz Biotechnology), followed by the addition of a mix containing 1 μL of annexin-V FITC and 5 μL of PI (Santa cruz Biotechnology). Data acquisition and analysis were performed using a BD Accuri™ C6 Plus personal flow cytometer. A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the parasites. Cells that were stained with annexin-V (PI-positive or -negative) were considered apoptotic, and cells that were only PI-positive were considered necrotic (Dorooodgar et al., 2016; Ghaffariifar et al., 2015).

**Animals and Ethics Committee**
BALB/c mice were kindly provided from the Institute Carlos Chagas/Fiocruz-PR, Curitiba, Brazil. The animals weighing approximately 25-30 g and aged 6-8 weeks were kept under sterile conditions and used according to protocols approved by the Institutional Animal Care and Committee. This study was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina (13134.2016.62).

**Viability of peritoneal macrophages**

The cytotoxic effects of *C. coriaceum* extracts in peritoneal macrophages were tested based on mitochondrial oxidation by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO, USA) assay as described by (Mosmann, 1983). Macrophages (5x10^5 cells/mL) was recovered from peritoneal cavity of Balb/c mice with cold PBS supplemented with 3% of FBS and then cultured in 24-well plates with 200 µL of RPMI 1640 medium (10% FBS) for 2 h (37°C, 5% CO2). Adherent cells were incubated with 25, 50 and 100 µg/mL of *C. coriaceum* extracts and cultured for 24 h under the same conditions. After this period, the culture was washed with PBS, and added MTT (5 mg/mL), followed by further incubation for 3 h. As control was used the culture under the same conditions without treatment, as vehicle was used DMSO 0.01% and as positive control H2O2 0.4%. The MTT product (formazan crystals) was diluted with 300 µL of dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA), transferred to 96 wells plates and reading with a spectrophotometer (Thermo Scientific, Multiskan GO) at 550 nm. The results were expressed as percentage of viability compared to the control group calculated with the following formula:

\[
\% \text{ Viable macrophages} = \frac{OD \text{ samples of extracts treated}}{OD \text{ sample untreated}} \times 100
\]

**Antiamastigote assay**

Peritoneal macrophages (5x10^5 cells/mL) were cultured in 24-well plates containing 13 mm glass coverslips and incubated with 200 µL of RPMI 1640 medium for 2h at 37°C and 5% CO2. The adherent macrophages were infected with *L. amazonensis* promastigotes of (1x10^6 cells/mL) for 2h. After infection, the non-internalized promastigotes were removed by washing with PBS and the cells treated with *C. coriaceum* extracts (25, 50 or 100 µg/mL), RPMI 1640 medium (control) or DMSO 0.01% (vehicle) and AMB 1 µM (positive control) for 24h (37°C, 5% CO2). Then, cells were stained with Giemsa (Laborclin, Pines-PR Brazil) and 20 fields analyzed by optical
microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan) (1000x magnification) to determine the % of infected macrophages and number of amastigote per macrophage. The supernatant was stored for measurement of cytokines and nitric oxide.

**Promastigote recovery test**

Promastigotes recovery assay was performed as previously described by (Silva et al., 2013). In brief, peritoneal macrophages were infected with *L. amazonensis* and treated with concentrations of *C. coteaeum* extracts in the same conditions described in antimastigote assay. It is known that viable amastigotes have the ability to differentiate into promastigotes when exposed to ideal conditions, so after 24 h of treatment the cell culture was washed with PBS and incubated with 199 culture media at 24°C in order to induce differentiation of intracellular viable amastigotes in promastigote free forms. Free promastigotes recovered (FPR) were counted in a Neubauer chamber for three consecutive days and the number of recovery promastigotes normalized as the follow equation:

\[
\% \text{ FPR} = \frac{\text{number of FPR of extracts treated}}{\text{number of FPR of untreated}} \times 100
\]

**Cytokines measurement**

The Cytometric Bead Array Assay (CBA, BD Biosciences®) was used to measure the levels TNF-α, MCP-1, IL-6 and IL-10 in supernatants from non-infected and *L. amazonensis*-infected macrophages following the manufacturer’s instructions. Data acquisition and analysis were performed using a BD Accuri™ C6 Plus personal flow cytometer.

**Determination of nitrite as estimative of NO levels**

Nitric oxide (NO) was determined by the Griess method. Briefly, supernatant aliquots (60 μL) of antimastigote assay supernatants were centrifuged at 5000 rpm for 2 min and a volume of 50 μL the supernatant was recovered and added 50 μL of Griess reagent (1% sulfanilamide and 0.1% of naf†iletlenodiamino-bictloridrato in orthophosphoric acid (H3PO4) 5%). After 10 min incubation at room temperature, the samples were placed in 96-well microplates. A calibration curve was made using dilutions of NaN02, and the absorbance was determined at 550 nm on microplate reader (Thermo
Measurement of Malondialdehyde Levels (MDA)

MDA is a lipid peroxidation metabolite derived from oxidative stress occurrence. Accordingly, the MDA levels were determined through High Performance Chromatography (HPLC) according to description by Victorino et al., 2013 with a few modifications. The analyses were conducted using an Alliance c2695 HPLC (Waters, Milford, USA) equipped with a SecurityGuard ODS-C18 (4 × 3.0 mm, Phenomenex), C18 reverse phase column (Eclipse XDBC18; 4.6 × 250 mm, 5 μm, Agilent) as well as a photodiode array detector (Photodiode Array Detector (PDA), 2998) using Empower 2 software (Waters, Milford, USA). The preparation of MDA standards used 1,1,3,3-tetraethoxyx propane (TEP). Aliquots containing 500 μL of cells + supernatants were deproteinized by adding 20% trichloroacetic acid, subsequently reacted with 1 mL of thiobarbituric acid. The mobile phase was 70% 10 mM KH2PO4 buffer, pH 7.0, and 40% HPLC-grade methanol. Readings were obtained at 532 nm, following an eight-minute isocratic flow at the rate of 1 mL/min. The results were expressed in nM MDA.

Peritoneal macrophage Reactive Oxygen Species (ROS) generation

To evaluate the ROS generation, L. amazonensis-infected macrophages (5 × 10⁴ cells/mL - infected and treated under the same conditions described in the antiamastigote assay) was washed in PBS (pH 7.4) and loaded with 2 μM of a permeant probe diacetate 2',7'-dichlorofluorescein (H₂DCFDA) (Sigma, St. Louis, MO, USA) diluted in DMSO, incubated in the dark for 30 min, 37°C, 5% CO₂. Reactive oxygen species (ROS) were measured as an increase in fluorescence caused by the conversion of non-fluorescent dye to highly fluorescent 20,70-dichlorofluorescein, with an excitation wavelength of 488 nm and emission wavelength of 530 nm in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

Determination of iron concentration and total bound iron

The determination of iron concentration in supernatants of anti-amastigote assay was performed utilizing the Dimension® automated system. The method is an adaptation of the direct assay for iron, developed by Smith et al., 1984 and is required the Flex® reagent cartridge for implementing the test. The dispensation, shaking and processing of
the samples are automatically performed by the Dimension® system. Briefly, in acidic conditions (pH 4.5) and in the presence of a reducing agent (ascorbic acid) occurs the release of iron bound to transferrin. The resulting product (Fe²⁺) forms a blue complex with 3-(2-piridil)-5,6-bis-2- (5-furyl sulfonic acid)-1,2,4-triazine (Ferene®). The absorbance of the complex is measured using a biocromatic endpoint technique (600, 700nm). The test principle for the total bound iron is similar, the samples are automatically mixed with a serum iron solution to saturate all available sites of iron binding in transferrin. In non-acidic conditions (pH 8.6), only saturated iron in excess, unbound, is available to be reduced to ferric iron by ascorbic acid and form the blue complex with Ferene®. The subsequent addition of acid (pH 4.5) releases the iron bound to transferrin, this supplemental iron is reduced to ferric iron by ascorbic acid, forming an increased amount of blue complex with Ferene®. The increase in absorbance during the change of pH 8.6 to pH 4.5 is proportional to the concentration of iron bound to transferrin.

### Relative Quantification of Nrf2, ferritin and HO-1 mRNA by Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The cell culture was performed as previously described in the anti-amastigote assay. RNA extraction was performed with 10⁶ cells using SV Total RNA Isolation System (Promega, USA) following the manufacturer’s procedure. RNA concentration was determined by absorbance (260 nm) measurements with a spectrophotometer (SynergyHT, Biotek, USA). Complementary DNA was synthesized using 500 ng of total RNA in a reverse transcription reaction by MMLV reverse transcriptase (Invitrogen, USA) following the manufacturer’s procedure. Real time RT-PCR quantitative mRNA analyses were performed in Rotor-Gene Q equipment (Qiagen, Germany) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA), in a final volume of 20 µl. The reaction mixture also contained 2 µM primers and 100 ng of cDNA template. The sequences of primers used for Nrf2 were Nrf2-F 5'-TCACACGAGATGACCTTAGGCGAA-3' and Nfr2-R 5'-TACAGTTCGTGCGCGACTTTAT-3', for heme-oxygenase-1 were HO-1-F 5'-CCCAAAACTGGCCTGTAAAA-3' and HO-1-R 5'-CGTGGTCAGTCAACATGGAT-3', for ferritin-L were ferritin-F 5'-TTCAGGATGTGCAGAAAGCC-3' and ferritin-R 5'-AAGAGGGCTGGATTCCAGCTTCC-3' and for β-actin were β-actin-F 5'-
AGCTGCGTTTACACCCTTT-3' and b-actin-R 5'-AAGCCATGCCAATGTTGTCT-3'. Cycling conditions were 10 minutes at 95 °C, and 40 cycles of 30 seconds at 95 °C, 30 seconds at 62 °C and 30 seconds at 72 °C, followed by melting curve analysis (70 to 95 °C at 0.5 °C/second). The calculation for determining the qualified levels of gene expression by reference to β-actin were achieved by the cycle threshold method.

**Statistical analysis**

Data were expressed as mean ± standard error of the mean. At least three independent experiments were performed, each with duplicate datasets. Data were analyzed using the GraphPad Prism statistical software (GraphPad Software, Inc., USA, 500.288). Significant differences between the groups were determined by one-way ANOVA, followed by Tukey’s test for multiple comparisons. Differences were considered statistically significant when p ≤ 0.05.

**RESULTS**

*C. coriaceum* extracts inhibit the proliferation of *L. amazonensis* promastigote forms

The antileishmanial effect of EAC and MET was evaluated by determining the proliferation of parasites. It was observed that all tested concentrations of the extracts significantly inhibited the proliferation of *L. amazonensis* from 24h when compared with control or vehicle groups, except for the 25 μg/mL of MET that has effect after 48h of treatment. The treatment for 48 and 72 h were able to reduce significantly the number of parasites to all tested extracts (Figure 1).

As we obtained similar results between the different extracts concentration in this assay, the next experiments investigating the effect of treatments on promastigote forms were performed with the intermediate concentration of EAC and MET (50 μg/mL). As the extracts in this concentration showed effect in 24 hours, all the experiments were performed in this period.
Figure 1 – Antipromastigote effect of *C. coriaceum* leaves extracts. *L. amazonensis* promastigotes forms were subjected to 25, 50 and 100 μg/mL of ethyl acetate (EAC) or methanolic (MET) leaf extract. The parasite viability was assessed at 0, 24, 48 and 72 h. As control was used non-treated parasites, as vehicle control was used DMSO 0.01% and as positive control was used amphotericin B (AMB) 1 μM. The values represent the mean ± SEM of three independent experiments performed in duplicate. ** Significant difference compared to control group (p ≤ 0.01), *** (p ≤ 0.001).

The *C. coriaceum* extracts induces loss of promastigote forms typical morphology and reduce the cell volume

Morphological alterations of *L. amazonensis* treated with EAC and MET were observed by scanning electron microscopy. The treatment caused rounding and reduction of parasite body size. MET treatment also showed rupture of the plasma membrane and cell lysing. This data was confirmed by flow cytometer analysis, where was observed a reduction of cell volume of EAC and MET treated parasites.
Figure 2 – Morphological changes of treated promastigote forms. Scanning electron microscopy images of *L. amazonensis* promastigote forms incubated in the absence (A) or presence of 50 µg/mL of ethyl acetate (EAC) (B) or methanolic (MET) (C) leaf extract for 24 h. Cell volume in promastigote forms of *L. amazonensis* treated with ethyl acetate (EAC) or methanolic (MET) (50 µg/mL). FSC-H was considered a function of cell size. The gray area corresponds to the control group (i.e., untreated parasites) and the white area corresponds to the treated group. Typical histograms of at least three independent experiments are shown.

The antipromastigote effect of *C. coriaceum* extracts is due mitochondrial depolarization, increase in ROS production, phosphatidylserine exposure and damage in plasma membrane of the protozoan

Once verified the antipromastigote effect of *C. coriaceum* extracts, we decided to understand the mechanism by which the parasites were being eliminated. Based on this, it was evaluated the mitochondrial membrane integrity in EAC and MET (50 µg/mL) treated parasites using TMRE, a fluorescent marker that complexes with active mitochondria. It was found that the extracts decreased total TMRE fluorescence intensity compared with the control group, indicating loss of integrity of this organelle (Figure 3A).

We also evaluated the effects of total ROS production in treated promastigotes.
using H$_2$DCFDA, a fluorescent probe that primarily detects H$_2$O$_2$ and hydroxyl radicals and fluoresces after forming dichlorofluorescein (Myhre et al., 2003). The results showed that all tested extracts increased total ROS production on promastigotes forms at concentration 50 μg/mL when compared with the control group (Figure 3B).

In addition, we investigated whether the cell death mechanism triggered by the treatment involved apoptosis by marking the externalization of PS. Our data show that treated parasites have increased annexin V labeling compared with the control group, indicating an increase in PS externalization (Figure 3C).

After these results, it was studied whether the mechanism of cell death triggered by also involves the plasma membrane integrity. For this, treated parasites were stained with PI, which diffuse across permeable membranes and bind to nucleic acids. As shown in Figure 3D, EAC and MET extracts increased PI stained in promastigotes forms.

To differentiate the cell death mechanism in necrotic, apoptotic or late-apoptotic process, we performed the annexin V/PI co-staining in treated promastigotes. Promastigotes with single label for annexin (annexin V+) was considered as apoptotics, PI+ as necrotic and the double mark for annexin V+/PI+ as late-apoptotic process. The percentage of promastigotes that were Annexin V+ was 16.8 and 11% after treatment with EAC and MET, respectively. The PI+ cells were 8.3% for EAC and 5.7% for MET. Lastly, the annexin V+/PI+ promastigotes were 69.2 and 14.6% for EAC and MET, respectively, indicating that the most of the parasites were in late apoptosis-like death in both conditions (Figure 3F, G).
Figure 3 – C. coriaceum-induced death mechanism in L. amazonensis promastigote forms submitted to a 24 h treatment with 50 μg/mL of the extracts. The following methods were used for the respective assessments: (A) TMRE assay for fluorometric
analysis of the mitochondrial membrane potential, (B) H2DCFDA probe for reactive species of oxygen measurement, (C) Annexin V labeling for phosphatidyserine exposition and (D) propidium iodide staining for the analyses of plasma membrane integrity. Data represent the mean ± SEM of three independent experiments performed in duplicate. ** Significant difference compared to control (p ≤ 0.01). Co-staining of EAC (F) or MET (G) treated promastigotes with PI and annexin V–FitC analyzed by flow cytometry. As control was used untreated parasites (E). Typical dot plots of at least three independent experiments are shown. EAC – ethyl acetate leaf extract; MET – methanolic leaf extract.

**Low concentrations of C. coriaceum extracts does not alter the viability of peritoneal macrophages**

In the attempt to verify whether the C. coriaceum extracts has toxic effect on murine macrophage, we performed the MTT assay and verified that the concentrations of 25, 50 and 100 μg/mL were not toxic in the period assessed (24 h) for both EAC and MET (Figure 4). For this reason, we chose these concentrations to proceed the follow experiments.

![Figure 4 – Citotoxicity of C. coriaceum extracts. Peritoneal BALB/c macrophages were submitted to a 24 h treatment using the 25, 50 and 100 μg/mL of ethyl acetate (EAC) or methanolic (MET) leaf extracts and viability analyzed through MTT assay. As control was used untreated parasites, as vehicle was used 0.01% DMSO and as positive control, 0.4% H2O2. Amphotericin B (AMB) was used at 1μM. The values represent the mean ± SEM of three independent experiments performed in duplicate.](image-url)
C. coriaceum extracts acts on intracellular amastigotes forms and decreases the recovery of promastigotes forms from infected macrophages.

To verify the effect of C. coriaceum extracts on amastigotes forms of L. amazonensis, it was performed the elimination assay which evaluated the percentage of infected macrophages and the number of amastigotes after 24 h of treatment. It was observed that the treatment with 25-100 μg/mL of EAC and MET could induce a significant reduction in the percentage of infected macrophages and number of amastigotes per macrophage (Figure 5A and B).

To confirm the reduction of infection, it was performed the recovery assay of the promastigotes forms in which the culture of infected macrophages was submitted to ideal conditions to the differentiation of viable amastigotes in free promastigote forms. It was verified that all tested concentrations promoted at least 50% of reduction in recovery promastigotes forms, from 48 and 72h of culture (Figure 5C).

There was no statistical difference between the different treatments (EAC, MET or AMB) nor between the different concentrations of the extracts (25, 50 and 100 μg/mL). Therefore, aiming for minor side effects in future therapeutic applications, the lowest concentration (25 μg/mL) was chosen for the following experiments.

Figure 5 – C. coriaceum extracts effect on L. amazonensis-infected macrophages. Infected cells were submitted to a 24 h treatment using 25, 50 and 100 μg/mL of the leaf extracts. (A) Assessment of the number of amastigotes per macrophage and (B)
percentage of infected macrophages. (C) 48 and 72 h of *L. amazonensis*-infected macrophages incubation 199 media at 24°C. The number of recovered parasites was measured in Neubauer chamber and the values converted as %. Dashed line indicates the control group (100%). Data represent the mean ± SEM of three independent experiments performed in duplicate. As control was used infected non-treated cells, as vehicle was used 0.01% DMSO. EAC – ethyl acetate extract, MET – methanolic extract, AMB – amphotericin B. * Significant difference compared to control (*p ≤ 0.05) ** (p ≤ 0.01), *** (p ≤ 0.001), **** (p ≤ 0.0001).

**C. coriaceum** leaf extracts induce cytokine secretion by macrophages

Once that treatment on infected macrophages resulted in a reduction in the infected cell number and recovery promastigotes, we direct the focus in the attempt to verify what mechanism of action triggered by these extracts treatment. It was verified that treatment with EAC and MET could significantly increase the levels of TNF-α, MCP-1, IL-6 and IL-10 when compared with *L. amazonensis*-infected non-treated cells (Control) (Figure 6A, B, C and D, respectively).

![Figure 6 - Effect of C. coriaceum extracts on cytokine production of L. amazonensis-infected macrophages](image)

The infected cells were submitted to 24 h treatment with the EAC
and MET extracts at 25 μg/mL and the cytokine levels were verified by CBA. (A) TNF-α, (B) MCP-1, (C) IL-6 and (D) IL-10. Uninfected represents the non-infected and non-treated cells, control represents infected non-treated cells. EAC – ethyl acetate leaf extract; MET – methanolic leaf extract. The values represent the mean ± SEM of three independent experiments performed in duplicate. ** Significant difference compared to control (p ≤ 0.01), *** (p ≤ 0.001).

The EAC and MET treatment reduces the amount of malondialdehyde and ROS, but not affect the NO levels

Knowing that the treatment induced immunomodulation, we investigated the two major microbicidal molecules synthesized by macrophages to contain the infection: ROS and NO. Our results demonstrated that the treatment with MET was capable of reduce the MDA levels (Figure 7A), a lipid peroxidation metabolite derived from oxidative stress occurrence. Furthermore, treatment with EAC and MET reduced the cleavage of H2DCFDA probe (Figure 7B), indicating a reduction of ROS generation. However, the treatment with the extracts was not able to affect the production of NO when compared with the infected control, while all the infected conditions showed reduced levels of NO when compared with the uninfected group (Figure 7C).
Figure 7 – Measurement of MDA, ROS and NO\textsuperscript{2-} of EAC and MET treated cells. The following methods were used to assess the \textit{L. amazonensis}-infected macrophages submitted to a 24 h of treatment using the ethyl acetate (EAC) and methanolic (MET) extracts at 25 \( \mu \text{g/mL} \): (A) MDA measurement by HPLC, (B) fluorescent probe \( \text{H}_2\text{DCFDA} \) for reactive oxygen species measurement and (C) Griess method for nitrite levels. The values represent the mean ± SEM of three independent experiments performed in duplicate. Uninfected represents the non-infected and non-treated cells, control represents infected non-treated cells. * Significant difference compared to control, ** (\( p \leq 0.01 \)). # Significant difference compared to uninfected group (\( p \leq 0.05 \)).

\textit{C. coriaceum} leaf extracts modulates iron pool of infected macrophages

Still with the objective to understand the pathway parasites death, we investigated the iron and total binding iron capacity, once this metal is essential for \textit{Leishmania} survival. The results showed that treatment with EAC and MET of \textit{L. amazonensis}-infected macrophages did not alter the total iron concentration (Fig. 8A) but increased iron bound to transferrin (Fig. 8B) when compared to control.

![Graph A](image1.png) ![Graph B](image2.png)

Figure 8 – Total iron concentration and total bound iron in \textit{L. amazonensis}-infected macrophages treated with EAC and MET. The determination of labile iron concentration in supernatants was performed utilizing the Dimension\textsuperscript{®} automated system. \textit{L. amazonensis}-infected macrophages treated or not with 25 \( \mu \text{g/mL} \) of EAC (ethyl acetate extract) or MET (methanolic extract) for 24h do not alter the iron concentration (A) but increased total iron bound capacity (B). Data represent mean ± SEM of three independent experiments. ** Significant difference compared to control (\( p \leq 0.01 \)), *** (\( p \leq 0.001 \)).

\textit{Caryocar coriaceum} treatment upregulated the Nrfl2, ferritin and heme oxygenase-1 (HO-1) expression
Previous studies have demonstrated the effect of Nrf2 as key regulator of the antioxidant response which plays a central role in up regulation of multiple genes, including HO-1 and ferritin involved in iron metabolism (Qaisiya et al., 2013). Therefore, we decided to investigate the Nrf2, ferritin and HO-1 expression on *L. amazonensis*-infected macrophages treated with the extracts, and we observed that the MET treatment significantly upregulated the expression of Nrf2 (Figure 9A) and both treatments significantly upregulated the expression of ferritin and HO-1 (Figure 9B and C).

**Figure 9** - Nrf2, ferritin and HO-1 expression in *L. amazonensis*-infected macrophages treated with EAC and MET. Were used uninfected (macrophages incubated with culture medium) and control (infected macrophages with *L. amazonensis*). EAC – ethyl acetate leaf extract; MET – methanolic leaf extract. Data represent mean ±SEM of three independent experiments. ***Significant difference compared to control (p ≤ 0.001), **** (p < 0.0001).

**DISCUSSION**

The current treatment against ACL is based on a highly toxic chemotherapy (Alvar et al., 2006) which drives the search for new alternative strategies, in which natural compounds have received considerable attention. Thus, the present study aimed to verify the leishmanicidal action of leaf extracts from a Brazilian cerrado plant, *C. coriaceum*. 
A previous study demonstrated the anti-\textit{L. amazonensis} promastigote action of the hydroethanolic extract from leaves of \textit{C. brasiiliense} (Paula-Ju et al., 2006b) also corroborating that the leishmanicidal activities where possible due the presence of phenols and flavonoids contents and their antioxidant properties (Rondon et al., 2011; Silva et al., 2016, 2014). Additionally, others studies also indicated that the presence of phenol content and flavonoids in \textit{C. brasiiliense} and \textit{C. coriaceum} were the main role of antioxidant capacity, even in a supercritical CO$_2$ extract (Amaral et al., 2014; Araruna et al., 2013).

As phenols and flavonoids content are mainly extracted at Ethyl Acetate and Methanol polarity, was verified the action of EAC and MET extracts of \textit{C. coriaceum} on \textit{L. amazonensis} promastigotes. Our results showed that the EAC and MET extracts were able to reduce the number of viable parasites as well induce morphological and physiological alterations on promastigote forms using hundred times smaller concentrations than that used by Paula-Ju et al. (2006).

With regard to the physiological changes, we observed that the extracts induced depolarization of the mitochondrial membrane of the parasite. It is known that maintenance of mitochondrial integrity is essential for parasite survival, once \textit{Leishmania} spp. have a unique mitochondrion that is a principal site for the generation of cellular ATP by oxidative phosphorylation and, thus, becoming a promising antiparasitic target (Fidalgo and Gille, 2011; Monzote and Gille, 2010).

It is know that the dysfunction of mitochondrial respiratory chain can produce the huge amount of ROS inside the organelle (Roy et al., 2008). As expected, treatment with all extracts was able to cause ROS increase in promastigote forms. Furthermore, the cascade triggered by loss of mitochondrial integrity followed by increased ROS production may lead to the death of the parasite by apoptosis-like mechanism (Fidalgo and Gille, 2011; Mehta and Shaha, 2004).

Our data showed that the treatment induces a late apoptosis-like death in majority of the population, characterized by double labeling with Annexin V and P1 (Doroodgar et al., 2016; Ghaffarifar et al., 2015). Doroodgar et al. (2016) and Ghaffarifar et al. (2015) verified similar effects of Tamoxifen and Artemisina treatment, respectively, on \textit{Leishmania major} promastigotes.

Once the effect of the extracts on promastigote forms had been proven, we aimed to understand the action of these treatments on intracellular amastigote forms. We found
that the treatment did not affect the viability of macrophages, the main host cells of *Leishmania* spp. (Liu and Uzonja, 2012b), but acted on the intracellular amastigotes. Since EAC and MET showed a killing effect on parasite, we investigated the potential mechanisms involved in amastigote elimination.

The *Leishmania* elimination involved the secretion of proinflammatory molecules, as IFN-γ, TNF-α, IL-6 and MCP-1 which activate macrophages to produce ROS and NO and eliminate the parasites (Bhattacharyya et al., 2002; de Santana et al., 2017; Liew et al., 1990; Murray and Nathan, 1999). Our data showed increase of proinflammatory cytokines but not of ROS and NO. This can be explained by the concomitant increase in IL-10 levels, which is responsible to control the inflammation and synthesis of proinflammatory mediators (Mosser and Zhang, 2008).

Besides that, the *Caryocar* sp. extracts were reported by their antioxidant capacity (Paula-Junior et al., 2006), which can act directly on reactive molecules and explain the decrease of lipid peroxidation and ROS levels. This antioxidant capacity can act directly by scavenging ROS or indirectly by activating pathways which promote ROS degradation (Poljsak et al., 2013).

Once that the mechanism of action of the extracts on the intracellular amastigotes forms did not involve the production of ROS or NO, we investigated pathways involved with the iron metabolism, since this metal is fundamental for the replication and survival of the intracellular parasite. The success or failure of iron uptake by the pathogen impacts the outcome of pathogenesis and is an important process in infection control (Zaidi et al., 2017).

Our data showed that EAC and MET did not alter the total iron pool, but increases the total iron bound to transferrin. Previous work showed that *L. donovani* uses only the labile iron pool for its survival and replication (Das et al., 2009), in this sense, the increase of transferrin iron binding unavailable the nutrient to the parasite.

Kedzierski et al., 2007 showed that antioxidant defense could help macrophages to eliminate *L. major*. As demonstrated previously, EAC and MET extracts acted in the reduce ROS production, MDA formation and iron availability, we evaluated genes responsible for the antioxidant response and iron metabolism.

A pivotal factor that regulates the antioxidant responses in a variety of disease models is the transcription factor Nrf2 (Paiva and Bozza, 2014). Nrf2 is responsible for regulating a battery of antioxidant and cellular protective genes, primarily in response to
oxidative stress and genes that contain ARE motifs, triggering H-ferritin and heme oxygenase 1 (HO-1) (Alam et al., 1999; Hintze and Theil, 2005). HO-1 is associated with regulation of labile iron pool, by increasing ferritin, a protective protein that acts sequestering iron ions (Lin et al., 2008). In addition, this protein can be translocated to the nucleus, activating antioxidant mechanisms (Gozzelino et al., 2010). We showed for the first time that EAC and MET extracts increased the expression of Nrf2, HO-1 and Ferritin in *L. amazonensis*-infected macrophages.

Taking these results together, it was demonstrated that EAC and MET were able to modulate the iron by increase the iron bound to transferrin, upregulate Nrf2 expression, which massively stimulate the transcription of proteins HO-1 and ferritin responsible for controlling the bioavailability of labile iron pool, impairing the uptake of this metal by the parasite.

The phytochemical analysis of *C. coriaceum* leaf extracts performed by Arraruna et al. (2013) revealed the presence of pyrogallic and hydrolisable tannins, phenolic compounds, flavonoids etc, showing a variety of compounds in the methanol extract, including gallic acid, chlorogenic acid, caffeic acid, rutin and quercetin. Rutin and quercetin are reported as possessing antileishmanial (Vila-nova et al., 2012) and antioxidant action (Chen and Ahn, 1998), then they probably are responsible in part for activities found in this work.

In conclusion *C. coriaceum* leaf extracts EAC and MET have substantial *in vitro* antileishmanial activity, possibly due their major phenolic contents, acting directly on promastigote forms leading to death by late apoptosis-like process; and in *L. amazonensis*-infected macrophages triggering an antioxidant response. The mechanism triggered by the extracts involves its capacity to activate responses mediated by Nrf2/HO-1/Ferritin, followed by a modulation of the labile iron pool, which culminates in a depletion of available iron for parasite replication and survival within macrophages.

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Caryocar coriaceum fruit extracts induce leishmanicidal action directly on promastigotes and indirectly on intracellular amastigotes by iron metabolism modulation

(Extratos de frutos de Caryocar coriaceum induzem ação leishmanicida diretamente em promastigotas e indiretamente em amastigotas intracelulares por modulação do metabolismo do ferro)

Fernanda Tomiotto-Pellissier, Daniela Ribeiro Alves, Selene Maia de Morais, Bruna Taciane da Silva Bortoleti, Manoela Daiele Gonçalves, Taylon Felipe Silva, Eliandro Reis Tavares, Lucy Megumi Yamauchi, Andrea Name Colado-Simão, Idessania Nazareth Costa, Ivete Conchon-Costa, Milena Menegazzo Miranda-Sapla, Wander Rogério Pavanelli

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CARYOCAR CORIACEUM FRUIT EXTRACTS INDUCE LEISHMANICIDAL ACTION DIRECTLY ON PROMASTIGOTES AND INDIRECTLY ON INTRACELLULAR AMASTIGOTES BY IRON METABOLISM MODULATION

Fernanda Tomiotto-Pellissier<sup>a,b,*</sup>, Daniela Ribeiro Alves<sup>c</sup>, Selene Maia de Morais<sup>c</sup>, Bruna Taciane da Silva Bortoleti<sup>a,b</sup>, Manoela Daiele Gonçalves<sup>d</sup>, Taylon Felipe Silva<sup>a</sup>, Eliandro Reis Tavares<sup>c</sup>, Lucy Megumi Yamauchi<sup>c</sup>, Andrea Name Colado-Simão<sup>a</sup>, Idessania Nazareth Costa<sup>a</sup>, Ivete Conchon-Costa<sup>a</sup>, Milena Menegazzo Miranda-Sapla<sup>a</sup>, Wander Rogério Pavanelli<sup>a,b,*</sup>

<sup>a</sup>Department of Pathology Science, Center of Biological Sciences, State University of Londrina, Londrina, Paraná, Brazil.
<sup>b</sup>Biosciences and Biotechnology Postgraduate Program, Carlos Chagas Institute (ICC), Fiocruz, Curitiba, Brazil.
<sup>c</sup>Department of Veterinarian Sciences, Ceará State University, Fortaleza, Ceará, Brazil.
<sup>d</sup>Department of Chemistry, Center of Exact Sciences, State University of Londrina, Londrina, Paraná, Brazil.
<sup>e</sup>Department of Microbiology, Center of Biological Sciences, State University of Londrina, Londrina, Paraná, Brazil.

* Corresponding author. Laboratory of Immunopathology of Neglected Diseases, Center of Biological Sciences, State University of Londrina, Celso Garcia Rod, PR-445, Km380, Londrina, Paraná, Brazil. Phone number: +55 43 33714539. Zip Code: 86057970.
E-mail address: fernandatomiotto@gmail.com / wanderpavanelli@yahoo.com.br

ABSTRACT

Leishmaniasis is a group of neglected diseases caused by parasites of Leishmania genus. The treatment of Leishmaniasis represents a great challenge, because the available drugs present high toxicity and none of them has been shown to be fully effective. Caryocar is a botanical genus rich in phenolic compounds, which leaves extracts have already been described by its antileishmanial action. Thus, we investigated the effect of pulp and peel extracts of the Caryocar coriaceum fruit on promastigote and amastigote forms of Leishmania amazonensis. Both extracts had antipromastigote effect after 24, 48 and 72 h and this effect was by apoptosis-like process induction, with reactive oxygen
species (ROS) production, damage to the mitochondria and plasma membrane, and phosphatidylycerine exposure. Knowing that the fruit extracts did not alter the viability of macrophages, the main host cells for Leishmania, we observed that the treatment with extracts reduced the infection of these cells. Thereafter, in the in vitro infection context, the extracts showed antioxidant properties, by reducing NO, ROS and MDA levels. In addition, both peel and pulp extracts up-regulated Nrf2/HO-1/Ferritin expression and increase the total iron bound in infected macrophages, which culminates in a depletion of available iron for *L. amazonensis* replication.

**INTRODUCTION**

Leishmaniasis is a group of diseases caused by parasitic protozoa of the *Leishmania* genus, which are transmitted through the bite of phlebotomine insects. It is recognized by the World Health Organization (WHO) as a neglected tropical disease, since 350 million people in 88 countries worldwide are at risk of developing one of the clinical forms of the disease (WHO, 2018).

Macrophages are the main host cells for *Leishmania* parasites, in which amastigote forms can survive, depending on the iron uptake of the host to its metabolism, virulence and multiplication (2,3). Macrophages have a machinery to eliminate the parasites, which are mainly composed by free radicals formation notably nitric oxide (NO) and reactive oxygen species (ROS), however, the parasite can evade the macrophages response, persist on host and develop the disease (reviewd in: Rossi and Fasel, 2018).

The treatment of ATL represents a great challenge because the available drugs present high toxicity and none of them has been shown to be fully effective. Relapses, therapeutic failure and resistance to treatment are factors that motivate the search for drugs that present leishmanicidal actions that are more effective and less toxic to the patient (5).

*Caryocar* is a botanical genus belonging to the Caryocaraceae family, distributed in South and Central America in the vegetation called Cerrado. The fruits are used in food, cosmetics and in folk medicine. Rich in phenolic compounds, these extracts have been described as potent antioxidants, besides having anti-inflammatory, antineoplastic, antimicrobial and leishmanicidal effects (6–11).

It has been demonstrated that leaves extracts of *Caryocar coriaceum* has
leishmanicidal activity, either directly in promastigotes forms of *Leishmania amazonensis*, inducing death by a late apoptosis process, or indirectly in macrophages infected by amastigotes, by activation of antioxidant mechanism that prevents the availability of free iron required for parasitic replication within the macrophage (12,13). However, nothing is known about the effect of pulp and peel extracts of this plant on *Leishmania*.

Given this context, this study aimed to investigate the leishmanicidal effect of pulp and peel extracts of the *Caryocar coriaceum* fruit on promastigote and amastigote forms of *Leishmania (Leishmania) amazonensis*.

**MATERIALS AND METHODS**

**Pulp and peel extracts of *Caryocar coriaceum***

The extracts of *C. coriaceum* were kindly supplied by Dr. Selene Maia de Morais of the State University of Ceará and obtained as previous described (13). Briefly, peel and pulp of *C. coriaceum* mature fruits were obtained at the Campus of the State University of Ceará (UECE) (lat.: −3.792222; long.: −38.556111), Fortaleza, Brazil. These plants were submitted and identified by Prisco Bezerra Herbarium under the code EAC57060. The extracts were obtained by cold maceration with 96% ethanol, at 12 h cycle of light for 7 days. Filtration of the supernatant and evaporation of the solvent at reduced pressure in a rotary evaporator led to crude ethanol extracts of *C. coriaceum* fruit pulp and fruit peel.

**Leishmania (Leishmania) amazonensis maintenance**

*L. (L.) amazonensis* (MHOM/BR/1989/166MJO) promastigotes forms of were maintained in culture medium 199 (GIBCO, Invitrogen, New York, USA) pH 7.18-7.22 supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen, New York, USA), 10 mM-HEPES buffer, 0.1% human urine, 0.1% L-glutamine, 10U/mL-penicillin and 10μg/mL-streptomycin (Invitrogen-GIBCO) and 10% sodium bicarbonate. The cell culture was maintained at 25°C in a 25 cm² culture flask. All experiments used promastigote forms at the stationary growth phase.

**Antipromastigote assay**

*L. amazonensis* promastigote forms (10⁶ cells/mL) were treated with *C. coriaceum*
extracts 25, 50 and 100 μg/mL. Parasites were counted on a Neubauer chamber after 24, 48 and 72h of treatment. *L. amazonensis* promastigote maintained in the culture medium was used as control, DMSO 0.01% was used as vehicle and amphotericin B (AMB) 1 μM was used as positive control.

**Scanning electron microscopy of promastigotes**

Scanning electron microscopy of promastigotes forms was performed according to (14). Briefly, the parasites (10⁶) were treated with 50 μg/mL of pulp and peel extracts for 24 h. After, were collected and subjected to 2.5% glutaraldehyde-fixation in 0.1 M of sodium cacodylate buffer containing 1 mM CaCl₂, collected and placed in poly-L-lysine treated coverslips and dehydrated with graded ethanol baths, CO₂ dry point, gold coated, and observed through scanning electron microscopy (FEI QUANTA 200 scanning electron microscope).

**Determination mechanism of action in promastigote forms**

To determine the mechanism of action in promastigote forms, the parasites (10⁶ cells/mL) were treated for 24h with 50 μg/mL of the extracts, as previous described by our group (7).

To assess the inner mitochondrial membrane potential, treated parasites were washed and incubated with 25 nM of tetramethylrhodamine ethyl ester (TMRE) (Sigma) for 30 min at 25°C and analyzed at excitation wavelength of 480 nm and an emission wavelength of 580 nm.

Phospholipids (PS) exposure was detected by Annexin-V FITC (Invitrogen, Eugene, USA). The parasites were resuspended in 100 μL of binding buffer (140 mM NaCl, 5 mM CaCl₂, and 10 mM HEPES-Na, pH 7.4), followed by the addition of 5 μL of Annexin-V FITC for 15 min. After the incubation, the binding buffer (400 μL) was also added. Data acquisition was performed at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

To determine the cellular membrane integrity, the promastigotes were directly incubated with propidium iodide (PI) (Sigma, St. Louis, MO, USA) (0.50 μg/mL) for 5 min. Immediately thereafter, the promastigotes were analyzed using an excitation wavelength of 480 nm and an emission wavelength of 580 nm.

All the analysis were performed on a fluorescence microplate reader (Victor X3,
PerkinElmer, Finland). To compare the different treatments, the fluorescent values obtained were normalized to the respective number of cells.

**Determination of reactive oxygen species (ROS) generation on L. amazonensis**

In order to assess the ROS generation, the promastigote forms of *L. amazonensis* (10^6 parasites incubated with 50μg/mL of extracts for 24h) were washed in PBS (pH 7.4) and loaded with 10 μM of a permeant probe diacetate 2',7'-dichlorodihorofluorescein (H2DCFDA) (Sigma, St. Louis, MO, USA) diluted in DMSO, and incubated in the dark for 45 min, at cell temperature. Reactive oxygen species (ROS) were measured with an excitation wavelength of 488 nm and emission wavelength of 530 nm on a fluorescence microplate reader (Victor X3).

**Co-determination of annexin V and propidium iodide label**

Promastigotes (10^6 cells/mL) under the same above-mentioned conditions were washed and resuspended in 100 μL of assay buffer 1x (Santa cruz Biotechnology), followed by the addition of a mix containing 1 μL of annexin-V FITC and 5 μL of PI (Santa cruz Biotechnology). Data acquisition and analysis were performed using a BD Accuri™ C6 Plus personal flow cytometer. A total of 10,000 events were acquired. The single label for annexin (annexin V+) was considered apoptotic death, the single label for propidium iodide (PI+) as necrotic death and the double mark for annexin V+/PI+ as a late-apoptotic process (15,16).

**Ethics Committee**

BALB/c mice were kindly provided by the Carlos Chagas Institute (ICC)/Fiocruz, Curitiba, Brazil. The animals weighing approximately 25-30 g and aged 6-8 weeks were kept under sterile conditions and used according to protocols approved by the Institutional Animal Care and Committee. This study was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina (13134.2016.62),170

**Viability of peritoneal exudate cells**

The cytotoxic effects of *C. coriaceum* extracts in peritoneal exudate cells (PEC) were tested based on mitochondrial oxidation of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide) (Sigma) assay (17). The total of 5x10^5 cells/mL were recovered from the peritoneal cavity of BALB/c mice with cold PBS supplemented with 3% of FBS and then cultured in 24-well plates with 200 μL of RPMI 1640 medium (10% FBS) for 2 h (37°C, 5% CO₂). Adherent cells were incubated with 25, 50 and 100 μg/mL of pulp or peel extract and cultured for 24 h under the same conditions. After this period, MTT (5 mg/mL) was added for 3 h. PEC under the same conditions without treatment were used as control; DMSO 0.01% was the vehicle and the positive control was H₂O₂ 0.4%. The plates were readed using a spectrophotometer (Thermo Scientific, Multiskan GO) at 550 nm. The results were expressed as a percentage of viability compared to the control group.

**Anti-amastigote assay**

PEC (5x10^5 cells/mL) were cultured in 24-well plates containing 13 mm glass coverslips and incubated with 200μL of RPMI 1640 medium for 2h at 37°C and 5% CO₂. The adherent cells were infected with *L. amazonensis* promastigotes of (1x10^6 cells/mL) for 2h. After infection, the non-internalized promastigotes were removed through PBS-washing and the cells treated with the extracts (25, 50 or 100 μg/mL), RPMI 1640 medium (control), DMSO 0.01% (vehicle) or AMB 1 μM (positive control) for 24h (37°C, 5% CO₂). Subsequently, the cells were stained with Giemsa (Laborclin, Pines-PR Brazil) and 20 fields analyzed through optical microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan) (1000x magnification) in order to determine the % of infected cells and amastigote number per cell. The supernatant was stored for the measurement of cytokines and nitric oxide.

**Promastigote recovery test**

Promastigotes recovery assay was performed as previously described by (18). In brief, adherent PEC were infected with *L. amazonensis* and treated with concentrations of *C. coriaceum* extracts in the same conditions described for the anti-amastigote assay. Viable amastigotes are known to have the ability to differentiate into promastigotes when exposed to ideal conditions; therefore, after 24 h of treatment the cell culture was washed with PBS and incubated with 199 culture media at 24°C to induce the differentiation of intracellular viable amastigotes into promastigote free forms. Free promastigotes recovered (FPR) were counted on a Neubauer chamber for three consecutive days and the
number of recovery promastigotes normalized using the following equation:

\[
\text{% FPR} = \frac{\text{number of FPR of extracts treated}}{\text{number of FPR of untreated}} \times 100
\]

**Malondialdehyde (MDA) Levels Measurement**

MDA levels were determined through a High-Performance Chromatography (HPLC) according to Tomiotto-Pellissier et al., 2018. Briefly, the analyses were conducted using an Alliance e2695 HPLC (Waters, Milford, USA) equipped with a SecurityGuard ODS-C18 (4 × 3.0 mm, Phenomenex), C18 reverse phase column (Eclipse XDBC18; 4.6 × 250 mm, 5 μm, Agilent) as well as a photodiode array detector (Photodiode Array Detector, 2998) using Empower 2 software (Waters, Milford, USA). The preparation of MDA standards used 1,1,3,3-tetraethoxypropane (TEP). After the treatment with EAC or MET, as described in anti-amastigote assay, aliquots containing 500 μL of cell suspension were deproteinized by adding 20% trichloroacetic acid, subsequently reacted with 1mL of thiobarbituric acid. The mobile phase was 70% 10 mM KH2PO4 buffer, pH 7.0, and 40% HPLC-grade methanol. Readings were obtained at 532 nm, following an eight-minute isocratic flow at the rate of 1 mL/min. The results were expressed in nM MDA.

**Determination of iron concentration and total bound iron**

The determination of iron concentration in supernatants of the anti-amastigote assay was performed utilizing the method developed by Smith et al. (1984) with modifications. Briefly, in pH 4.5 and in the presence of ascorbic acid, it occurs the release of iron bound to transferrin. The resulting product (Fe^{2+}) forms a complex measured using a biochromatic endpoint technique (600, 700nm). The test principle for the total bound iron is similar: all available sites of iron binding in transferrin were saturated. In pH 8.6, only saturated iron in excess, unbound, is available to be reduced to ferric iron by ascorbic acid forming a complex. The subsequent addition of acid (pH 4.5) releases the iron bound to transferrin, this supplemental iron is reduced to ferric iron by ascorbic acid, forming an increased amount of complex. The increase in absorbance during the change of pH 8.6 to pH 4.5 is proportional to the concentration of iron bound to transferrin.

**Relative quantification of Nrf2, ferritin and HO-1 mRNA**

The mRNA expression levels quantification for nuclear factor erythroid 2-related
factor 2 (Nrf2), ferritin and heme-oxygenase 1 (HO-1) genes were performed in accordance to Tomiotto-Pellessier et al., 2018. Briefly, total RNA from cellular culture in the same condition as the anti-amastigote assay were extracted by using SV Total RNA Isolation System kit (Promega, Madison, USA) following the manufacturer's procedure. RNA concentration was determined by absorbance (260 nm) measurements with a spectrophotometer (Synergy HT, Biotek, Winooski, USA). 500 ng of total RNA was used to perform complementary DNA synthesis by reverse transcription reaction using 2 μM Oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen, Carlsbad, USA) following the manufacturer's procedure. Real-time PCR was performed in Rotor-Gene Q equipment (Qiagen, Hilden, Germany). In a final volume of 20 μL, was added 50 ng of cDNA template, 2 μM of Nrf2 (Forward-TCACACGAGATGAGGTTAGGGCAA and Reverse-TACAGTTCTGGGCGGGACCTTTAT), Ferritin (Forward-TTCCAGGATGTGCAGAAAGCC and Reverse-AAGAGGGCTGATTCCAGGTTC) and HO-1 (Forward-CCCAAAACTGGCCTGTAAAA and Reverse-CGTTGTCAGTCAACATGGAT) primers, and QuantiNova SYBR green qPCR kit (Qiagen, Hilden, Germany). The profile of the assay consisted of 10 min at 95°C and 40 cycles of 30 s at 95°C, 30s at 60°C, and 30s at 72°C, followed by melting curve analysis (60–95°C at 0.5°C/s). After the amplification, the cycle threshold values were normalized and used to determine the significant gene expression levels using REST2009 software (Qiagen, Hilden, Germany). β-actin (Forward-AGCTGCGTTTACACCCTTT and Reverse-AAGGCCATGCCAATGTGTCT) was used as reference gene for comparative cycle threshold method analyzes. Assays were carried out in triplicate with three different experiments.

**Statistical analysis**

Data were expressed as a mean ±SEM. At least three independent experiments were performed, each with duplicate datasets. Data were analyzed using the GraphPad Prism statistical software (GraphPad Software, Inc., USA, 500.288). Significant differences between the groups were determined through one-way ANOVA, followed by Tukey’s test for multiple comparisons. Differences were considered statistically significant upon p ≤ 0.05.

**RESULTS**
Peel and pulp extracts exerts anti-promastigote effect on *L. amazonensis*

The antileishmanial effect of pulp and peel extracts was assessed by determining the number of parasites. All tested concentrations of the extracts proved significant reduction of *L. amazonensis* proliferation from 24 h (p ≤ 0.01), 48 h (p ≤ 0.001) and 72 h (p ≤ 0.001) in relation to the control or vehicle groups (Figure 1A).

Difference between the concentrations are showed in Figure 1B. The dose of 100 µg/mL was more effective in the reduction of *L. amazonensis* proliferation than 25 µg/mL in 24 and 48 h (p ≤ 0.05 and p ≤ 0.01, respectively) for pulp extract, and in 24, 48 and 72 h for peel extract (p ≤ 0.05, p ≤ 0.01 and p ≤ 0.01, respectively). After 48 h of pulp extract treatment, 100 µg/mL was different from 25 and 50 µg/mL (p ≤ 0.01) and after 72 h, there was no difference between concentrations of this extract. The same concentration of the two different extracts did not differ.

Due similar results the next experiments investigating the effect of treatments on promastigote forms were performed with the intermediate concentration (50 µg/mL) at 24 h.

![Graph A and B](image)

Figure 1 – Antipromastigote effect of C. coriaceum leaves extracts. Promastigote forms of *L. amazonensis* were treated with peel and pulp C. coriaceum extracts (25, 50, 75 and 100 µg/mL) and evaluation of parasite viability at 0, 24, 48 and 72 h. Amphotericin B (AmB) 1 µM, was used as the positive control and DMSO (0.1%) as diluent for extracts, and only culture media as control. Values represent the mean ± SEM of three independent experiments performed in duplicate. ** Significant difference in relation to extracts and AMB treatments (p ≤ 0.01), **** (p ≤ 0.0001) (A). Detailed statistical analysis between the concentrations of each treatment (B).
Apoptosis-like mechanism induced by *C. coriaceum* extracts

Once verified the leishmanicidal action of the extracts, we aim to elucidate the death mechanisms induced by this treatment. We verified that pulp and peel extracts were able to reduce the mitochondrial membrane potential (ΔΨm) (*p* ≤ 0.0001), increase the ROS levels (*p* ≤ 0.001), induce the exposition of PS (*p* ≤ 0.0001) and damage the plasma membrane (*p* ≤ 0.001) (Figure 2A-D). The increase in ROS levels, induction of PS exposition and PI labeling were higher in peel-treated than in pulp-treated parasites (*p* ≤ 0.0001).

To differentiate the cell death mechanism, we performed an annexin V/ PI costaining in treated promastigote forms. The annexin V+ parasites had an increase of 10.6 and 7.34% after treatment with pulp and peel, respectively, when compared to control. The PI+ cells had an increase of 7.8 for pulp extract and 1.48% for peel extract. Lastly, the annexin V+/PI+ promastigotes were 28.4 and 24.9% higher in the pulp and peel treated parasites, respectively, when compared to control, indicating that most of the promastigote forms were undergoing late apoptosis-like death in both conditions (Fig. 2F and G).
Figure 2 – Effect of pulp and peel C. coriaceum extracts (50 μg/mL) in L. amazonensis promastigote forms. (A) TMRE labeling for analysis of the mitochondrial membrane potential, (B) H<sub>2</sub>DCFDA probe for ROS measurement, (C) Annexin V labeling for analysis of phospholipids exposition, and (D) propidium iodide staining for the analyses of plasma membrane integrity. Data represent the mean ± SEM of three independent experiments performed in duplicate. *** Significant difference in relation to control (p ≤ 0.001), **** (p ≤ 0.0001). Co-treating of pulp (F) or peel extract (G) treated promastigotes with PI and annexin V–FITC analyzed by flow cytometry. As control was used untreated parasites (E). Typical pseudocolor plots of at least three independent experiments are shown.

Moreover, we found that extracts acted on the morphology of promastigote forms: pulp extract induced the blebbing formation on parasite membrane (Figure 3B) and peel extract rounding and shrinking of Leishmania (Figure 3C) after 24h of 50 μg/mL treatment.
Figura 3 – Promastigote forms changes verified by scanning electron microscopy. Images of *L. amazonensis* promastigote forms incubated either in the absence (A) or presence of 50 μg/mL of pulp (B) or peel (C) *C. coriaceum* extracts for 24 h. Scale bar of 10 μM for images with 8000x (A and B) and 5 μM for images with 10500x (C) of magnification.

*C. coriaceum* extracts act in intracellular amastigote forms without cytotoxicity to host cells

Knowing the effects of *C. coriaceum* pulp and peel extracts on promastigote forms, we performed experiments to verify if they also acted on intracellular amastigotes.

First, we found that the tested concentrations did not interfere with the viability of host cells (macrophages) (Figure 3A) (p ≥ 0.99). Then, all tested concentrations (25 to 100 μg/mL) of both extracts were able to significantly reduce the percentage of infected macrophages (Figure 3B) and amastigote forms by macrophage (Figure 3C) when compared to control (p ≤ 0.05).

To confirm the reduction of infection, we performed a recovery assay of promastigotes forms in which the culture of amastigote-infected macrophages was submitted to ideal conditions for the differentiation of viable amastigotes in free promastigote forms. All tested concentrations reduced the percentage of recovery promastigotes forms, after 48 and 72h of culture (Figure 3D and E) (p ≤ 0.05).

As the different concentrations tested had similar effect, we chose the lowest concentration for the execution of the following experiments.
Figure 4 – Effect of pulp and peel *C. coriaceaum* extracts (50 µg/mL) in macrophages and intracellular amastigotes. Peritoneal BALB/c macrophages were submitted to a 24 h-treatment using the 25, 50 and 100 µg/mL of pulp or peel extracts and viability analyzed through MTT assay (A). Infected cells were submitted to the treatment and accessed the amount of amastigotes per macrophage (B) and percentage of infected macrophages (C). Percentage of recovery promastigotes after 48 and 72 h of *L. amazonensis*-infected macrophages incubation 199 media at 24°C (D and E). Dashed line indicates the control group. Vehicle – DMSO 0.01%, AMB – amphotericin B. Data represent the mean ± SEM of three independent experiments performed in duplicate. * Significant difference in relation to control (p ≤ 0.05) ** (p ≤ 0.01), *** (p ≤ 0.001).

Peel and pulp *C. coriaceaum* extracts acts as antioxidant in *L. amazonensis*-infected macrophages
With the objective of understanding the mechanisms involved in the elimination of intracellular parasites, we analyzed the production of reactive species, important in the response against *Leishmania*. Surprisingly, our results showed that *C. coriaceum* pulp and peel extracts acted as antioxidants, reducing the amount of NO₂⁻, ROS and MDA (Figure 4A, B and C, respectively) produced by infected macrophages, compared to control (*p ≤ 0.05*).

![Graphs showing effect of pulp and peel extracts on NO₂⁻, ROS, and MDA levels.](image)

**Figure 5 – Effect of pulp and peel *C. coriaceum* extracts in the synthesis of oxidant mediator by *L. amazonensis* infected-macrophages.** Infected cells were submitted to the extracts treatment (25 μg/mL) and accessed the amount NO₂⁻ (A), ROS (B) and MDA (C). Data represent the mean ± SEM of three independent experiments performed in duplicate. * Significant difference in relation to control (*p ≤ 0.05*), ** (*p ≤ 0.01*), *** (*p ≤ 0.001*), **** (*p ≤ 0.0001*)

*C. coriaceum* pulp and peel extracts modulate iron pool of infected macrophages

Still aiming to understand the parasites death pathway, we investigated the levels of iron and total binding iron capacity. The results showed that the treatment with pulp and peel extracts did not alter the total iron concentration of *L. amazonensis*-infected macrophages (Figure 5A) (*p ≥ 0.2*) but increased iron bound to transferrin (Figure 5B) when compared to control (*p ≤ 0.001*), decreasing the labile bioavailability of iron. The higher increase in total iron bound capacity was verified in peel treatment (*p ≤ 0.001*).
Figure 6 — Total iron concentration and total bound iron in *L. amazonensis*-infected macrophages treated with pulp and peel *C. coriaceum* extracts. *L. amazonensis*-infected macrophages treated or not with 25 µg/mL of pulp and peel extracts for 24h. Iron concentration (A), total iron bound capacity (B). Data represent mean ± SEM of three independent experiments. ** Significant difference compared to control (p ≤ 0.01), *** (p ≤ 0.001).

**C. coriaceum** fruit extracts induces Ferritin, HO-1 and Nrf2 expression

Knowing that the extracts treatment induced an antioxidant action and concomitant increase of iron bound to transferrin, we investigated the cascade involving the ferritin protein, the HO-1 enzyme and the transcription factor Nrf2. Our results showed that both pulp and peel extracts were able to up-regulate the expression of the three genes investigated (ferritin, HO-1 and Nrf2) when compared to the untreated control (p ≤ 0.01). In addition, it was observed that the pulp extract caused more pronounced effects than the peel extract in the expression analysis of HO-1 and Nrf2 (p ≤ 0.01).
Figure 7 - Relative mRNA expression levels of *L. amazonensis*-infected macrophages treated with pulp and peel *C. coriaceum* extracts. Real-time RT-PCR quantitative mRNA analyses were performed to quantify the ferritin, HO-1 and Nrf2 expression. Uninfected macrophages were used as reference (dashed line). Data represent mean ± SEM of three independent experiments. ** Significant difference compared to control or between treatments when indicated (p ≤ 0.01), *** (p ≤ 0.001), **** (p ≤ 0.0001).

**DISCUSSION**

In the past few decades, the emergence of limitations in the use of synthetic drugs such as high toxicity, side effects and high costs caused an increased interest in the ethnobotanical research (20–22). In the case of leishmaniasis, current therapy can provoke serious side effects such as hepatotoxicity, nephrotoxicity, cardiotoxicity (5), which arouses interest in natural compounds, targeting a minor damage to the patient. Recent studies, by our group and other researchers, have shown that extracts from leaves of *Caryocar* genus plants act on different microorganisms, including *Leishmania amazonensis* (7,8,13). Our group also showed an inhibitory effect pulp and peel *C. coriaceum* extracts on promastigote forms of *Leishmania amazonensis* (13), however, until this now nothing was known about the possible action mechanisms of these extracts on the extra and intracellular parasite.

In the present work we showed that the range of 25 to 100 μg/mL of the *C. coriaceum* ethanol extracts acts in *L. amazonensis* promastigotes, corroborating with our
previous study, where we defined an IC₅₀ of 30 (±5) and 38 (±13) µg/mL, for pulp and peel extracts respectively (13). In addition, we demonstrated that the extracts were able to induce the depolarization of the mitochondrial membrane of the promastigote forms. The mitochondrial integrity is essential for parasite survival, once trypanosomatids have a single mitochondrion (23,24). The mitochondria is an important source of ROS, molecules with different cellular functions, that when in excess can give the start to the cellular death (23–25).

Knowing that mitochondrial dysfunction and the production of reactive oxygen species can lead to cell death by apoptosis, we investigated this process of death by annexin V labeling. We found that, similar to the effects of extracts leaves (7), the fruit extracts are able to increase both, annexin V and PI marking.

To differentiate the death type in necrotic, apoptotic and late-apoptotic, we performed the co-staining analyzed by flow cytometry. We verified that most of the parasites were in the double-positive zone, indicating that the late-apoptotic process was predominant. This death type was previous described in Leishmania parasites treated with different compounds (7,15,16,26,27). These results are reinforced by the morphological changes found in the parasite, since bleb formation and cellular shrinkage are typical signs of apoptotic death (28).

In the next experiments set, we aimed to elucidate whether the fruit extracts would be able to act in intracellular amastigote forms, which are more resistant and a challenging target, because the compound needs to diffuse through the host cell structures, to act on the parasite (29).

First, we proved that the pulp and peel *C. coriaceum* extracts were not toxic to the macrophages, the major host cell for *Leishmania*. Our data are in accordance to previous results, that showed and selectivity index (macrophage/ promastigote forms) of approximately 9 and 12 to pulp and peel, respectively (13).

After, we demonstrated that the extracts were capable to improve the elimination of intracellular amastigotes. This effect was similar to those found to *C. coriaceum* leaves extracts (7). Then, we inquire which mechanisms are involved in this elimination.

It is known that the main effector molecules in the intracellular pathogens elimination are reactive nitrogen species (RNS) and reactive oxygen species (ROS) (4,30), on the other hand, RNS and ROS also responsible for the tissue damage in leishmaniasis (31,32). Given the importance of these molecules in the infection course, we investigated their
effects in the studied model. Surprisingly, both ROS and RNS had decreased levels in the presence of treatment.

This action is in accordance with our previous results that showed an antioxidant effect of the extracts of *C. coriaceum* fruit in the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (13). In addition, antioxidant capacity is intrinsically related to the presence of phenols and flavonoids, as isouercitrin, quercetin and rutin, which are also present in the studied extracts (13).

[Figura em anexo no email]

Figure. Chemical structures of isouercitrin, quercetin and rutin with the chelating sites evidenced by dotted circles.

Isoquercitrin, quercetin and rutin are widely known as natural antioxidants in literature. We can observe in figure X that these compounds present an α-dihydroxy structure in the B ring, and 3- and 5-OH groups in conjugation with the 4-oxo function. These molecules structure generates at least two possibilities for the coordination with metal ions. All these characteristics contribute for the high radical scavenging potential and metal quelating properties.

Once we discarded the hypothesis of being the oxidative stress responsible for the elimination of the parasite, we investigated the iron metabolism. *Leishmania* parasites uses the labile iron pool from host and depend on the uptake of this metal from for its replication and survival (2,3). As Fe⁵⁺ usually act as prooxidant, these compounds in the extracts probably chelate these ions leading to a low intracellular concentration of iron, which disrupt the parasite metabolism. This means that the use of *C. coriaceum* extracts may facilitates the Fenton reaction. Also it is known that the presence of these flavonoids nearby the surface of the phospholipid structures are ideally for the removal of free radicals. Thus, the increase in transferrin binding, caused by *C. coriaceum* fruit extracts treatment, made iron unavailable to the parasite, leading to a reduction in the rate of macrophage infection.

To confirm this reasoning, we verified the cascade involving Nrf2/HO-1/ferritin complexes, since they are directly related to both the antioxidant capacity and iron metabolism (33). While on the one hand the activation of Nrf2 may be a weapon of *L. amazonensis* to block oxidative stress and persist in the host (34), on the other hand, it can induce transcription of downstream genes, such as HO-1, which acts in iron metabolism by inducing ferritin expression. Finally, ferritin plays an important role in the sequestration of free iron, reducing the availability of this metal to intracellular parasites
(33,35). The overall antioxidant balance can still be a powerful ally in protecting against tissue injury caused by leishmaniasis (36).

A previous study of our group showed that methanolic and ethanolic extracts of *C. coriaceum* leaves are also able to up-regulate the transcription of Nrf2, HO-1 and ferritin genes. However, when oxidative mediators were evaluated, fruit and peel extracts appeared to be more effective, as they reduced MDA and ROS levels more effectively, as well as being able to reduce NO formation, whereas leaf extracts did not influence the production of this mediator (12).

**CONCLUSION**

Taking together our data showed that extracts from the *C. coriaceum* fruit, probably due their major compounds, can induce death of *L. amazonensis* promastigotes by apoptose-like mechanisms and to act on intracellular amastigote forms by activating the Nrf2/ HO-1/ ferritin pathway, and consecutively reducing the availability of iron for the survival of the parasite. In addition, the treatment induced an antioxidant response, important in protecting against tissue damage that occurs in leishmaniasis.

**REFERENCES**


In vitro leishmanicidal and fungicidal activities of endophytic fungal enzyme extracts

(Atividades leishmanicida e fungicida in vitro de extratos de enzimas fúngicas endofíticas)


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In vitro leishmanicidal and fungicidal activities of endophytic fungal enzyme extracts

Daniela Ribeiro Alves¹,², Selene Maia de Morais¹*, Fernanda Nogueira Tomiootto Pelissier³, Fábio Roger Vasconcelos₄, Isaac Neto Goes da Silva¹, Alan Henrique Depieri Cataneo³, Milena Menegazzo Miranda⁴, Gustavo Adolfo Saavedra Pinto², Wander Rogério Pavanelli³, Ivete Conchon-Costa³, Arlindo de Alencar Araripe Noronha⁴, Francisco das Chagas Oliveira Freire².

¹ Veterinarian Sciences Post Graduation Program, State University of Ceará, Av. Dr. Silas Munguba, 1700, Campus Itaperi, Fortaleza, Ceará, Brazil
² Brazilian Agricultural Research Corporation (EMBRAPA) - Tropical Agroindustry, Rua Sara Mesquita n 2270 Planalto do Pici, Fortaleza, Ceará, Brazil
³ Pathological Sciences, State University of Londrina; Rodovia Celso Garcia Cid, Pr. 445, Km 380, Campus Universitário, Londrina, Paraná, Brazil.
⁴ Animal Physiology Laboratory, Department of Animal Science, Federal University of Ceará, Fortaleza, Brazil

Author to whom correspondence should be addressed:
selenemaiademorais@gmail.com*; Tel.: +55-85-988938523

Abstract

This work describes the production of lipases from endophytic fungi Vermisporium-like, Emericella nidulans, Dichotomosphora portulaeae and D. boerhaviae and determination of biological potential evaluating the activity against the dermatophytes Malassezia sp and Microsporum canis, the parasite Leishmania amazonensis and macrophages derived from bone barrow murine cells. All fungal enzymes extract showed lipolysis action. The proteomic analysis of fungal enzymes exhibits several molecules mostly ranging in size from 220 to 20 kDa with clear differences in protein profile’s yield. Dichotomosphora enzymes demonstrate best fungicidal action with MFC of 14.65 µg.mL⁻¹. All fungal enzymes were effective against promastigote form of Leishmania amazonensis at 5 mg.mL⁻¹. The effect of fungal lipases of Vermisporium-like, E. nidulans, D. portulaeae and D. boerhaviae against amastigote forms, at the same concentration, caused a decrease respectively of 78.88, 39.65, 63.17 and 98.13% of infected macrophages. The current results lead to a selectivity index of 19.56, 30.68, 18.09 and 20.99 respectively, demonstrating the potential of these fungal enzyme extracts against dermal pathogens.

Keywords: leishmanicidal; antifungal; antioxidant; acetylcholinesterase
inhibitors; fungal enzymes

Introduction

Leishmaniasis and fungal dermatophytosis are common diseases in veterinary clinics. Complementary and alternative veterinary medicine includes plant natural products for the healing of animals committed of such diseases (SANTOS et al., 2013; SILVA et al., 2015). Usually, plants are more often reported as remedies nevertheless a new approach is envisaged in this work on the use of endophytic fungal enzymes in the control of parasites and fungal dermatophytes (DE LIMA et al., 2014; SOARES et al., 2015a).

Leishmaniasis is a zoonotic disease transmitted by protozoa of the genus *Leishmania*, which are intracellular pathogens that infect macrophages, neutrophils, and dendritic cells. These pathogens are endemic to reach about 98 countries and putting at risk 350 million people. *Leishmania* spp. can induce two different forms of the disease, the visceral form, and the cutaneous/mucocutaneous form (WORLD HEALTH ORGANIZATION, 2010). There is a lack of efficient drugs to treat this disease and the major Brazilian governmental program is eliminating ill animals (BRASIL, 2013c).

Another health problem, which commits domestic animals, are skin pathogens and *Microsporum, Malassezia* and *Trichophyton* represent the most prevalent and may infect the home environment. Traditionally the options for fungal treatment are the azoderivatives but in the same way that with *Leishmania* spp. presents a high cytotoxicity.

The fungal cell membranes are the most important targets for the development of drugs (LOPES et al., 2013). The therapeutic properties of many fungi metabolites lead to the search of active compounds produced from various species. Endophytic fungi are those identified within plant systems, which were previously considered sterile environments. These are essential for biological control in the production of secondary metabolites, changes in animals, genetic vectors, thus having great potential for exploitation. It is believed that oleaginous plant endophytic fungi can be a potential source of lipases.

Current biodiversity in the Northeastern Brazil has a latent potential through the genetic heritage that it has to offer as a promising source of bioactive metabolites of pharmacological importance. This work aims to contribute to the discovery of leishmanicidal and fungicidal products from endophytic fungal enzyme extracts to fulfill the current demand.
The identification of the toxicological activities of fungal enzyme extracts on pathogens, is a contribution to intervention methods, altering the metabolism of the organism and so preventing infection by protozoa and toxic fungi and interrupting the course of correspondent diseases.

Material and Methods

Fungi isolation

Fresh seeds of *Jatropha curcas* L. were acquired at Beckman seeds®, Fortaleza, the state of Ceará. 5 (five) seeds with or without disinfestation were added to 30 (thirty) Potato Dextrose Agar (PDA) disposable plates. After three (3) days, strains of *Vermisporium*-like and *Emericella nidulans* were isolated, identified using spore morphology and submitted to microorganism collection from Brazilian Agricultural Research Corporation (EMBRAPA®) - Tropical Agroindustry under No CMIAT232 and CMIAT233, respectively. *Dichatomosphthora boerhaaviae* (CMIAT 235) was obtained from EMBRAPA endophytic fungi collection and strains of *Dichatomosphthora portulacae* (CBS 149.94) was obtained from CBS® Fungal Biodiversity Centre collection.

Animal welfare

The ethics committee of Londrina State University approved the protocol for animal use (162/2016).

Enzymatic activity plate assay - Determination of Hydrolases

After 5 days of culture, pellets with 6.5 mm in diameter of each fungus was inoculated in an enzymatic media, which contained as an inductor of enzyme activity 1% Tween 20 (T20) (v/v); or 1% Tween 80 (T80) (v/v). The medium compounds for “T20” and “T80” solution’s preparation were peptone 6.0 g.L⁻¹, NaCl 3.0 g.L⁻¹, CaCl₂·2H₂O 0.06 g.L⁻¹ and Agar 10.8 g.L⁻¹, pH 7.4 (BUSSAMARA et al., 2010; LI; CHENG; CHEN, 2012).

The medium “W”, as called by the author (COLENI; JUNQUEIRA; MORAESSANTOS, 2006), contained: 5.0 g.L⁻¹ (NH₄)₂SO₄, 2.0 g.L⁻¹ (NH₄)₂CO, 1.0 g.L⁻¹ MgSO₄·7H₂O, 1.0 g.L⁻¹ NaCl, 15.0 g.L⁻¹ bacteriological agar, 10.0 mL of ethanolic solution of 0.1% Victoria blue B, 15.0 g.L⁻¹ olive oil.
Medium “K” was different from the others due to the inclusion of potassium ion, as proposed by Kamimura et al. (KAMIMURA et al., 1999). This modified medium is composed by: 1.0 g L\(^{-1}\) Yeast extract, 1.0 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.5 g L\(^{-1}\) MgSO\(_4\).7H\(_2\)O, 1.0 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) and 20.0 g L\(^{-1}\) bacteriological agar.

All the media was sterilized at 121 °C for 15 minutes and the medium “K” was cooled to 60 °C and then the following compounds were added: 10.0 g L\(^{-1}\) of egg lecithin, CaCl\(_2\) 100.0 mM, and 2.0 mL of aqueous solution 0.1% Rhodamine B (previously sterilized by filtration through 0.22 μm syringe filter). Each media was emulsified with intense shaking by 10 minutes and then 20.0 mL distributed in each 90 mm diameter Petri plates.

The fungi were inoculated in the center of Petri dishes with the aid of a sterile loop. These plates were maintained in a bacteriological incubator for 10 days at 27 °C. The enzymatic activity of fungi was observed through the formation of precipitate crystals of calcium salts or the appearance of clear different color halos around the colonies, using stereomicroscope and microscope. Daily growths were observed and measured the size of the colony and the activity halo, when present. The bacterial strains *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as a positive control in the detection of lipase’s presence test.

**Enzymes extraction assay**

After 5 days of culture of each fungus were selected and inoculated 5 pellets 6.5 mm in diameter in Erlenmeyer with 250 mL of “W” media composition without agar and pH adjusted to 8.0. The flasks were then incubated at 30 °C for 7 days. It was established that *Vermisporium*-like crude extract corresponds to fungal enzyme “A”, *E. nidulans* crude extract to “B”, *D. portulaceae* crude extract to “C” and *D. boerhaaviae* crude extract to “D”. After the incubation period, the mycelial mass was separated by filtration through Whatman filter paper # 1 (KAMIMURA et al., 1999). The filtrates were lyophilized for later dilution at a concentration of 2.0 mg mL\(^{-1}\) and used as enzymatic extracts for analysis. Three bottles were inoculated for each strain.

**Enzymatic Index (EI)**

The potential lipolytic activity, for the fungi assay, was measured using the
Enzymatic Index. This index described by Hankin and Anagnostakis (HANKIN; ANAGNOSTAKIS, 1975) it was determined by the colony diameter ratio over the halo diameter of calcium crystals, after 03 days of subculture. Also, for the extract assay activity can be determined by the well diameter radio by the well diameter radio over the halo after 1 hour of activity. If the EI content is greater than 1, the better the production of the enzyme of interest.

**Total Protein quantification and 1D SDS-PAGE**

The Bradford assay (BRADFORD, 1976) was carried out to determine the concentration of solubilized protein using Bradford reagent (Sigma-Aldrich, St Louis, MO) using bovine serum albumin (BSA) as a standard and the extracts kept at -80°C. A protein standard (BSA) was prepared in seven dilutions. The range of the protein assay was between 0 and 2.0 mg.mL^-1. Determination of protein concentration was performed in triplicate. Spectrophotometer Reader Nanodrop 2000 (Thermo-Fisher, Waltham, EUA) was used to measure the absorbance at 595 nm.

The crude extracts were lyophilized and suspended in 2 mL of distilled water. Five volumes of ice-cold acetone were added to the crude extracts overnight at -20°C. After centrifugation at 14000 rpm for 30 min at 4°C, the collected pellets were dialyzed, further lyophilized for 30 min and suspended in in sample buffer (7 M urea, 2 M thiourea, 4% 3-
[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS), 2% free ampholytes [IPG buffer, pH 4–7 (GE LifeSciences)], 40 mM dithiothreitol (DTT) to 25 mL distilled (H2O). Insoluble material was removed by centrifugation. Proteins extracts were evaluated according to (i) the diversity of bands; (ii) the amount of protein extracted as well as (iii) the integrity of samples. Approximately 10 μg of protein preparations were resolved by one-dimensional electrophoresis on a denatured dodecyl-sulfate-polyacrylamide gel (12% 1D-SDS-PAGE). Staining was done by using coomassie brilliant blue G250 (sigma) (BURNIE et al., 1989).

**Lipolytic activity determination of extracts**

The lipase presence determination was based on the determination of the capacity of the extracts to induce lipolysis in the media that contain long carbon chains. The crude extracts were first submitted to diffusion into K and W agar gel. After agar solidification, wells of 6.5 mm diameter were prepared and 20 μL of samples were added to each well
in triplicate and incubated at 30 °C for 18 h. Lipolytic activity was identified by a light blue color halo, for the W agar and a light halo with at least one color or more for the K agar. The El was measured and compared for each sample in both media (COLEN; JUNQUEIRA; MORAES-SANTOS, 2006).

**Fungicidal Assay**

The minimum concentration capable of inhibiting 100% fungi growth (MIC100) was determined by the dilution technique, according to CLSI method (CLINICAL AND LABORATORY STANDARDS INSTITUTE, 2008). Six strains were tested (3 *Malassezia* sp. and 3 *Microsporum canis*), isolated from infected domestic animals, identified and stocked at the Microbiology section and kindly donated by VETTINGS®.

The spore suspension solution for the initial inoculation was prepared from filamentous fungi cultivated on Potato Dextrose Agar (PDA) and incubated at a temperature at 28±2°C for 7 days. The spore count was performed in a Neubauer chamber to achieve the concentration of 10^5 to 10^6 cells.

In laminar flow cabinet, 100.0 μL of RPMI medium were distributed into each well of a 96-well microplate. 100.0 mL extract were added and serial dilutions performed from 2500.0 to 2.44 μg.mL⁻¹. Well intervals were prepared to evaluate the sterility control of the medium; to control of fungal growth; by testing the fungicide (TECTO® RC Thiabendazole; Syngenta®), the fungicide concentration was in accordance with manufacturer instructions (4.0 mg.L⁻¹). Finally, 50.0 μL of the fungal suspension were added to all wells except the lines intended for the control of the sterile medium.

The readings were taken by checking the MIC100, with the aid of a stereoscopic checking the lowest concentration of the samples capable of inhibiting the growth of the microorganism, after 5 days of incubation. The plates were also inspected under an inverted microscope to assure growth of the controls and sterile conditions.

The Minimal fungicide concentration (MFC100), as considered as minimum concentration capable of killing 100% fungi, were measured by transfer 50.0 μL from wells without fungal growth and inoculate on PDA. MFC100 was established according to the fungus growth after incubation under the same conditions for 5 days.

**Leishmania Parasite**

*Leishmania (Leishmania) amazonensis* (MHOM/BR/1989/166MJO) was used in
promastigote forms, in the stationary growth phase (day 5 of culture). The parasites were obtained from popliteal lymph nodes of *L. amazonensis*-infected BALB/c mice and maintained in 199 culture medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 1M HEPES Biological Buffer (AMRESCO), 1% human urine, 1% L-glutamine (SYNTH), penicillin (10 U.mL\(^{-1}\)) and streptomycin (10 \(\mu\)g.mL\(^{-1}\)) (GIBCO) and 10% sodium bicarbonate (SYNTH). Cell cultures were incubated at 25°C in 25-cm\(^2\) flasks. All parasites were from a culture that was serially passed for less than 5 weeks.

**Viability of *L. amazonensis*-promastigote forms**

The direct effect of fungal extracts against *L. amazonensis* was performed in 24-well microtiter plates, each well containing 1000 µl of 199 supplemented culture medium with 1 x 10\(^6\) promastigote forms in stationary phase with or without the fungal extracts of interest (A, B, C and D) at final concentration of 5, 2.5, 1.25 and 0.625 mg.mL\(^{-1}\). Viable promastigote concentration was determined in a CASY model TT cell counter and analyzing system (ROCHE) after 24h of treatment. In the stock solutions of fungal extracts, 1% dimethyl sulfoxide (DMSO) (GIBCO) was used as a vehicle. However, DMSO concentration did not exceed 0.5% in all experiments. Untreated parasites and vehicle only (0.5% DMSO) were included as negative controls. The plates were also inspected under an inverted microscope to assure growth of the controls and sterile conditions.

**Bone marrow-derived macrophages (BMDMs)**

Bone marrow-derived macrophages were obtained as previously described by Modolell and Munder (MODELELL; MUNDER, 1994). Briefly, BALB/c mice were euthanized and femur and tibia bones were dissected out. Bones were trimmed at each end, and marrow was flushed out with 3.0 mL of Dulbecco’s Modified Eagle Medium-F12 (DMEM-F12) (SIGMA) containing 100 U.mL\(^{-1}\) penicillin (GIBCO) supplemented with 10% FBS. Bone marrow was dispersed and cultured in 6-well microtiter plates at 37°C and 5% CO\(_2\) in DMEM-F12 supplemented with 30% of L-929 fibroblasts culture supernatant.

**BMDMs viability assay**

The viability of BMDMs treated with fungal extracts was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously
described by Mosmann (1983). BMDMs (5 × 10^5 U.ml⁻¹) were cultured in 24-well plates and preincubated with 200 μL of DMEM/F12 medium for 2 h for adherence at 37°C and 5% CO₂. The cells were washed with PBS and then, adherent cells were incubated with different concentrations of fungal extracts A, B, C and D (5, 20 and 50 mg.ml⁻¹) or with vehicle (0.5% DMSO) and maintained in culture for 24 h at 37°C and 5% CO₂. After incubation with extracts, the BMDMs culture was washed with PBS and added MTT at a final concentration of 5 μg.ml⁻¹ in each well, followed by incubation for an additional 4 h at 37 °C/ 5% CO₂. The MTT formazan product was solubilized with DMSO, plates were read at 570 nm in a spectrophotometer. The results were expressed as a percentage of MTT reduction relative to the control group calculated as the following formula: (viable macrophages)% = (OD of drug-treated sample/OD of the untreated sample) x 100.

Selectivity index (SI)

The effects of fungal extracts on *L. amazonensis* promastigote forms and BMDMs cytotoxicity were expressed as the percentage of viable cells compared to control, and the 50% inhibitory concentration (IC₅₀) was calculated by non-linear regression (GraphPad Software, v. 5.00). The degree of selectivity of fungal extracts was expressed as SI = IC₅₀ of fungal extracts on BMDMs/IC₅₀ of the same fungal extracts on promastigotes. Higher SI reveals greater extract’s selectivity up the parasite, rather the host. The experiments were tested in triplicate.

Phagocytic assay

BMDMs (5 × 10^5 U.ml⁻¹) were cultured in 24 well plates containing 13.0 mm diameter glass coverslips and incubated with 200 μL of DMEN F-12 medium for 2 h for adherence at 37 °C/ 5% CO₂. Adherent macrophages were infected with promastigote forms (10:1) for 2 h. After infection, free promastigotes were removed by washing with PBS, and the infected cells were treated with A, B, C and D fungal extracts (5.0 mg.ml⁻¹) and incubated for 24 h at 37°C and 5% CO₂(MOSMANN, 1983). DMEM F-12 medium was used as a control. *L. amazonensis*-infected BMDMs were stained with Giemsa and 20 fields analyzed using a light microscope (magnification of 1000x, OLYMPUS, model CX31RBSFA) to determine the phagocytic index by % of infection and the number of parasites/macrophage.
Statistical Analysis

Analyses were performed as triplicate data sets, from 3 independent experiments. Results were analyzed as a mean ± standard deviation. Data were analyzed by ANOVA (t-Student), followed by Tukey’s test for multiple comparisons, using the software Prism GraphPad (GraphPad Software, v. 5.00). Differences were considered statistically significant at p<0.01.

Results and Discussion

The long chain of oleic acid is present in the group of ceramides that are current on the plasma membrane and play the main role of many cellular events. As the maintenance and virulence of Leishmania spp. is directly depending on the presence of these carbon chains, in the parasites’ membrane (ALI, 2014) and in the host (MAJUMDER et al., 2012), changes in control of these ceramides in Leishmania spp. seems to be a promising target to kill this parasite.

The enzyme extract of Vermisporium-like corresponds to fungal enzymes “A”, E. nidulans to “B”, D. portulacea to “C” and D. boerhaaviae to “D”. The proteomic analysis exhibits several molecules mostly ranging in size from 220 to 20 kDa with clear differences in protein profile’s yield of the A, B, C and D samples as seen in Figure 1.

Figure 1 – Electrophoretic protein patterns of standard markers (S), D. portulacea (1), Vermisporium-like (2), D. boerhaaviae (3) and E. nidulans (4) extracts, respectively, by SDS-PAGE method with 12% separating gel.
The growth of the endophytic fungi in Tween 20 and Tween 80 media with the production of enzymes is indicated by the calcium deposition. The fungal enzymes A, B, C and D are good producers of hydrolases as seen in Table 1. It was found at least two groups of enzymes produced by these fungi since each media contain different length carbon chains that lead to different promoter sites of reaction.

### Table 1. Enzymatic Index of extracts

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Fungi growth analysis</th>
<th>Crude extract analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T20</td>
<td>T80</td>
</tr>
<tr>
<td>A</td>
<td>$1.53^\pm 0.11$</td>
<td>$1.68^\pm 0.12$</td>
</tr>
<tr>
<td>B</td>
<td>$1.23^\pm 0.20$</td>
<td>$1.77^\pm 0.20$</td>
</tr>
<tr>
<td>C</td>
<td>$1.21^\pm 0.10$</td>
<td>$1.75^\pm 0.21$</td>
</tr>
<tr>
<td>D</td>
<td>$1.26^\pm 0.21$</td>
<td>$1.65^\pm 0.11$</td>
</tr>
</tbody>
</table>

Similar lower case letters indicate significant similarities between rows, which means sample similarities between media reactivity in different analysis. ($p < 0.001$, ANOVA followed by Tukey test). Fungal enzyme extracts of *Vermisporium-like* corresponds to "A", of *E. nidulans* to "B", of *D. portulaceae* to "C" and of *D. boerhaavieae* to "D".

The fungal growth produced enzymes more able to react with a high carbon chain (T80), then adapt to react with a low carbon chain (T20). Tween 20 has a chain of C_{12} characterized as lauric acid and Tween 80 has a C_{18} characterized as oleic acid. The similarity between the high carbon chain (T80) and lecithin carbon chains was demonstrated to be essential for the development of the assay.

The presence of the enzymes that react with "T80" and "K" media was evidently superior to another produced enzymes, as they are great suppliers of C_{18} chains. The El values for all fungal crude extracts showed greater potential for the hydrolysis of ceramides, leading to the fungicidal activities displayed in Table 2.
### Table 2. Fungicidal activities of fungi extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Malassezia sp.</th>
<th>M. canis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC / MFC</td>
<td>MIC / MFC</td>
</tr>
<tr>
<td>A</td>
<td>4.88 ± 0.06 / 78.25 ± 0.88</td>
<td>4.88 ± 0.06 / 19.53 ± 1.08</td>
</tr>
<tr>
<td>B</td>
<td>4.88 ± 0.13 / 156.25 ± 0.18</td>
<td>4.88 ± 0.01 / 19.53 ± 1.79</td>
</tr>
<tr>
<td>C</td>
<td>4.88 ± 0.06 / 14.65 ± 6.91</td>
<td>4.88 ± 0.06 / 43.94 ± 3.45</td>
</tr>
<tr>
<td>D</td>
<td>4.88 ± 0.01 / 644.53 ± 5.96</td>
<td>4.88 ± 0.03 / 14.65 ± 6.91</td>
</tr>
</tbody>
</table>

Similar lower case letters indicate significant similarities between rows and upper case letters indicate significant similarities between columns (p < 0.001, ANOVA followed by Tukey test). Fungal enzyme extracts of *Vermisporium-like* corresponds to “A”, of *E. nidulans* to “B”, of *D. portulaceae* to “C” and of *D. boerhaaviae* to “D”.

The antifungal results observed in this study demonstrated that the crude extracts behave similarly with respect to their inhibitory activities (Table 2) at a concentration of 4.88 µg.ml⁻¹. Regarding the fungicidal activities, it was observed that extracts from *Dichotomophthora* species showed the better results against skin diseases of animal pathogens. The “C” crude extract was effective against *Malassezia* sp. and “D” crude extract was effective against *M. canis* at a concentration of 14.65 µg.ml⁻¹.

Holetz et al. (HOLETZ et al., 2002) affirm IC₅₀ values below 100 µg.ml⁻¹ indicates a good antimicrobial activity and above 500 weak activity, and between these values the activity is considered moderate. Weak activity denotes not relevant for use in pharmaceutical treating of fungal infections.

Using these IC₅₀ parameters the extract “A” also had a good antifungal action against *Malassezia* sp. with a medium concentration of 78.25 µg.ml⁻¹. The extract “B” has a moderate activity at a concentration of 156.25 µg.ml⁻¹. The “D” extract demonstrated a weak activity with IC₅₀ equal 644.53 µg.ml⁻¹.

With regard to fungicidal capacity against *M. canis*, all the extracts demonstrated good activity. The “A” and “B” extracts with an IC₅₀ of 19.53 µg.ml⁻¹ and the “C” extract at a concentration with an IC₅₀ of 43.94 µg.ml⁻¹.

As the most dermatophytes demonstrate lipid-addict, during an infection there is a great expression of pathogen’s lipases (ZAHUR et al., 2014). Besides the mechanism of action of several compounds during the disease caused by a dermal pathogen remains
unknown (LOPES et al., 2013) making the lipase an effective target, as observed in this work.

The use of external lipases, produced by another microorganism, can be a way to disrupt the course of infection, by causing a gradual decline of pathogen homeostasis, toward disorder or lack of pathogen systematic reactions.

In the same way, *Leishmania* spp. are reported as capable of developing cutaneous and mucocutaneous forms (WORLD HEALTH ORGANIZATION, 2010). Similarly, it is known that the leishmanial infection is directly dependent on the lipases action by the membrane’s maintenance mechanism with the rafts synthesis and disruption. The production of extracellular phospholipases increases the virulence of *Leishmania* by stimulation of phosphatidylcholine membrane rafts disruption (LECOEUR et al., 2013) leading to the differentiation between of amastigote and promastigote forms.

The maturation of the promastigote form to amastigote is through the transformation of the parasite since it is highly adapted to survive in phagolysosomes, that are rich in triglyceride phospholipids and other lipolytic bioproducts (ROSENZWEIG et al., 2007). This mechanism leads the lipase production and the course of the infection in macrophages and neutrophils, allowing the entrance of pathogen toward the defense cells.

In the first set of experiments, the antileishmanial effect of fungi crude extracts was investigated against the promastigote forms of *L. amazonensis*. All the extracts prevented cell proliferation using 5 mg/mL for 24 h (*p*<0.0001) (Figure 2), showing the equal antiproliferative effect of standard Glucantine (250 µg/mL) after 24h. After 96 and 168 h, all the extracts in all concentrations reduced cell proliferation (*p*<0.0001).
Figure 2 – Effect of fungal extracts on *L. amazonensis* promastigote growth and BMDMs viability. (A) Growth kinetics of *L. amazonensis* promastigote forms (1 x 10^6) after treatment with fungal extracts (5mg.mL^-1) or vehicle for 24h. (B) BMDMs (5 x 10^5/well) were plated in 24-well plates, treated with fungal extract (5mg.mL^-1) or vehicle and incubated for 24h at 37°C and 5% CO₂. DMEM F-12 medium was used as positive control. Data represent the mean ± standard deviation of three independent experiments. *** Significantly different from control (p < 0.0001, ANOVA followed by Tukey test). Fungal enzyme extracts of *Vermisporium-like* corresponds to “A”, of *E. nidulans* to “B”, of *D. portulacea* to “C” and of *D. boerhaaviae* to “D”

Cutaneous leishmaniasis present lacks of specificity and high costs in the treatment, that leads the intervention to be based by the ratio risk-benefit for each patient (MIRANDA et al., 2015b). Finding new drugs that are selective to *Leishmania* spp. and do not attack the host is the main patch to establish a new protocol of intervention.

Searching for new intervention protocol, tests were performed to verify the integrity of the BMDMs before and after the presence of the fungi extracts to evaluate their selectivity potential. Bone marrow was then dispersed and cultured. At the fifth day of culture were added 3.0 mL of L-929 supernatant to each well to monocyte/macrophage differentiation and at the seventh day of culture, most cells were positive for CD11b and F4/80, a monocyte/macrophage marker (Figure 3).
Figure 3 - Flow cytometry characterization of bone marrow-derived macrophages (BMDM) Cells were isolated from BALB/c bone marrow and cultured as described (n=5 mice). Upon reaching optimal differentiation, macrophages were stained with CD11b-PECy7 and F4/80-PE and analyzed by flow cytometry. Results are representative of three independent experiments.

After detecting that BMDMs cells reached optimal differentiation to macrophages, all crude extracts was tested at concentrations of 50, 20, and 5 mg.mL⁻¹ (Figure 4). All fungi crude extracts at a concentration of 5 mg.mL⁻¹ do not reduce the macrophage viability (Figure 2 and 4). The crude extract "C" also does not show a significant difference from the Control at a concentration of 20 mg.mL⁻¹.
Figure 4 – Effect of fungal extracts on BMDMs viability. BMDMs (5 x 10^5 per well) were plated in 24-well plates, treated with fungal extract (50, 20 and 5 mg.mL⁻¹) or vehicle and incubated for 24h at 37°C and 5% CO₂. DMEM F-12 medium was used as positive control. Data represent the mean ± standard deviation of three independent experiments. *** Significantly equal to control (p < 0.0001, ANOVA according to Tukey test). Fungal enzyme extracts of*Vermisporium-like* corresponds to “A”, of *E. nidulans* to “B”, of *D. portulaceae* to “C” and of *D. boerhaaviae* to “D”.

These tests result for fungal enzymes “A”, “B”, “C” and “D” respective IC₅₀ values of 1.389, 1.120, 1.151 and 0.834 mg.mL⁻¹. If they were compared to plant extracts, all fungi extracts demonstrate medium to low activity, as example *Musa paradisiaca* and *Spondias mombin* whose IC₅₀ values varied from 1.70 to 915 μg.mL⁻¹ (ACCIOLY et al., 2012). Therefore, a lower concentration (5 mg.mL⁻¹) was chosen for testing the 24 h treatment of infected macrophages, since the same concentration demonstrated pharmacological results in promastigote forms, in order to obtain the Selectivity Index (SI).

Then, the amastigote infected-BMDM’s cells were treated with the fungal extracts (A, B, C and D) at a concentration of 5.0 mg.mL⁻¹ for 24 h. In order to establish the phagocytic index, macrophages and amastigotes were scored, indicating the number of parasites per macrophage as the extent of infection (Figure 5).
Figure 5 – Effect of fungal extracts on *L. amazonensis*-infected BMDMs. BMDMs were infected with *L. amazonensis* and treated with fungal extracts (5mg.mL⁻¹) or vehicle for 24h at 37°C and 5%CO₂. (A) Percentage of infected macrophages; (B) a mean number of amastigotes per macrophage. (C) Representative frame images from the effect of each fungal extract on *L. amazonensis*-infected BMDMs, where DMEM F-12 was used as a control, *Vermisporium*-like crude extract as “A”, *E. nidulans* crude extract as “B”, *D. portulaceae* crude extract as “C” and *D. boerhaaviae* crude extract as “D”. Data represent the mean ± standard deviation of three independent experiments. **p<0.001, ***p<0.0001 Significantly different from control, according to Tukey test.

Although of “B” crude extract had been slightly higher than fifty percent, concerning the percentage of infected macrophages, all the extracts were significantly different from control with p<0.0001. These results demonstrate that the enzymes were not only capable of entering into the macrophages. The enzymes also present in crude extracts were capable of acting directly in the course of infection of *L. amazonensis* causing decreases of 78.88, 39.65, 63.17 and 98.13 % of infected macrophages, respectively (Figure 5).

Besides, not only the number or macrophages was reduced but also the intensity of the infection per BMDMs. Even with mean values of “B” crude extract being greater than two units of amastigotes per macrophage, the crude extracts were capable of reducing the number of parasites.
Moreover, all results of antileishmanial and cytotoxic effects of fungal extracts were confronted to each other, by the Selectivity Index score (Table 3). Their IC₅₀ values were evaluated against promastigotes of *L. amazonensis* and BMDMs. The lower and better IC₅₀ values were from “D” crude extract showed a result of 20.985 for SI.

**Table 3. Leishmanicidal and cytotoxic effects of fungal extracts**

<table>
<thead>
<tr>
<th>Fungal Extracts</th>
<th><em>L. amazonensis</em> promastigote forms</th>
<th>BMDM</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.388± ± 0.0005</td>
<td>27.175± ± 0.005</td>
<td>19.571c</td>
</tr>
<tr>
<td>B</td>
<td>1.120± ± 0.0005</td>
<td>34.360± ± 0.005</td>
<td>30.665a</td>
</tr>
<tr>
<td>C</td>
<td>0.834± ± 0.2436</td>
<td>18.58± ± 1.07</td>
<td>23.554b</td>
</tr>
<tr>
<td>D</td>
<td>1.151± ± 0.001</td>
<td>22.065± ± 1.235</td>
<td>19.187c</td>
</tr>
<tr>
<td><strong>Pentamidin</strong> [26]</td>
<td>23.71c (18.44–30.50)</td>
<td>17.9c (0.002–0.026)</td>
<td>0.75d</td>
</tr>
<tr>
<td><strong>Glucantine</strong> (SILVA et al., 2015)</td>
<td>13.95b (± 2.06)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

IC₅₀ - 50% inhibitory concentration; SI = BMDM IC₅₀ / promastigote IC₅₀

Similar lower case letters indicate significant similarities between columns (p < 0.001, ANOVA followed by Tukey test). Fungal enzyme extracts of *Vermisporium-like* corresponds to “A”, of *E. nidulans* to “B”, of *D. portulacea* to “C” and of *D. boerhaaviae* to “D”

The antileishmanial results are in other cases superior to many isolated and/or modified compounds [27–29], but the better SI demonstrated in this work was obtained by the “B” crude extract, with an SI of 30.679. The “A” and “C” crude extracts can also be considered as presenting good leishmanicidal activities with SI of 19.561 and 18.097, respectively. “A” crude extract presented the higher and worst results with regard to IC₅₀ value in *L. amazonensis* promastigotes inhibition and of BMDMs.
Conclusions

This work evidences the presence of lipases in crude extracts in cultures of endophytic fungi *Vermisporium*-like, *Emericella nidullans* (present in *J. curcas* seeds) and *Dichotomophthora portulaceae*, besides the plant pathogen *D. boerhaaviae*. The proteomic analysis points several molecules with clear differences in protein profiles. The antifungal potential of these extracts was demonstrated against six strains of animal dermatophytes fungi (*Malassezia* sp and *Microsporum canis*). In a general, *Dichotomophthora* species produces the most effective enzymes against the studied fungi. In the second place, *Vermisporium*-like presented the lowest MIC/MFC for both studied dermatophytes.

Concerning the promastigote and amastigote forms, the leishmanicidal activities of the enzyme extracts, *Dichotomophthora* species also showed to be the most effective, followed by *E. nidullans*, which presented the lowest IC₅₀ for *L. amazonensis* promastigote forms, and *Vermisporium*-like with the lowest IC₅₀ values against amastigote forms inside infected-BMDMs.

As far as the cytotoxic activities to BMDMs are concerned, all extracts at 5 mg.ml⁻¹ demonstrated toxicological activities, leading to best results of selectivity. *Emericella nidullans* stands out as the most selective crude extract at 30.679 SI, what means that its potential of developing side effects is low if compared with the problems caused by the use of standard pentavalent antimonials. In second, *D. boerhaaviae* at 20.985 reaffirms the genus potential, followed *Vermisporium*-like and *D. portulaceae*.

Although the mechanism of action of these enzymes was not completely elucidated, there was evidence pointing to some effect on membrane lipids in all pathogens. Nevertheless, further investigation is necessary to understand how these protein products potentially act in pathogen’s enzymes through *de novo* synthesis and membrane modifications. These membrane modifications are seen as a virulence factor, which plays an important role at cell metabolism maturation in the host.

Conflict of interest

The authors declare that there is no conflict of interest and certify the impartiality of the reported research.

Author’s Contributions

Conceived and designed the experiments: DRA; SMM; FCOF; FRV; GASP.
Performed the experiments: DRA; FRV; FNTP; AHDC; MMM. Analyzed the data: DRA; SMM; FCOF; FRV; INGS; FNTP; AHDC; MMM. Contributed reagents/materials/analysis tools: SMM; FCOF; GASP; WRP; ICC; INGS; FRV; AAANM; DRA. Wrote the paper: DRA; SMM; FCOF; FRV; WRP.

Acknowledgments

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References


17. Mosmann T. Rapid colorimetric assay for cellular growth and survival:


11 CONCLUSÕES

Em virtude dos fatos mencionados podemos inferir que foram encontrados como componentes principais os flavonoides quercetina, rutina e isoquercetina em extratos de várias partes vegetais (folha, casca e polpa do fruto) de Caryocar coriaceum, popularmente conhecido como Pequizeiro, modificando-se apenas suas porcentagens de expressão.

Os mesmos extratos foram avaliados quanto sua atividade biológica, onde pôde observar-se que extratos da folha de Caryocar coriaceum exercem efeito leishmanicida atuando em formas promastigotas por mecanismo semelhante à apoptose e amastigotas intracelulares pela resposta dependente da ferrugina Nrfr/HO⁻¹/ferritina e depleção de ferro. Já extratos de casca e frutos de Caryocar coriaceum induzem ação leishmanicida de forma direta em promastigotas e indireta em amastigotas intracelulares por modulação do metabolismo do ferro, o que nos leva a acreditar que ocorreu possivelmente devido ao seu maior conteúdo fenólico.

Foi ainda avaliada a diversidade de fungos endofíticos na caatinga. Após determinado o potencial de produção de metabolitos primários e secundários, estes foram separados e então avaliadas suas atividades estes demonstraram grande potencial bioativo. Evidenciamos também a presença de proteínas em extratos brutos em culturas de fungos endofíticos Vernisporium-like (S. eucalypti), Emericella nidullans, Dichotomosphora portulacea e D. boerhaaviae. A análise proteômica aponta várias moléculas com claras diferenças nos perfis de proteínas.

De modo geral, as espécies de Dichotomosphora produzem as enzimas mais eficazes contra as células estudadas. Com relação às atividades antileishmaniais, contra as formas promastigota e amastigota, dos extratos enzimáticos, as espécies de Dichotomosphora também se mostraram as mais eficazes, seguidas por E. nidullans, que apresentou o menor IC₅₀ para as formas promastigotas de L. amazonensis e Vernisporium-like com menor de IC₅₀ contra formas amastigotas dentro de BMDMs infectados.

No que diz respeito às atividades citotóxicas para as BMDMs, Emericella nidullans se destaca como o extrato bruto mais seletivo a 30.679 SI, o que significa que seu potencial de desenvolver efeitos colaterais é baixo se comparado aos problemas causados pelo uso padrão de antimoniais pentavalentes. Em segundo, D. boerhaaviae em
20.985 reafirma o potencial do gênero, seguido de *Vermisporium*-like e *D. portulaceae*.

Embora o mecanismo de ação dessas enzimas não tenha sido completamente elucidado, houve evidências apontando algum efeito sobre os lipídios da membrana dos patógenos. No entanto, é necessária uma investigação mais aprofundada para entender como esses produtos proteicos agem potencialmente nas enzimas dos protozoários por meio de síntese *de novo* e modificações na membrana. Essas modificações de membrana são vistas como um fator de virulência, que desempenha um papel importante na maturação do metabolismo celular do parasito no hospedeiro.

Este estudo produziu uma pesquisa exploratória e dada a magnitude do tema considera-se que muito há ainda que investigar sendo, implicando portanto, um campo fértil de trabalho. Avaliando os resultados/dificuldades, recomenda-se a adaptação das metodologias aqui expostas para permitir maior rendimento dos extratos, facilitando a identificação e o isolamento de novos compostos. Embora dos esforços para diagnosticar e conter a doença, novas investigações são fundamentais para que se possa identificar compostos, com atividade antibiótica, capazes de tratar as cepas mais resistentes e/ou amenizar os efeitos colaterais dos tratamentos atuais, como os aqui descritos.

Diante do exposto, os dados obtidos nessa tese comprovam a importância do estudo de metabolitos secundários obtidos de vegetais e de fungos endofíticos da caatinga, abrindo perspectivas ao uso desses extratos naturais, e substâncias isoladas, como agentes terapêuticos capazes de colaborar ou agir favoravelmente ao combate a infecções por protozoários intracelulares.
12 PERSPECTIVAS

Dada a natureza do trabalho realizado no âmbito desta tese, as perspectivas de desenvolvimento que se apresentam são diversas. Metabólitos naturais têm se revelado relevantes, demonstrando diversas e extensas atividades biológicas relevantes, dentre elas destacamos atividades antileishmaniais, antibacteriana, antifúngica, antiacetilcolinesterásica, antioxidante, dentre outras.

Aqui foi demonstrado como de fundamental importância investigar o papel de compostos de *C. coriaceum*, bem como de fungos endofíticos de plantas da caatinga, a fim de aferir seus potenciais biológicos isolados. Na questão metodológica, existem ainda melhorias e modificações a fazer, sobretudo na perspectiva de tornar a extração um processo contínuo, com todas as etapas integradas.

Por último, mas não menos importante, se fazem necessários estudos mais detalhados sobre o efeito farmacológico dessas substâncias como alternativa aos fármacos comumente usados, minimizando toxicidade ao paciente, custos e eventuais resistências.

Refira-se que se encontram atualmente em curso projetos de investigação, resultantes desta tese e nos quais a autora se encontra envolvida, conducentes à realização de grande parte destas perspectivas de desenvolvimento.
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APÊNDICES
Flavonoid composition and biological activities of ethanol extracts of *Caryocar coriaceum* Wittm, a native plant from Caatinga Biome
APÊNDICE B - Comprovante de publicação do artigo (Capítulo 03)

Caryocar coriaceum extracts exert leishmanicidal effect acting in promastigote forms by apoptosis-like mechanism and intracellular amastigotes by Nrf2/HO-1/ferritin dependent response and iron depletion Leishmanicidal effect of Caryocar coriaceum leaf extracts
Caryocar coriaceum fruit extracts induce leishmanicidal action directly on promastigotes and indirectly on intracellular amastigotes by modulation of iron metabolism.
APÊNDICE D - Comprovante de publicação do artigo (Capítulo 05)

*In vitro* leishmanicidal and fungicidal activities of endophytic fungal enzyme extracts


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APÊNDICES