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AVALIAÇÃO DO POTENCIAL BIOLÓGICO DO ÓLEO ESSENCIAL DA CASCA DO FRUTO DE *Myrciaria floribunda (*H. WEST EX WILLD.) O. BERG

Recife
2020

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de Mestre em Ciências Biológicas.

Área de concentração: Biotecnologia

Orientadora: Prof.ª Dr.ª Maria Tereza dos Santos Correia.

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Aprovada em: 19/02/2020.

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Gratidão

Enfrente-se.
Desafie-se. Vença-se.
Busque, e o que não
Encontrar, torne-se

Wandy Luz, 2019.

RESUMO

Óleos essenciais (OE's) são compostos voláteis obtidos de plantas aromáticas que podem ser promissores em atividades biológicas. *Myrciaria floribunda* (H. West ex Willd.) O. Berg é uma espécie nativa do Brasil pertencente à família Myrtaceae cujos OE's são descritos por apresentar uma ampla variedade de propriedades farmacológicas. A busca por alternativas terapêuticas eficazes e com baixa toxicidade para o tratamento de doenças não-transmissíveis e infecciosas, dentre elas as doenças neurodegenerativas e negligenciadas, respectivamente, ainda é um desafio. Visto que, os fármacos disponíveis para o tratamento apresentam várias limitações. Neste contexto, o presente estudo teve por objetivo avaliar o potencial biológico do óleo essencial da casca do fruto de *M. floribunda* (MfEO). Assim, foi determinada a composição química do óleo e investigado seus parâmetros farmacocinéticos e toxicológicos através de ensaios *in sílico*. Posteriormente, testes de interações molecular entre os principais compostos majoritários do MfEO com as enzimas acetilcolinesterase e esterol 14-alfa desmetilase foram avaliados. Também, foram analisadas as atividades citotóxicas, de inibição enzimática e antiparasitária do OE. Os resultados revelaram a presença de 26 compostos (96,86%) por cromatografia gasosa acoplada a espectrometria de massa, dos quais os principais componentes majoritários foram: δ-Cadineno (26.8%), γ-Cadineno (15.69%), γ-Muuroleno (6.21%), α-Selineno (6.11%), α-Muuroleno (6.11%) e (E)-Cariofileno (5.54%). Os compostos majoritários do óleo apresentaram propriedades favoráveis para a interação com as enzimas avaliadas e o OE foi capaz de inibir a acetilcolinesterase, demostrando resultados promissores através do ensaios *in silico* e *in vitro*. MfEO apresentou concentração citotóxica (CC50) de 260.9 e 332.9 µg/ml para célula Vero e macrófago J774A.1, respectivamente. MfEO demonstrou significativa atividade leishmanicida para as formas promastigotas (IC50: 55,84 µg/ml) e amastigotas de *Leishmania amazonensis* (IC50: 7,54 µg/ml), com um índice de seletividade (44,1) para amastigotas intracelulares do parasita. Este estudo foi pioneiro na descrição da composição química e do potencial biotecnológico do óleo essencial das cascas do fruto de *M. floribunda*, proporcionando uma nova perspectiva para o uso das cascas do fruto no desenvolvimento de aplicações farmacológicas de baixo custo.

Palavras-chave: Cambuí. *Myrciaria floribunda*. Fitoterapia.

ABSTRACT

Essential oils (OE's) are volatile compounds obtained from aromatic plants that can be promising in biological activities. *Myrciaria floribunda (*H. West ex Willd.) O. Berg is a species native to Brazil belonging to the family Myrtaceae whose OE's are described for having a wide variety of pharmacological properties. The search for new effective therapeutic alternatives with low toxicity for the treatment of non-communicable and infectious diseases, including neurodegenerative and neglected diseases, respectively, is still a challenge. Since, the drugs available for treatment have several limitations. In this context, the present study aimed to assess the biological potential of the essential oil of the peel of the fruit of *M. floribunda* (MfEO). The chemical composition of the oil was determined and its pharmacokinetic and toxicological parameters were investigated through silica tests. Subsequently, tests for molecular interactions between the major majority compounds of MfEO with the enzymes acetylcholinesterase, and sterol 14-alpha demethylase. Also, the cytotoxic, enzymatic and antiparasitic inhibition activities of the OE were analyzed. The results revealed the presence of 26 compounds (96.86%) by gas chromatography coupled with mass spectrometry, of which the main major components were: δ-Cadinene (26.8%), γ-Cadinene (15.69%), γ-Muurolene (6.21%), α-Selinene (6.11%), α-Muurolene (6.11%) and (E)-Karyophylene (5.54%). The major compounds of the oil showed favorable properties for the interaction with the evaluated enzymes and OE was able to inhibit acetylcholinesterase, showing promising results through *in silico* and *in vitro* assays. MfEO showed cytotoxic concentration (CC50) of 260.9 and 332.9 µg/ ml for Vero cell and macrophage J774A.1, respectively. MfEO demonstrated significant leishmanicidal activity for the promastigotes (IC50: 55.84 µg/ml) and amastigotes of *Leishmania amazonensis* (IC50: 7.54 µg/ml), with a selectivity index (44.1) for intracellular amastigotes of the parasite. This study was a pioneer in the description of the chemical composition and biotechnological potential of the essential oil of the peels of the fruit of M. floribunda, providing a new perspective for the use of the peels of the fruit in the development of low-cost pharmacological applications.

Keywords: Cambuí. *Myrciaria floribunda*. Phytotherapy.

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LISTA DE ABREVIATURAS E SIGLAS

ACh	Acetilcolina
AChE	Acetilcolinesterase
AChEIs	Inibidores de acetilcolinesterase
ADMET	Absorção, distribuição, metabolismo, excreção e toxicidade
$CaCl_2$	Cloreto de cálcio
CAS	Sítio ativo catalítico
DA	Doença de Alzheimer
DH	Doença de Huntington
DP	Doença de Parkinson
DN	Doenças Neurodegenerativas
DTN	Doenças tropicais negligenciadas
ELA	Esclerose Lateral Amiotrófica
EM	Esclerose Múltipla
IC_{50}	Índice de concentração inibitória de 50%
MfEO	Óleo essencial da casca do fruto de *Myrciaria floribunda*
MG	Miastemia gravis
OE's	Óleos essenciais
ODS	Objetivos de desenvolvimento sustentável
OMS	Organização Mundial de Saúde
LC	Leishmaniose Cutânea
LCL	Leishmaniose cutânea localizada
LCD	Leishmaniose Cutânea Difusa
LMC	Leishmaniose mucocutânea
LV	Leishmaniose Visceral
mAChRs	Receptores muscarínicos de acetilcolina
nAChRs	Receptores nicotínicos de acetilcolina

SUMÁRIO

1 INTRODUÇÃO

O estudo sobre as propriedades dos óleos essenciais (OE's) tem sido crescente ao longo dos anos, levando em consideração que a indústria tende à adaptar suas formulações para as exigência de diferentes públicos e normas específicas para o comércio de produtos (IRSHAD et al., 2019). Devido as suas propriedades, os OEs apresentam vantagens por serem de fácil obtenção, sustentáveis e biodegradáveis, tornando-os produtos bem aceitos entre os consumidores (FIGUEIREDO, PEDRO & BARROSO, 2017).

O Brasil é um país rico pela sua diversidade de plantas, fator consideravelmente importante para a realização de estudos e aplicações biotecnológicas utilizando os OE's (MENDES et al., 2016). Dentre as famílias que compreendem as espécies vegetais produtoras de óleos, a família Myrtaceae se destaca pela ampla variedade de espécies. No País, diversos gêneros pertencentes a esta família apresentam grande importância econômica como por exemplo, *Plinia* (jabuticabeira), *Eugeni*a (pitangueira), *Psidium* (goiabeira) e *Myrciaria* (cambuizeiro) (SERAGLIO et al., 2018).

Myrciaria floribunda (H. West ex Willd.) O. Berg. é uma planta nativa do Brasil, conhecida popularmente como cambuí, seus frutos podem ser consumidos "*in natura"* ou após o processamento, na forma de licores, geleias e doces, ressaltando assim, o valor econômico da espécie (WU, LONG & KENNELLY, 2013; TNG et al., 2019). Além da importância econômica, essa espécie tem chamado atenção da comunidade científica por conta das suas propriedades no campo biomédico, como ação antimicrobiana, inseticida (TIETBOHL et al., 2014; TIETBOHL et al., 2019) e de inibição enzimática (TIETBOHL et al., 2017; OLIVEIRA et al., 2018; FEDER et al., 2019). No entanto, a investigação do potencial biológico do óleo essencial de *M. floribunda* ainda é rara e nenhum relato sugere a utilização da casca do fruto para investigação científica, que por vezes é descartada como material sem utilidade.

A utilização da casca do fruto de *M. floribunda* como possibilidade de aplicação biotecnológica é inovadora e compreende um campo aberto para estudo nas áreas alimentícia, cosmética e farmacêutica. No campo farmacêutico, a busca por alternativas terapêuticas para o tratamento de doenças crônicas não-transmissíveis e infecciosas ainda é necessária, visto que os medicamentos utilizados nos tratamentos convencionais apresentam limitações como diminuição da efetividade medicamentosa a longo prazo e efeitos colaterais (MENEZES et al., 2015; BURČUL et al., 2019), alto custo, resistência (SAVOIA, 2015), entre outros. Nesse contexto, as doenças neurodegenerativas, como doença de Alzheimer e Parkinson; e doenças tropicais negligenciadas, como a Leishmaniose Cutânea merecem destaque, pelo alto índice de

indivíduos acometidos e medidas terapêuticas ineficazes e tóxicos. Dessa forma, este estudo investigou o potencial biológico do óleo essencial da casca do fruto de *Myrciaria floribunda* (H. WEST EX WILLD.) O. BERG.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Avaliar o potencial biológico do óleo essencial da casca do fruto de *Myrciaria floribunda* (MfEO).

1.1.2 Objetivos específicos

• Obter e caracterizar o óleo essencial;

• Determinar os parâmetros farmacocinéticos e toxicológicos *in silico* e docking molecular dos compostos majoritários do óleo;

• Verificar o potencial de inibição enzimática da AChE;

• Determinar a citotoxicidade do OE em linhagens celulares;

• Verificar o efeito do óleo sobre promastigota e amastigota de *Leishmania amazonensis* e descrever às alterações ultraestruturais.

2 REFERENCIAL TEÓRICO

2.1 ÓLEOS ESSENCIAIS

Óleos essenciais (OE's) são um complexo de compostos funcionais de origem natural que podem ser extraídos de diferentes partes da planta, como: flores, folhas, cascas e frutos (PENG, BISHOP & QUEK, 2019). De uma forma geral, os OE's são constituídos principalmente pelos compostos terpênicos e de acordo com a distribuição de sua sequência química, eles podem ser classificados em: monoterpenos ($C_{10}H_{16}$), sequisterpenos ($C_{15}H_{24}$), diterpenos ($C_{20}H_{32}$) e triterpenos ($C_{30}H_{40}$) (CHOUHAN et al., 2017). Além das classes de terpenos, outros grupos funcionais como álcool, aldeído, éster, éter, cetona, ésteres e óxidos fenólicos são encontrados em OE's (BURČUL et al., 2019), essa diversidade de compostos químicos oferecem uma amplitude de aplicações na indústria de alimentos, cosméticos e farmacêutica, sendo de grande importância o estudo e identificação de suas propriedades (WINKELMAN, 2018; KHARBACH et al., 2019).

OE's são obtidos a partir de um produto vegetal que serve de matéria-prima para sua extração. Alguns dos métodos convencionais utilizados para obtenção destes são: hidrodestilação, destilação a vapor e extração por solvente (DHIFI et al., 2016), outros métodos relatados como inovadores e promissores no processo de obtenção são extração por fluído supercrítico, extração assistida por micro-ondas e extração por ultrassom (JING et al., 2019). Ao longo dos anos, a investigação de aplicações dos OE's em saúde, agricultura e meio ambiente tem aumentado (MANION & WIDDER,2017; BAHMANI & SCHMIDT, 2018; HASSAN, MANZOOR & SALEEM, 2020).

Diversos estudos demonstram que os OE's podem ser promissores em várias atividades biológicas, entre elas: antimicrobiana (CORREA et al., 2019; RUIZ-RICO, MORENO & BARAT, 2020), antibiofilme (ARTINE et al., 2018; GAO et al., 2020), larvicida (PANDIYAN, MATHEW, and MUNUSAMY 2019; BENELLI et al., 2020), leishmanicida (SILVA et al. 2018; SAMPAIO et al., 2019), tripanocida (AZEREDO and SOARES 2013; PEREIRA et al., 2017), fungicida (MEDEIROS et al., 2016; SANTOS et al. 2018), anti-doenças autoimune (COCK and CHEESMAN, 2019), anti-inflamatória (CHOU et al. 2018; ASCARI et al., 2019); moduladora do sistema neurológico (ARRUDA et al., 2012; ZARRAD et al., 2015); Inibição enzimática (OLIVEIRA et al., 2019; FRATERNALE, FLAMINI & ASCRIZZI, 2019), antioxidante (GEDIKOĞLU, SÖKMEN & ÇIVIT, 2019; DENG et al.,2020; HENNIA et al., 2020) e aromaterapia (ALI et al., 2015; HASSAN, MANZOOR & SALEEM, 2020).

2.1.1 Família Myrtaceae

Myrtaceae é uma das grandes famílias de angiospermas que abrange cerca de 145 gêneros e cerca de 5970 espécies, distribuídas nas regiões tropicais e subtropicais do mundo (THE PLANT LIST, 2013). No Brasil, é uma das famílias mais representativas, apresentando cerca de 1000 espécies, das quais 22 são nativas e está entre as 10 famílias com maior riqueza de espécies no país (MENDES et al., 2016).

A família Myrtaceae caracteriza-se por apresentar desde arbustos a árvores altas e ser uma das principais famílias de plantas frutíferas, com numerosos exemplos de frutos comestíveis e de sabor raro (STEFANELLO, PASCOAL & SALVADOR, 2011). Alguns exemplos de gêneros que pertencem a família cujos frutos são comercializados incluem, espécies dos gêneros *Psidium* (goiabas), *Eugenia* (pitangas), *Plinia* (jabuticabas) e *Syzygium* (jambo e jamelão). Além de especiarias como o gênero *Syzygium* (cravo-da-índia); *Eucalyptus* e *Corymbia* são importantes fontes de madeira e óleos essenciais que contribuem no desenvolvimento econômico (REYNERTSON et al., 2008; SERRALHO et al., 2018; FARIAS et al., 2020).

Ao longo dos anos, diversos estudos relatam o potencial dos óleos essenciais de espécies da família Myrtaceae como uma fonte promissora de compostos bioativos e propriedades biológicas, como demonstra a tabela 1 nos últimos cinco anos.

Tabela 1 – Óleos essenciais da família Myrtaceae e suas atividades biológicas

Espécie	***Parte da planta***	***Propriedades***	***Referência***
Calyptranthes restingae	Folhas	Antinoceptivo	Passos et al., 2016
Calyptranthes grandifolia *Calyptranthes tricona*	Folhas	Leishmanicida	Kauffmann et al., 2016
Eugenia arenosa *Eugenia pyriformis*	Folhas	Leishmanicida	Kauffmann et al., 2016
Eugenia dysenterica	Folhas	Antidiarréico	Galheigo et al., 2016
Eugenia hiemalis Cambess.	Folhas	Antimicoplasmático	Zatelli et al., 2016

Myrcia lundiana	Folhas	Antifúngico	Alves et al., 2016
Myrcia sylvatica	Folhas	Antimicrobiano	Silva et al., 2016
Pimenta racemosa	Folhas	Antimicrobiano	Contreras-Moreno et al., 2016
Syzygium zeylanicum	Folhas	Larvicida	Govindarajan & Benelli, 2016
Syzygium lanceolatum (Lam.)	Folhas	Antimicrobiano e antioxidante	Muthumperumal et al., 2016
Eucalyptus camaldulensis	Folhas	Antifúngico	Yangui et al., 2017
Eugenia brejoensis	Folhas	Tripanocida	Souza et al., 2017
Eugenia candolleana	Folhas	Larvicida	Neves et al., 2017
Eugenia caryophyllata	Flores	Anti-inflamatório	Han & Parker, 2017
Eugenia dysenterica	Folhas	Antioxidante e anticolinesterásico	Feitosa et al., 2017
Eugenia klotzschiana	Folhas e flores	Antimicrobiano e antioxidante	Carneiro et al., 2017
Eugenia pitanga	Folhas	Leishmanicida	Kauffmann et al., 2017
Leptospermum citratum	Flores	Inseticida	Park et al., 2017
Myrcia splendens	Folhas	Antitumorais e antimicrobiano	Scalvenzi et al., 2017
Myrtus communis	Folhas	Antifúngico	Yangui et al., 2017
Blepharocalyx salicifolius	Folhas	Broncodilatador, Antitussígeno e antiespasmódico	Hernández et al., 2018
Eucalyptus citriodora	Folhas	Antimicrobiano	Tolba et al.,

			2018
Eugenia uniflora	Folhas	Antifúngico	Santos et al., 2018
Melaleuca alternifolia	Folhas	Antioxidante e antimicrobiano	Zhang et al., 2018
Myrciaria floribunda	Frutos	Antioxidante	Oliveira et al., 2018
Myrcia oblongata	Folhas	Antimicrobiano	Santana et al., 2018
Myrcia rostrata	Folhas	Antinociceptivo	Silva et al., 2018
Psidium guineense	Folhas	Antioxidante, anti-inflamatório, antiproliferativo e antimicobactéria	Nascimento et al., 2018
Syzgium guineense	Folhas	Antioxidante e antimicrobiano	Okhale et al., 2018
Campomanesia guazumifolia	Folhas	Antioxidante	Santos et al., 2019
Corymbia citriodora *Myrciaria dubia*	Folhas	Inseticida	Negrini et al., 2019
Eucalyptus globulus	Folhas	Antibacteriano e anticâncer	Adnan, Mohd. 2019
Eucalyptus globulus *Melaleuca citrina*	Folhas	Inibidor de acetilcolinesterase	Petrachaianan et al., 2019
Eugenia dysenterica	Folhas	Antifúngico	Silva et al., 2019
Eugenia pyriformis	Partes aéreas	Antifúngico, Leishmanicida e antitumoral	Durazzini et al., 2019
Myrciaria floribunda	Folhas	Inseticida	Tietbohl et al., 2019
Myrciaria plinioides	Folhas	Antileishmania	Kauffmann et al., 2019
Psidium myrtoides	Folhas	Antibacteriano	Dias et al.,

		e antitumoral	2019
Syzygium aromaticum	Folhas	Antifúngico	Tančinová et al., 2019
Eucalyptus cloeziana *Eucalyptus umbellata* *Eucalyptus benthamii*	Folhas	Repelente, inseticida, antibacteriano	Tian et al., 2020
Eugenia caryophyllus	Flores	Inseticida	Matos et al., 2020
Eugenia stipitata	Folhas	Antinociceptivo, anti-inflamatório e antipirético	Costa et al., 2020
Melaleuca sp.	Folhas	Antibacteriano e antioxidante	Siddique et al., 2020

2.1.2 *Myrciaria floribunda* (H. West ex Willd.) O. Berg

Myrciaria floribunda (H. West ex Willd.) O. Berg. conhecida popularmente como cambuí ou camboin é uma espécie nativa do Brasil, encontrada nos domínios fitogeográficos da Amazônia, Floresta Atlântica, Caatinga e Cerrado (MAIA, 2019). Em relação aos seus aspectos botânicos trata-se de um arbusto com altura variando de 3 – 16 m, folhas elípticas e flores sésseis com brácteas, seus frutos são bagas que pode variar de 0,4 - 0,8 cm (Figura 1) e apresentam-se nas colorações verde vermelho, vinho, ou laranja de acordo com o grau de maturação (SANTOS et al., 2018).

Os frutos de *M.floribunda* podem ser apreciados tanto "*in natura",* como processados na forma de licores, geleias e doces (WU, LONG & KENNELLY 2013; TNG et al., 2019). Segundo estudos de Santos et al. (2017) e Oliveira et al. (2018) os frutos *M. floribunda* são fontes nutricionais que apresentam concentrações significativas de β-criptoxantina (pró-vitamina A) e ácidos fenólicos relatados por apresentarem potencial antioxidante.

Figura 1 – Folhas, flores e frutos de *Myrciaria floribunda*

1A – Ramos com flores e frutos (Desenhado por: Nolán Iglesias Martínez. Fonte: Ramos, Pendás & Urra, 2015); 1B – flor (Fonte: Ramos, Pendás & Urra, 2015); 1C - frutos de *M. floribunda* (Fonte: Autor).

Estudos avaliando as propriedades farmacológicas de extratos das folhas de *M. floribunda* relataram potencial antitumoral (TIETBOHL et al., 2017), antioxidante e antimicrobiana (AZEVEDO et al., 2019). Enquanto que, o óleo essencial das folhas demonstrou-se como um promissor inseticida (TIETBOHL et al., 2014; TIETBOHL et al., 2019; FEDER et al., 2019) e apresentou leve inibição da enzima acetilcolinesterase (TIETBOHL et al., 2012). No entanto, ainda são raros os estudos avaliando as propriedades biológicas da espécie e nenhum relato sugere a utilização da casca do fruto para investigação, que por vezes é desprezada como matéria sem utilidade. Dessa forma, pesquisas científicas que forneçam informações sobre o potencial de espécies naturais e seus resíduos em aplicações biotecnológicas são de grande importância para o desenvolvimento de produtos sustentáveis que ofereçam vantagens econômicas e ambientais (TREICHEL et al., 2020).

2.1.3 Frutos e seus benefícios para a saúde

As frutas e vegetais contêm uma variedade de micronutrientes especifícos para a função física e mental. São altamente recomendados, tendo em vista as contribuições benéficas que esses alimentos oferecem para uma vida saudável (BROOKIE et al., 2018). Eles apresentam uma grande variedade de constituintes fitoquímicos, entre eles minerais e vitaminas que desempenham um papel fundamental na proteção e bom funcionamento do corpo (YAHIA, GARCÍA-SOLÍS & CELIS, 2019).

As vitaminas (C, A e E), flavonoides, antocianinas e compostos fenólicos presentes em frutas são componentes descritos como potenciais antioxidantes, que atuam neutralizando espécies reativas de oxigênio e nitrogênio, responsáveis pelo estresse oxidativo (BARBOSA, SILVA & CORREIA, 2019). O estresse oxidativo é a causa e/ou progressão de várias doenças, entre elas: doenças cardiovasculares, câncer, doença inflamatória crônica, formas de depressão e doenças neurodegenerativas (PATEL et al., 2019).

Estudos comparativos avaliando as propriedades nutricionais das cascas e polpas de frutas relatam que, as cascas são fontes promissoras de compostos bioativos (flavonoides, antocioaninas, minerais e vitaminas) e em grande parte dos casos excedem o valor nutricional da polpa (ABBASI et al., 2017**;** SAIDANI et al., 2017). No entanto, no consumo de frutas muitas vezes as cascas são descartadas como resíduos não utilizáveis e de pouco valor, porém, deve ser dada atenção às suas propriedades como alimento funcional e uma fonte de matéria-prima para as indústrias alimentícia e farmacêutica (CZECH et al., 2019). Vários produtos naturais obtidos de plantas medicinais, frutas e vegetais demonstram ser potenciais agentes terapêuticos no desenvolvimento de novas formulações para o tratamento de doenças, dentre elas doenças neurodegenerativas (SOLAYMAN et al., 2017; Silva & Pogačnik, 2020) e doenças tropicais negligenciadas (CHEUKA et al., 2017; VARELA & FERNANDES, 2019).

2.2 DOENÇAS NEURODEGENERATIVAS

Distúrbios mentais como demência e depressão são condições altamente prevalentes na população mundial, além de serem achados comuns em doenças neurodegenerativas (RÉUS et al., 2016; KOVASC, 2018; GALTS et al., 2019). De acordo com a organização mundial de saúde (OMS) cerca de 50 milhões de pessoas no mundo apresentam demência e projeta-se que esse valor triplique até 2050, para um valor de 152 milhões de casos (WHO, 2017). Estimativas recentes, abordam que mais de 264 milhões de pessoas vivem com depressão no mundo (WHO, 2020). Ambos transtornos mentais costumam ser fatais e resultam em altos gastos para o sistema de saúde (WHO, 2017; POHL & LIN, 2018; KAMAl et al., 2019).

As doenças Neurodegenerativas (DN) são uma das causas mais comuns de morbidade e mortalidade em todo mundo, principalmente em idosos (Erkkinen et al., 2018). A neurodegeneração é um fenômeno de perda progressiva da estrutura ou função dos neurônios, resultando em comprometimentos mentais e funcionais, devido à alterações no processo de neurotransmissão e a incapacidade de renovação celular (WINNER & WINKLER, 2015; CHEN, ZHANG & HUANG, 2016).

O diagnóstico precoce em combinação com o tratamento adequado são de grande importância para retardar a progressão da doença, que geralmente é diagnosticada com base na avaliação neuropatológica (KOIKKALAINEN et al., 2016). As DN podem ser classificadas de acordo com as características clínicas primárias (demência, parkinsonismo, distúrbios em neurônios motores), distribuição anatômica de neurodegeneração (degeneração frontotemporais, distúrbios extrapiramidais ou degenerações espinocerebelares) ou anormalidades moleculares (KOVACS & BUDKA, 2010; DUGGER & DICKSON, 2017). Contudo, a etiologia das DN permanece em investigação.

Doença de Alzheimer (SPIERS-JONES, ATTEMS & THAL, 2017), Doença de Parkinson (FU, HARDY & DUFF, 2018), Doença de Huntington (COLGAN & TABRIZI, 2018), Esclerose Lateral Amiotrófica (HARDIMAN et al., 2017), Esclerose múltipla (NIEDZIELSKA et al., 2016) e Miastemia grave (PHILLIPS & VINCENT, 2016) são exemplos de DN. O quadro 1 resume as principais características encontradas nestas doenças, que estão relacionadas à distúrbios no sistema nervoso central (SNC), sistema nervoso periférico (SNP) e acometimento do sistema colinérgico.

Quadro 1 – Doenças Neurodegenerativas: principais características e sintomas

Doenças Neurodegenerativas	Características	Principais sintomas
Doença de Alzheimer (DA)	Atrofia da substância cinzenta, acúmulo de placas amiloides e emaranhados neurofibrilares	Demência
Doença de Parkinson (DP)	Perda de substância nigra e de neurônios dopaminérgicos	Perda de movimentos
Doença de Huntington (DH)	Perda de neurônios GABAérgicos	Distúrbios cognitivos, motores e psiquiátricos
Esclerose Lateral Amiotrófica (ELA)	Perda de neurônios motores superiores e inferiores	Fraqueza e paralisia muscular
Esclerose Múltipla (EM)	Distúrbio desmielinizante imuno-mediado do SNC	Alterações na mobilidade, equilíbrio, visão e cognição
Miastemia Grave (MG)	Doença autoimune que afeta a junção neuromuscular	Fraqueza e fadiga muscular

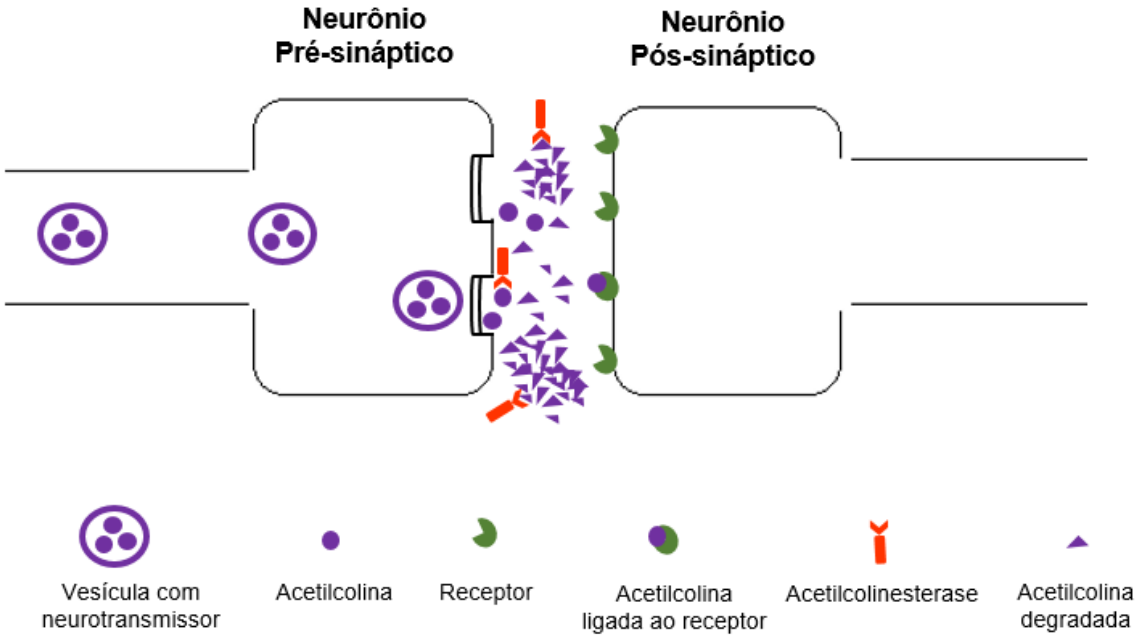
Fonte: Elaborado pelo autor

2.2.1 Sistema Colinérgico

O sistema colinérgico está envolvido em importantes funções do organismo, incluindo cognição; aprendizado e memória; processamento sensorial e motor; sono e estresse; processamento e regulação emocional (OFEK & SOREQ, 2013; SZUTOWICZ ET AL., 2013; PEPEU & GIOVANNINI, 2017). Ele atua sobre o sistema nervoso periférico (SNP), incluindo nervos motores, órgãos do sistema autônomo e sistema nervoso central (SNC), na propagação excitatória e comunicação entre células nervosas (MU & HUANG, 2019). O sistema colinérgico é formado por uma rede de neurônios que sintetizam e liberam acetilcolina (ACh), neurotransmissor que desempenha papel crucial na manutenção da homeostase e funções cerebrais (BAXTER & CRIMINIS, 2018).

A molécula de ACh é constituída a partir de um éster de colina e ácido acético por ação da acetiltransferase. Após sua síntese, a ACh é armazenada em vesículas sinápticas e encaminhada para a membrana do neurônio pré-sináptico (Figura 2) quando um impulso nervoso é propagado há liberação da ACh na fenda sináptica (BROWN, 2019). Esse neurotransmissor irá atuar sobre o neurônio pós-sináptico, através da ligação com receptores colinérgicos, assim, o impulso nervoso é regenerado e propagado para o neurônio seguinte, resultando na transmissão da mensagem excitatória (AKAIKE et al., 2018).

Figura 2 – Síntese e liberação de acetilcolina na fenda sináptica



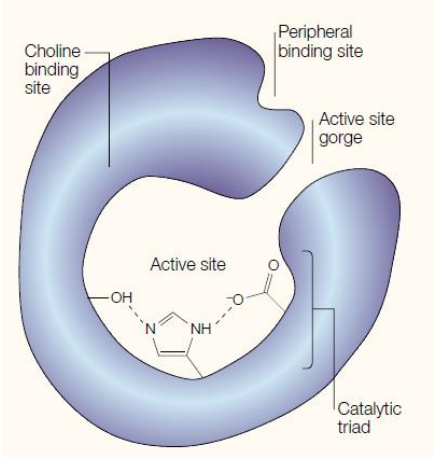
Fonte: Elaborado pelo autor

Os receptores de ACh de uma forma geral são divididos em receptores nicotínicos (nAChRs) e receptores muscarínicos (mAChRs) (WESSLER & KIRKPATRICK, 2008). Os nAChRs são formados por combinações de subunidades α e β, encontrados no cérebro, junções neuromuscular, regiões ganglionares e células não-neuronais (WONNACOTT et al., 2018; ZOLI ET AL., 2018). Os mAChRs são receptores acoplados a proteína G, classificados em cinco subtipos (M1, M2, M3, M4 e M5) e são encontrados no SNP; órgãos autônomos e glândulas exócrinas (LANGMEAD et al., 2008). Após, a condução da mensagem excitatória, a ACh é degradada rapidamente pela ação da acetilcolinesterase (AChE), que desempenha um papel essencial na neurotransmissão (AKAIKE et al., 2018).

AChE é uma serino-hidrolase responsável pela finalização da transmissão dos impulsos nervosos nas sinapses colinérgicas, através da hidrólise da ACh presente na fenda sináptica em colina e ácido acético (CONCEIÇÃO PETRONILHO et al., 2011). É uma das enzimas mais eficientes do sistema nervoso, com capacidade de hidrolisar $6x10^5$ moléculas de ACh por minuto. Encontra-se presente em tecidos condutores, nervos, músculos, SNC e SNP; fibras motoras e sensoriais; sistema simpático e parassimpático; sistema colinérgico e não-colinérgico; e junções neuromusculares (TRIPATHI & SRIVASTAVA, 2010).

A estrutura tridimensional da AChE demonstra que a enzima possui dois sítios para ligação, um sítio ativo catalítico (CAS) localizado inferiormente a região de abertura e o sítio aniônico periférico (PAS) localizado a cerca de 15 Å acima do CAS, Figura 3 (SOREQ & SEIDMAN, 2001; SAXENA & DUBEY, 2019). O sítio catalítico é subdividido em dois subsítios: esterático e aniônico. O subsítio esterático é composto por uma tríade catalítica de ácido glutâmico (E202), serina (S203) e histidina (H447). Enquanto que, o subsítio aniônico é constituído por triptofano (W86) (PETRONILHO, PINTO & VILLAR, 2011). A tríade catalítica é a responsável pela hidrólise da acetilcolina, enquanto o nitrogênio quaternário da fração colina interage com o triptofano (W86) no subsítio aniônico. Já o sítio aniônico periférico (PAS), associa-se a compostos bis-quaternários e pode estar envolvido na ação de inibidores da enzima ou na inibição por excesso de substrato (SCHMATZ, 2009; SAXENA & DUBEY, 2019**).**

Figura 3 – Estrutura característica da enzima acetilcolinesterase



Fonte: Soreq & Seidman (2001)

2.2.2 Acetilcolinesterase e neurodegeneração

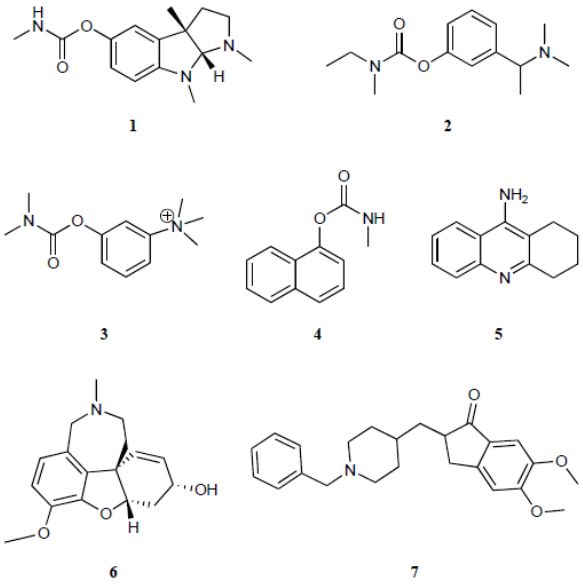
As vias de sinalização colinérgicas tem sido estudadas em doenças neurodegenerativas com a finalidade de melhorar o quadro fisiopatológico nesses distúrbios neurológicos e psiquiátricos (VENTURA et al., 2010; PEPEU & GIOVANNINI, 2017). Para restaurar os níveis de ACh nas sinapses colinérgicas e melhorar a função cognitiva e motora, consequentemente a qualidade de vida, tem sido retratado efeitos benéficos de inibidores de acetilcolinesterase (AChEIs) no tratamento da Doença de Parkinson (MATSUNAGA et al., 2016) e Doença de Alzheimer (DA) (WANG & ZHANG, 2019).

Na DA à degeneração dos neurônios colinérgicos tem sido um achado primário e constante em pacientes (STANCIU et al., 2020). Outros acometimentos como alterações genéticas na expressão de receptores muscarínicos e nicotínicos; diminuição da síntese e liberação de ACh e acúmulo relativo de AChE na fenda sináptica são problemas relacionados ao sistema colinérgico que comprometem a neurotransmissão (OZ, PETROIANU & LORKE, 2016). Os AChEIs tem sido preconizados no tratamento farmacológico da DA por apresentarem propriedades neuroprotetoras e anti-inflamatórias, além de reduzir as fibrilas tóxicas na DA e restaurar os níveis de ACh (SANTOS et al., 2018; BENFANTE et al., 2019). Atuam também no tratamento dos sintomas comportamentais e psicológicos, como a demência, reduzindo a necessidade de psicotrópicos e ansiolíticos, principalmente em doses altas (TAN et al., 2020). A Figura 4 apresenta as estruturas químicas de alguns AChEIs utilizados no tratamento da DA.

Medicamentos como rivastigmina, neostigmina, tacrina e donepezil são utilizados no tratamento da disfunção cognitiva e de memória associada a DA. Contudo, estes compostos

cursam com efeitos adversos como, alterações gastrointestinais, náuseas, vômito e problemas associados a sua biodisponibilidade, como por exemplo, baixa seletividade (BURČUL et al., 2019). Diante disso, existe a necessidade de novos compostos que apresentem menos efeitos adversos. Nesse contexto, os produtos naturais são candidatos promissores para a investigação de compostos bioativos que possam atuar no combate e/ou prevenção de doenças neurodegenerativas (KHATOON, REHMAN & RAHMAN, 2018).

Figura 4 - Estruturas de inibidores de acetilcolinesterase



Legenda: 1 – fisostigmina, 2 - rivastigmina, 3 - neostigmina, 4 - carbaril, 5 - tacrina, 6 - galantamina, 7 – donepezil.
Fonte: Burčul et al. (2019).

2.2.3 Produtos naturais *versus* doenças neurodegenerativas

Os produtos extraídos de plantas podem atingir diferentes vias de sinalização metabólicas envolvidas na patogênese de doenças, devido a ação de vários fitoconstituintes que encontram-se presentes em produtos naturais (DEY et al., 2016). Estudos abordam que compostos bioativos presentes em produtos naturais como antocianinas, carotenoides, resveratrol, alfa tocoferol, quercitina, 7,8-di-hidroxifavona entre outros compostos da classe de polifenois apresentam efeitos benéficos para a prevenção de doenças neurodegenerativas (SOLANKI et al., 2015; ELUMALAI & LAKSHMI, 2016; SHAHIDI & YEO, 2018; MAHER, 2019). Alguns mecanismos apresentados pelos produtos naturais na neuroproteção são: redução da fosforilação da proteína Tau (ESSA et al., 2012**)**, inibição de agregação proteica

(CUANALO-CONTRERA & MORENO-GONZALEZ, 2019), potencial antioxidante, ação sobre a neuroinflamação (JIN et al., 2019) e inibição da acetilcolinesterase (SILVA & POGACNIK, 2020).

A galantamina (alcaloide natural) e a rivastigmina (alcaloide sintético) são inibidores de acetilcolinesterase aprovados clinicamente que possuem como base produtos naturais (GONZÁLEZ et al., 2019). Atualmente, não existe terapias eficazes para a maioria das doenças neurodegenerativas que baseiam-se em reduzir os sintomas comportamentais e cognitivos, sem alterar de forma significativa a progressão da doença (CUNY, 2012; SATHEESHKUMAR et al., 2016).

Novas terapias mais eficazes que possa prevenir a progressão destas doenças são necessárias, nesse contexto, os óleos essenciais são ótimas estratégias para o planejamento de novos medicamentos e avaliação das atividades cerebrais (XU et al., 2014; RANJAN & KUMARI, 2017). Estudos com óleos essenciais demonstraram ser eficazes em modelos de doenças neurodegenerativas *in vitro* e *in vivo*, atuando como neuroprotetores e na redução do comprometimento neuronal (AYAZ et al., 2017; AUMEERUDDY-ELALFI et al., 2018**;** PETRACHAIANAN et al., 2018**;** POHL & LIN, 2018; BENNY & JAYA, 2019; EFTEKHARI et al., 2019).

2.3 DOENÇAS TROPICAIS NEGLIGENCIADAS (DTN)

Doenças tropicais negligenciadas são um grupo diversificado de doenças transmissíveis prevalentes em países tropicais e subtropicais. Atualmente, a OMS reconhece 17 infecções relacionados à DTN (HOTEZ et al., 2016) quadro 2. Estas doenças continuam sendo um problema de saúde pública mundial, que afeta mais de um bilhão de pessoas, principalmente populações que residem em condições de pobreza, falta de saneamento básico e em contato próximo com vetores infectados (WHO, 2020).

Medidas com o propósito de combater as DTN estão entre os desafios estabelecidos pela ONU. De acordo com os objetivos de desenvolvimento sustentável (ODS), até 2030 países desenvolvidos e em desenvolvimento devem realizar intervenções para cobertura universal das DTN na atenção primária à saúde, principalmente em áreas de extrema pobreza. E também, realizar intervenções que impeçam a transmissão de doenças negligenciadas, cuja finalidade de ambas medidas é promover equidade e eliminar problemas de saúde relacionados à pobreza (UNITED NATIONS, 2015; ENGELS & ZHOU, 2020).

Quadro 2 – Lista de doenças tropicais negligenciadas consideradas pela OMS

Doenças Tropicais Negligenciadas (DTN)	
Úlcera de Buruli	Tracoma
Teníase e Neurocisticercose	Micetoma
Doença de Chagas	Bouba
Dracunculíase	Oncocercose
Dengue	Hanseníase
Equinococose	Esquistossomose
Raiva	Tripanossomíase humana africana
Filariose Linfática	Helmintíase transmitida pelo solo
Leishmaniose	

Fonte: adaptado de Santos (2019)

Fatores importantes estão envolvidos no surgimentos e disseminação das DTN, dentre elas a Leishmaniose. Condições socioeconômicas (pobreza), ambientais (desmatamento e mudanças climáticas), migrações para áreas endêmicas, resistência a medicamentos, falta de controle dos reservatórios e vetores são exemplos de fatores que estão diretamente envolvidos com o aumento de casos de Leishmaniose (ORYAN & AKBARI, 2016).

2.3.1 Leishmaniose

A leishmaniose é uma doença tropical negligenciada causada por protozoários do gênero *Leishmania* que continua sendo uma epidemia recorrente, responsável por casos de morbidade e mortalidade (VALERO & URIARTE, 2020). Apresenta distribuição mundial, sendo encontrada principalmente em países da Europa, Ásia, África e América. Anualmente, cerca de um milhão de novos casos são relatados por ano e em média 65.000 mortes (WHO, 2019). No Brasil, a Leishmaniose Cutânea (LC) e Leishmaniose Visceral (LV) estão incluídas na lista nacional de doenças e agravos de notificação compulsória, de acordo com dados do Sistema de Informação de Agravos de Notificação (SINAN) em 2018 foram registrados 17.119 casos de LC e 3.376 casos de LV (SINAN, 2018).

2.4 AGENTE ETIOLÓGICO, CICLO DE VIDA E VETOR

Os protozoários do gênero *Leishmania* pertencem a ordem Kinetoplastida e a família Trypanosomatidae são os agentes etiológicos da Leishmaniose. Estes podem ser classificados em dois subgêneros: *Viannia e Leishmania,* de acordo com o local de desenvolvimento do protozoário no hospedeiro. As espécies responsáveis por causar infecções pertencem a estes dois grupos e cerca de 20 espécies são conhecidas por causarem infecções em humanos, sendo 10 delas de grande importância para a saúde pública, tabela 2 (HURREL et al., 2016**;** STEVERDING, 2017).

Tabela 2 – Principais formas clínicas de leishmaniose cutânea

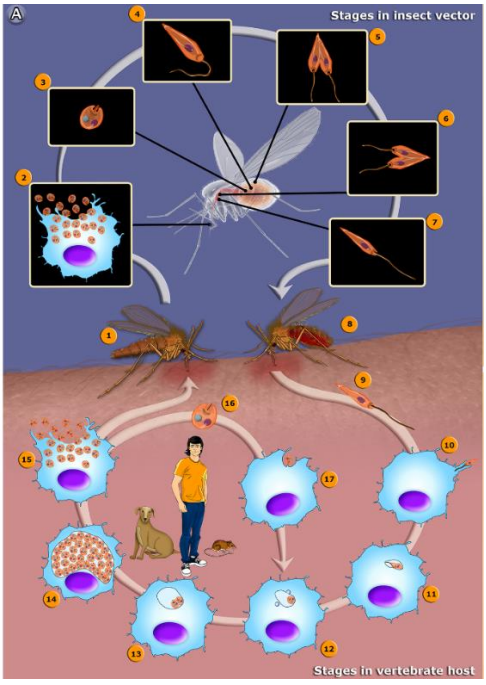
Síndrome Clínica	Espécies[1]	Manifestações Clínicas
Leishmaniose Cutânea Localizada (LCL)	*L. (L.) major* *L. (L.) mexicana* *L. (L.) amazonensis* *L. (V.) braziliensis* *L. (L.) tropica* *L. (L.) aethiopica* *L. (V.) panamanensis* *L. (L.) infantum* *L. (L.) donovani*	Número único ou limitado de lesões; úlceras formadas podem ser molhadas ou secas com borda crateriforme elevada. Cargas moderadas de parasitas em biópsias da borda da úlcera; resposta DTH[2] positiva
Leishmaniose Cutânea Difusa (LCD)	*L. (L.) amazonensis* *L. (L.) mexicana* *L. (V.) pifanoi* *L. (L.) aethiopica* *L. (L.) major*	Lesões nodulares múltiplas, disseminadas e não ulcerativas; muitos parasitas em lesões; resposta DTH ausente (anergia)
Leishmaniose Mucocutânea (LCM)	*L. (V.) braziliensis* *L. (V.) panamanensis* *L. (V.) guyanensis* *L. (L.) amazonensis*	Lesões altamente inflamatórias envolvendo membranas de mucosas; pode ser desfigurante. Formas raras de parasitas presentes em biópsias; forte resposta de DTH

[1] (L.) denota o subgênero *Leishmania*. (V.) denota o subgênero *Viannia*. [2] DTH refere-se a uma resposta de hipersensibilidade tardia ao antígeno de *Leishmania*, um teste que também é chamado de teste cutâneo de *Leishmania* (LST) ou teste de Montenegro.Fonte: Adaptado de Scorza, Carvalho & Wilson, 2017.

Os parasitos causadores da leishmaniose apresentam ciclo heteroxênico e duas formas evolutivas distintas: promastigota e amastigota. A promastigota é a forma extracelular, flagelada e móvel. Enquanto que, a forma amastigota é obrigatoriamente intracelular, imóvel e sem flagelo livre (figura 5). A transmissão da leishmaniose para homem ocorre através da picada de flebotomíneos fêmeas do gênero *Lutzomyia* (novo mundo) e *Phlebotomus* (velho mundo), que atuam como vetores no processo de infecção.

Quando os flebotomíneos infectados realizam o repasto sanguíneo, injetam a forma promastigota metacíclica (forma infectante) no hospedeiro vertebrado. O sistema imune inato do hospedeiro na tentativa de combater o processo infeccioso, recruta macrófagos que fagocitam os parasitas. Estes permanecem internalizados no vacúolo parasitóforo, região em que ocorre a conversão da forma promastigota para amastigota e multiplicação celular (SUNTER & GULL, 2017). Após, intensa multiplicação, a membrana do macrófago é rompida e os amastigotas são liberados no tecido. Esses podem ser fagocitados por novos macrófagos ou serem ingeridos por flebotomíneos durante novo repasto sanguíneo. Caso seja ingeridos pelo vetor, no intestino médio do inseto, as formas amastigotas se diferenciam em promastigota metacíclica e migram para a região estomacal, onde ocorre a diferenciação para a forma infectante, quando o flebotomíneo se alimentar de um novo hospedeiro o ciclo evolutivo recomeça (TEIXEIRA et al., 2013).

Figura 5 – Ilustração do ciclo evolutivo da *Leishmania amazonensis*

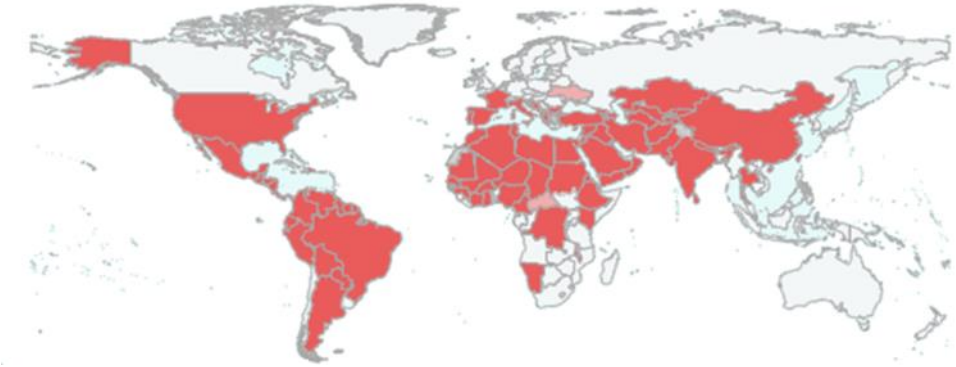


Fonte: Teixeira et al. 2013

2.4.1 Leishmaniose Cutânea (LC)

A Leishmaniose Cutânea (LC) também denominada de Leishmaniose Tegumentar (LT) foi descrita pela primeira vez no Velho Mundo pelos pesquisadores Lewis e Cunningham, em 1876. Mundialmente, 89 países são endêmicos para LC e 90 % dos casos ocorrem no Afeganistão, Argélia, Brasil, Paquistão, Peru, Arábia Saudita, Arábia e Síria, figura 6 (REITHINGER et al., 2007; WHO, 2019).

Figura 6 - Áreas endêmicas de Leishmaniose Cutânea



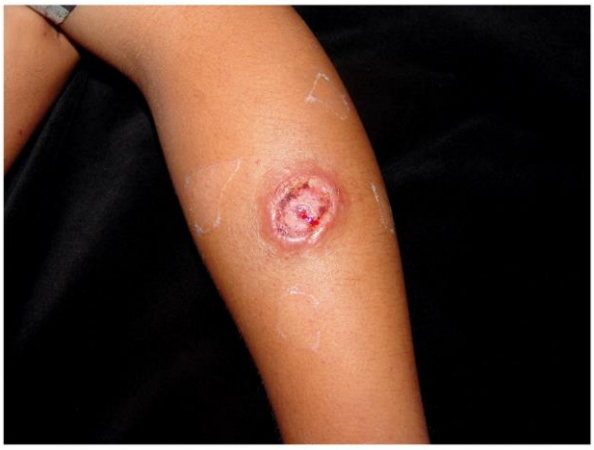
Fonte: WHO, 2018

As espécies comumente responsável pelos casos de leishmaniose cutânea no Brasil são a *Leishmania brasilienses* e *Leishmania amazonensis*. As diferentes apresentações clínicas da doença variam de acordo com a resposta imune do hospedeiro e aspectos do parasita; e com base nas formas clínicas a LC pode ser classificada em: localizada, difusa ou mucocutânea (MASMOUDI et al., 2013).

2.4.2 Leishmaniose cutânea localizada (LCL)

A LCL caracteriza-se por uma pápula eritematosa assintomática, com tamanho variando de 1 a 10 mm de diâmetro, apresentando região ulcerada e bordas nodulares grossas ou pontiagudas (figura 7). As regiões do corpo mais acometidas são: orelhas, nariz, lábios, bochechas, pernas, mãos e antebraços, cujo período de incubação pode variar de 1 a 4 semanas (TORRES-GUERRERO, 2017).

Figura 7 - Lesão de Leishmaniose Cutânea Localizada



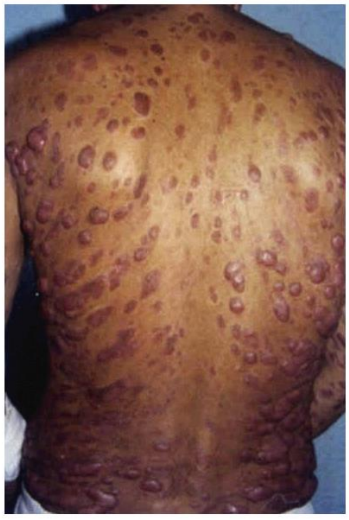
Fonte: Torres-Guerrero et al. (2017)

A LC pode curar espontaneamente em até 4 anos, quando isso acontece, a cura progride da periferia para o centro da lesão. A cicatrização espontânea deixa uma placa com pigmentação irregular e cicatrizes retráteis, bem como deformidade local devido à grande extensão do dano tecidual. Por outro lado, quando não há uma resposta imune celular do hospedeiro frente à infecção pode haver uma piora do quadro e estabelecimento da Leishmaniose Cutânea Difusa (TORRES-GUERRERO et al.2017; ANVERSA et al., 2018).

2.4.3 Leishmaniose Cutânea Difusa (LCD)

A LCD é decorrente da ausência de resposta imune frente aos antígenos do parasita, o que resulta na disseminação tecidual, linfática e sanguínea. As lesões são caracterizadas por múltiplos nódulos ou pápulas que se desenvolvem lentamente e sem ulcerações, envolvendo todo corpo e em alguns casos mucosas. Nessa forma clínica de leishmaniose não há cura espontânea e casos de recidivas são frequentes após o tratamento, figura 8 (HAILU, DAGNE & BOELAERT, 2016**).**

Figura 8 – Lesão de Leishmaniose Cutânea Difusa

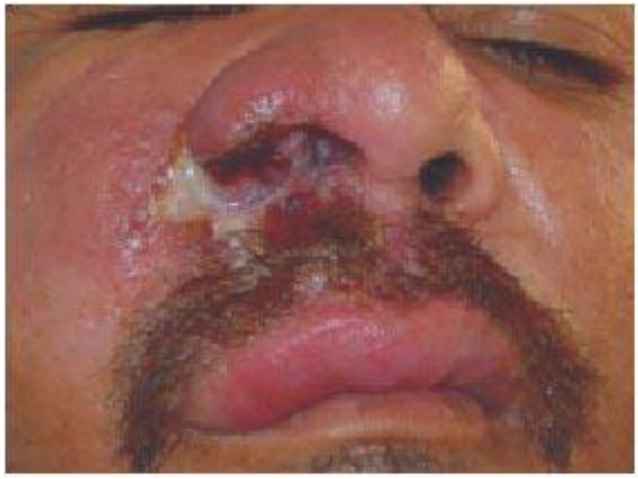


Fonte: Torres-Guerrero et al. (2017)

2.4.4 Leishmaniose mucocutânea (LMC)

A LMC têm como principal característica o acometimento de mucosas, principalmente a mucosa nasal e oral. Os sintomas iniciais são inespecíficos, dificultando o diagnóstico. A princípio as lesões são superficiais e progridem lentamente formando crostas com aspecto necrótico que levam a desfiguração facial do palato, septos nasais e lábios (Figura 9), ocorre também linfadenopatia acentuada e, quando não tratada, pode levar à morte (GOTO & LINDOSO, 2012; STEVERDING, 2017).

Figura 9 – Lesão de Leishmaniose Mucocutânea

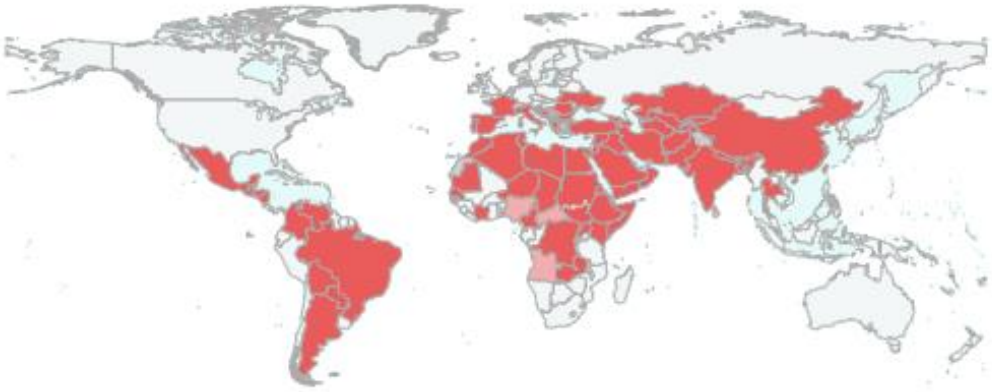


Fonte: Gomes et al. (2004)

2.5 LEISHMANIOSE VISCERAL – LV

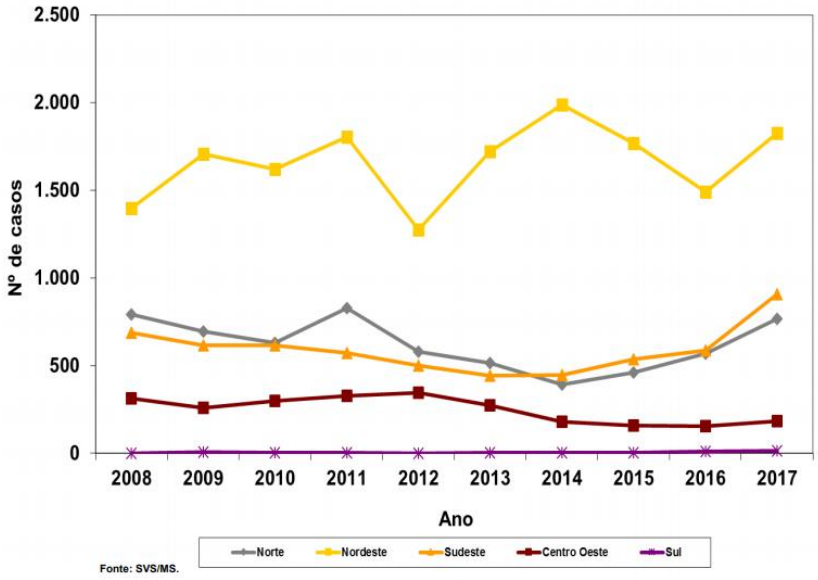
A LV também conhecida como Calazar é a forma mais grave da leishmaniose, na maioria dos casos é fatal. Cerca de 76 países são endêmicos, e no continente americano em pelo ao menos 12 deles (Figura 10). No Brasil é descrita em vários munícipios, com média de 3500 casos registrados anualmente e coeficiente de incidência de 2,0 casos/100.000 habitantes. De acordo com dados do Ministério da Saúde (OMS), a região nordeste apresenta uma grande prevalência de caso ao longo dos anos, gráfico 1 (MINISTÉRIO DA SAÚDE, 2017; WHO, 2018).

Figura 10 - Áreas endêmicas de Leishmaniose Visceral



Fonte: WHO (2018).

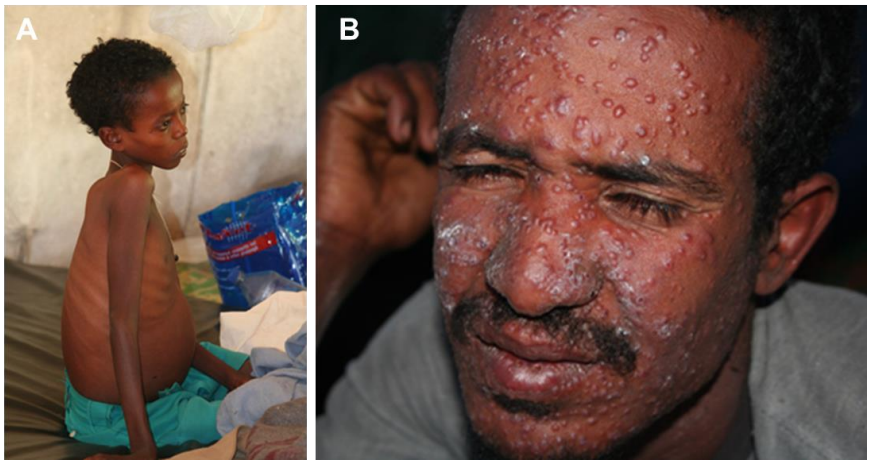
Gráfico 1- Casos de Leishmaniose Visceral por regiões brasileiras, 2008 a 2017



Fonte: Ministério da Saúde (2017).

A LV é causada pelas espécies *Leishmania donovani (*na Índia e África) e *Leishmania infantum* (Mediterrâneo e Américas). Manifesta-se como uma doença sistêmica, que, quando não tratada, pode evoluir para óbito em mais de 90 % dos casos (MINISTÉRIO DA SAÚDE, 2016). As manifestações clínicas caracteriza-se por febre prolongada, pancitopenia, linfadenopatia, perda de peso, hepatomegalia, esplenomegalia, anemia e globulinemia (SUDAN et al., 2019). A LV pode evoluir, se não tratada para uma forma crônica denominada leishmaniose dérmica pós-kala-azar (PKDL) caracterizada por nódulos endurecidos ou máculas despigmentadas, Figura 11 (SUNDAR & CHAKRAVARTY, 2018).

Figura 11 - Leishmaniose Visceral e Leishmaniose dérmica Pós-kala-azar



A - Leishmaniose Visceral; B - Leishmaniose dérmica Pós-kala-azar
Fonte: WHO (2020).

2.6 DIAGNÓSTICO E TRATAMENTO

O diagnóstico da leishmaniose envolve aspectos clínicos e laboratoriais. O diagnóstico laboratorial direto é realizado através da identificação microscópica de amastigotas em esfregaços de biópsias, raspagens ou impressões corados com Giemsa. Os testes sorológicos para detecção de anticorpos *anti-leishmania* como a reação intradérmica de Montenegro, Elisa, imunofluorescência, citometria de fluxo, testes rápidos e testes de aglutinação são métodos imunológicos para detecção do parasito. A detecção de DNA do parasita por PCR é um método bastante sensível, no entanto, não é uma prática em países em desenvolvimento (ELMAHALLAWY et al., 2014). O tratamento da Leishmaniose baseia-se em três classes de

medicamentos: antimoniais pentavalentes, pentamidina e anfotericina B. No entanto, todos os quimioterápicos disponíveis apresentam limitações, dentre elas: alta toxicidade, reações adversas e casos de resistência, como demonstra a tabela 3 (MENEZES et al., 2015; SAVOIA, 2015; KASHANI, KHAMESIPOUR & FIROOZ, 2018).

Tabela 3 – Medicamentos utilizados no tratamento da Leishmaniose

Medicamento	Via de Administração	Toxicidade	Comentário
Antimoniais pentavalentes	IM IV IL	Cardiotoxicidade grave, Pancreatite, Nefrotoxicidade, Hepatotoxicidade	Medicamentos de primeira linha, mas com altas incidências de resistência; resposta variável em diferentes espécies que causam LC
Anfotericina B	IV	Nefrotoxicidade grave, Reações relacionadas à infusão, hipocalemia, febre alta	Toxicidade grave; necessidade de hospitalização prolongada; medicamento de primeira linha para LV na Índia, onde há resistência antimonial
Anfotericina B lipossomal	IV	Rigidez e calafrios leves durante a infusão Nefrotoxicidade leve (infreqüente e leve)	Alto custo
Miltefosina	Oral	Vômitos e diarréia, nefrotoxicidade, hepatotoxicidade, teratogenicidade	Eficaz por via oral, mas sua meia-vida longa pode incentivar o surgimento de resistência em uso prolongado; eficaz para LV e contra algumas espécies que causam LC;

			contraindicado na gravidez como teratogênico em ratos
Paromomicina	IM (LV) ou tópico (LC)	Nefrotoxicidade grave, Ototoxicidade, Hepatotoxicidade	Baixo custo; falta de eficácia na África Oriental; formulação tópica disponível para CL
Pentamidina	IM	Alta taxa de hiperglicemia, como resultado de dano pancreático; hipotensão, taquicardia e alterações eletrocardiográficas	Apenas para formas específicas de CL na América do Sul; primeira linha de tratamento de CL na Guiana Francesa

IV: administração intravenosa; IM: administração intramuscular; IL: administração intralinfática.

Fonte: Adaptado de Menezes et al. (2015)

Não existe vacinas para a leishmaniose e a terapia medicamentosa apresenta muitas limitações. Nesse contexto, a busca por alternativas terapêuticas eficazes, menos tóxicas e de baixo custo para o tratamento da CL é necessária. Assim, a prospecção de produtos naturais para o tratamento de doenças parasitárias consiste numa medida promissora para investigação e desenvolvimento de formulações úteis no tratamento da CL (Luna et al., 2019; Soni et al., 2019).

3 METODOLOGIA

3.1 OBTENÇÃO DO ÓLEO ESSENCIAL

Os frutos de *Myrciaria floribunda* foram coletados na cidade de Exú, estado de Pernambuco, Brasil, em 2018 (Latitude:-7.355809029182327; longitude:-39.887568331218304). A espécie foi identificada pelo botânico Dr. Alexandre Gomes da Silva e o voucher 92722 foi depositado no Herbário do Instituto Agronômico de Pernambuco (IPA). A extração do MfEO foi realizada pelo método de hidrodestilação (aparelho de Clevenger). Para obtenção do óleo 200 g da casca do fruto foram misturados a 2,5 L de água destilada e submetido a hidrodestilação por 3 horas. Após a extração, o óleo foi armazenado a 5ºC.

3.2 CARACTERIZAÇÃO QUÍMICA

A análise química do óleo essencial foi realizada por cromatógrafo gasoso Thermo Trace GC Ultra (Thermo Scientific, Milão, Itália), através de um detector de ionização de chama (FID). Uma coluna capilar de sílica fundida VB-5 5 (ValcoBond 30 m × 0,25 mm x 0,25 mm) foi utilizada para separação dos constituintes (Valco Instruments Company Inc., Houston, TX, EUA) e nitrogênio a uma vazão de 1 L/min e pressão de entrada de 30 psi foi utilizado como gás de arraste. O gradiente inicial de temperatura foi 40ºC durante 2 min, e aumento de 4ºC/min até 230ºC foi mantido durante 5 min. As temperaturas do injetor e detector foram ajustadas para 250 °C e 280 °C, respectivamente. A amostra (1µl) foi injetada e a quantidade relativa de cada componente estimada pela área do pico correspondente e expressa como uma porcentagem da área total do cromatograma. Os espectros de massa foram realizados a 70 eV (no modo EI) com uma velocidade de 1.0 varreduras de m / z 35-350. A identificação dos componentes individuais foi realizada em comparação com os valores de índices de retenção de uma série de n-alcanos de acordo com a literatura (VAN DEN DOOL E KRATZ, 1963); os dados disponíveis na biblioteca espectral de massa do sistema GC-MS (MassFinder 4, Hochmuth, Hamburgo, Alemanha); Biblioteca Espectral de Massa NIST08 (ChemSW Inc. Fairfield, CA, EUA); Wiley Registry ™ da Relação Espectral de Massa 9ª Edição (Wiley, Hoboken, NJ, EUA) e dados espectrais de massa publicados (ADAMS, 2017).

3.3 ANÁLISES *IN SILICO*

3.3.1 ADMET

Para prever as propriedades farmacocinéticas semelhantes a drogas dos compostos majoritários do MfEO, foram utilizadas as seguintes plataformas on-line: Swiss ADME, Swiss Target Prediction (SIB., Lausanne, Suíça) PROTOX-II Server (Universidade Charité, Berlim, Alemanha), Molinspiration (Molinspiration Chemin formatics, Nova Ulica, República Eslovaca), Osiris Property Explorer, Osiris Data Warrior (Actelion Pharmaceuticals Ltd., Allschwil, Suíça) e pKCSM (Pires, Blundell e Ascher 2015). Informações sobre o medicamento de referência, a Neostigmina (PuChem CID: 4456), utilizada como parâmetro comparativo para as análises foram obtidas no banco de dados PubChem (https://pubchem.ncbi.nlm.nih.gov/).

3.3.2 Docking Molecular

A abordagem de docking molecular utilizada neste estudo teve como objetivo identificar a ligação dos compostos majoritários com enzimas envolvidas em doenças neurodegenerativas (acetilcolinesterase PDB:4PQE) e Leishmaniose (esterol 14-alfa desmetilase PDB:3L4D). As estruturas correspondentes às proteínas foram obtidas no Protein Data Bank (Berman, Henrick and Nakamura 2003) e os resultados sobre os valores de energias de ligação, eletrostática, intermolecular e superfície de interação foram determinadas e analisadas usando o algoritmo genético Lamarckiano (López-Camacho et al. 2015).

3.4 ATIVIDADE DE INIBIÇÃO ENZIMÁTICA

A avaliação do potencial de inibição enzimática da acetilcolinesterase foi determinada em microplaca por método espectrofotométrico (ASSIS et al., 2012; SOUZA et al.,2018). As enzimas utilizadas para os ensaios foram acetilcolinesterase (AChE) extraída de *Crassostrea rhizophorae* e AChE comercial *Electrophorus electricus* (Sigma). A incubação foi realizada adicionando 10 µl da AChE e 10 µl do MFEO em diferentes concentrações (0,0001 a 1 mg/ml) considerando-se a ausência de óleo (0,0 mg/mL) como controle negativo (100% de atividade). Foram utilizados como branco DMSO + tampão (Tris-HCl 0,5 mM pH 8,0); e Neostigmina como controle positivo (inibidor padrão). A placa foi incubada por 1h em temperatura de 25ºC. Após, o período de incubação foram adicionados 200µl do reagente cromogênico DNTB

(0,25mM) e 20 µl do substrato (ASCh) a leitura foi realizada em comprimento de onda de 405 nm nos tempos 0 e 180 segundos. O percentual de inibição enzimática foi determinado com base nos controles. O ensaio foi realizado em quadruplicata e os cálculos utilizando o programa Microsoft Excel.

3.4 ENSAIO DE CITOTOXICIDADE

O potencial citotóxico do MfEO foi avaliado de acordo com o protocolo estabelecido por Aliança, et al. (2017) com pequenas modificações. Resumidamente, os modelos de células Vero e macrófago J774A.1 foram plaqueados ($5x10^5$ células/poço) em placa de 96 poços em meio 100 µL de meio RPMI, suplementado com 10% SFB inativado e incubado após 3h a 37º C em CO_2 a 5%. Após esse período, as células não aderentes foram removidas, as células Vero e os macrófagos aderidos foram cultivados por 48 h em RPMI na ausência ou presença de diferentes concentrações do MfEO (31.25 a 500 µg/mL). Posteriormente, as células tratadas e não tratadas foram lavadas e incubadas em meio de cultura RPMI com 5 mg/mL de brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT, Sigma-Aldrich, St. Louis, MO, EUA), por 3 horas a 37 ° C. Em seguida, o precipitado de cristais de formazan derivado da redução do MTT foi solubilizado em solução de isopropanol (100 µL / poço) e a leitura das absorbâncias da placa foi avaliada em espectrofotômetro a 540 nm. A concentração citotóxica do MfEO (CC_{50}) foi determinada por análise de regressão utilizando o software SPSS 8.0.

3.5 ATIVIDADE LEISHMANICIDA

3.5.1 Efeito do MfEO em formas promastigotas

Para avaliar os efeitos do MfEO nas formas promastigotas de *Leishmania amazonensis* (LTB0016), 10^6 parasitas/mL foram cultivados em meio Schneider suplementado com 10 % SFB, na ausência ou presença de diferentes concentrações do MfEO (25 a 200 µg/mL) e incubados à 26 ºC por 48 h. A viabilidade celular dos parasitas foi avaliada pelo kit CellTiter-Glo® Luminescent Cell Viability Assay nos tempos 24 e 48 h, e a concentração do MfEO capaz de inibir 50 % do crescimento celular (IC_{50}) foi determinada por análise de regressão usando o software SPSS 8.0.

3.5.2 Efeito do MfEO em formas amastigotas

Para verificar a ação do MfEO sobre as formas amastigotas intracelular de *L. amazonensis,* os macrófagos J774A.1 (1 x 10^6 células/mL) foram plaqueados em placa de 24 poços contendo meio RPMI com 10 % SFB. Deixou-se os macrófagos aderirem durante 3 horas a 37°C em 5% de CO_2, e posteriormente foram infectados com promastigotas de *L. amazonensis* utilizando uma razão 20:1 de parasitas/ célula a 37 °C durante 14 horas. Os parasitas não-interiorizados foram removidos por lavagem e as células aderidas foram incubados por 24 h nas condições de incubação estabelecidas anteriormente com 1/8 X, ¼ X, ½ X e 1X o valor do IC_{50} do MfEO para as formas promastigotas. Células não tratadas e células tratadas com os fármacos de referência (antimoniato de meglumina e anfoterina B) foram utilizados como controles. Após, o período de incubação, as células foram lavadas em PBS, fixadas com álcool e coradas com coloração panótica; e a porcentagem de células infectadas foi determinada pela contagem de 150 macrófagos aleatoriamente em duplicada. O índice de sobrevivência foi determinado pela multiplicação da porcentagem de macrófagos infectados pelo número médio de parasitas por célula infectada e a IC_{50} do MfEO para as formas amastigotas foi determinada por análise de regressão como já descrito. O índice de seletividade (SI) do MfEO para promastigotas e amastigotas foi determinado pela razão da CC_{50} em macrófagos e a IC_{50} para as duas formas dos protozoários.

3.6 ANÁLISE ULTRAESTRUTURAL

Para verificar as alterações na ultraestrutura, promastigotas de *L. amazonensis* foram tratadas com 1x e 2x o valor da IC50, por 48h. Como controle, foram utilizadas células não tratadas. Posteriormente, as células foram fixadas por 2h em temperatura ambiente em uma solução contendo 2.5% de glutaraldeído / 4% paraformaldeído em 0,1 M de tampão fosfato pH 7.2. Após lavagem no mesmo tampão, as células foram pós-fixadas durante 1 h com 1% de tetróxido de ósmio / 0.8% de ferricianeto de potássio / $CaCl_2$ 5 mM em tampão cacodilato 0.1 M, pH 7.2. Após lavagem com tampão cacodilato, as células foram aderidas em lamínulas revestidas com poli-L-lisina. As amostras foram desidratadas em etanol, secas em ponto crítico HCP-2 (Hitachi, Tóquio, Japão), revestidas com uma camada de ouro de 20 nm de espessura e observadas em um Microscópio Eletrônico de Varredura JEOL T-200.

4 RESULTADOS

O resultado desta pesquisa gerou um segundo artigo que será submetido em um Journal de Fitoterapia.

4.1 The biological activity of essential oil from fruit peel of *Myrciaria floribunda* (H. West ex Willd.) O. Berg on *Leishmania amazonensis*

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Abstract

Cutaneous Leishmaniasis (CL) is a neglected disease characterized by high rates of morbidity and mortality worldwide. The treatment available for CL has several limitations including serious side effects and resistance to treatment**.** Herein we aimed to evaluate the activity of essential oil from the peel of *Myrciaria floribunda* fruits (MfEO) on *Leishmania amazonensis*. The cytotoxic potential of MfEO on host mammalian cells was evaluated by MTT. *In vitro* leishmanicidal effects of MfEO were investigated on promastigote and intracellular amastigote forms. The ultrastructural changes induced by MfEO were evaluated by Scanning Electron Microscopy (SEM). Molecular docking of δ-cadinene and γ-cadinene, the major compounds of MfEO onto the enzyme sterol 14-alpha demethylase (CYP51) was analyzed. Our results showed that MfEO presented a CC_{50} of 260.9 and 332.9 µg/mL for Vero and macrophages, respectively. The MfEO inhibited the growth of promastigote and the survival of intracellular amastigotes, with IC_{50} of 55.84 and 7.54 µg/mL, respectively. MfEO presented high selectivity towards amastigote forms, being 44.1 times more toxic for this form than to macrophages. The Molecular docking analysis showed that both major compounds interacted with the active site of *Leishmania* CYP51, suggesting a putative role of cadinenes as inhibitor of CYP51 of this parasite.

Keywords: Cutaneous Leishmaniasis; *Leishmania amazonensis;* essential oil; *Myrciaria floribunda;* phytotherapy; molecular docking

1. Introduction

Leishmaniasis, caused by protozoa of the genus *Leishmania*, are severe neglected tropical diseases (NTDs), responsible for high rates of morbidity and mortality. These diseases are dispersed worldwide, being present in 89 countries, with 90% of cases occurring in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia and Syria (WHO, 2020; Valero & Uriarte, 2020). Depend on the species of *Leishmania* involved and the immune response of the host, leishmaniasis can assume different clinical manifestations ranging from the localized cutaneous to the visceral form with potentially fatal outcomes (Torres-Guerrero et al, 2017). Cutaneous Leishmaniasis (CL) stands out for its high prevalence and potential to cause physical deformities (Holanda et al. 2019). In Brazil, *Leishmania amazonensis* is considered the most relevant epidemiological specie, due to its wide geographical distribution and its ability to cause different forms of cutaneous leishmaniasis (Christensen et al., 2019).

Currently, there are no vaccines for leishmaniasis and the chemotherapy for the treatment of this illness is still based on the parenteral administration of pentavalent antimonials (Menezes et al., 2015). Meglumine antimoniate and sodium stibogluconate are the first-line drugs. However, these drugs have various disadvantages, such as a long therapeutic regimen, variations in effectiveness, appearance of resistance (Sundar, Chakravarty & Meena, 2018) and severe side effects, including cardiotoxicity, hepatotoxicity and nephrotoxicity (Scott & Novais, 2016; Taheri, Rad & Molkara, 2019; Marques et al., 2019). Pentamidine and Amphotericin B have been used as second-line drugs. However, these drugs also present high toxicity and adverse side effects that limit their use (Iqba et al., 2016; Oliveira et al., 2017). In this regard, the search for effective, less toxic and low-cost therapeutic alternatives for the treatment of CL is still necessary.

Natural products have been used for the treatment of infectious disease since ancient times, especially in developing countries (García et al., 2017). Among them, Essential Oils (EOs) have been shown promising bio/pharmacological properties, including antimicrobial (Santana et al., 2018; Correa et al., 2019), anti-inflammatory (Han & Parker, 2017), antioxidant (Santos et al., 2019; Hennia et al., 2020) and leishmanicidal (Medeiros et al., 2011; Ramos et al., 2014) activities. Furthermore, the hydrophobic nature of EOs constituents makes them permeable to cells, which is a desirably feature for developing agents against intracellular pathogens (García et al., 2017). EOs are a complex mixture of compounds obtained from different plants parts, such as flowers, leaves, fruits, roots and seeds. EOs extracted from the

Myrtaceae family have attracted attention due to their promising pharmacological properties (Durazzini et al., 2019; Kauffmann et al., 2019).

Myrciaria floribunda (H. West Ex Willd.) O. Berg, a plant from Myrtaceae family, popularly known as Cambuí, presents insecticidal (Tietbohl et al., 2014; Tietbohl et al., 2019), antibacterial and antioxidant activities (Azevedo et al., 2019). This plant also has demonstrated to inhibit the enzymes acetylcholinesterase from *Electrophorus electricus* and *Crassostrea rhizophorae* (Tietbohl et al., 2012; Barbosa et al., 2020). In a previous study we have elucidate the chemical composition of EO from fruit peels of *Myrciaria floribunda* (MfEO) and demonstrated the good pharmacokinetic and toxicological profiles of their major constituents (Barbosa et al., 2020). However, the knowledge on the biological potential of the MfEO against trypanosomatids is lacking and, at the best of our knowledge, there is no reports on the use of MfEO against leishmaniasis. Therefore, in this study we investigated for the first time, the leishmanicidal activity *Myrciaria floribunda* essential oil on promastigote and amastigote forms of *L. amazonensis*. We also used molecular docking approach to model putative interactions of their main compounds onto the sterol 14-alpha demethylase, a key enzyme for *Leishmania* survival and replication.

2. Material and Methods

2.1 Essential Oil - *Myrciaria floribunda* fruits were collected at Exú municipality, in the state of Pernambuco, Brazil (voucher number: 92722). The MfEO was extracted from fruit peels by hydrodistillation using a Clevenger apparatus, as described previously (Silva et al., 2015). The chemical characterization was performed through GC/MS analysis, as described by Barbosa et al. (2020). For *in vitro* analysis, MfEO was initially dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA), at a concentration of 50 mg/mL and stored at -20°C, protected from light. This stock solution is diluted again to obtain a work solution, at final concentration of DMSO never exceeding 0.1 % v/v that is considered as nontoxic for parasites and mammalian cells.

2.2. Mammalian Cells - Vero Cells and macrophages were maintained by weekly passages in RPMI medium supplemented with 10% inactivated bovine fetal serum (iFBS) and 1% streptomycin-penicillin solution (Sigma-Aldrich, USA), at 37 ° C and 5% CO_2 atmosphere.

2.3 Cytotoxicity Assay - The cytotoxic potential of MfEO was evaluated according to the protocol established by Aranda-Souza, et al. (2019). Briefly, Vero cells or macrophages were seeded in 96-well plates (5×10^5 cells/well) containing 100 µL of RPMI medium, supplemented with 10% of iFBS, and incubated for 3 hours at 37 °C in 5% CO_2. After this time, non-adherent cells were washed out and the remaining adhered cells were incubated for 48 hours in RPMI in the absence or presence of the different concentrations of MfEO (31.25 to 500 µg/mL). Treated and untreated cells (negative control) were washed and incubated in fresh RPMI culture medium, containing 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA), for additional 3 h at 37 °C. After the incubation, the cells were solubilized in DMSO (100 µL/well) and the formazan precipitate derived from MTT reduction was determined spectrophotometrically at 540 nm. The concentration able to reduce the cell viability by 50% (CC_{50}) was determined by regression analysis using the software SPSS 8.0 for Windows.

2.4 Leishmanicidal Activity Assay - Promastigote forms of *Leishmania amazonensis* (LTB0016), were cultured in Schneider's medium supplemented with 10% iFBS, in the absence (control) or presence of different concentrations of MfEO (25-200 µg/mL) and incubated at 26 ºC for 48 hours. The cell viability of the parasites was evaluated by the CellTiter-Glo® Luminescent Cell Viability Assay kit at 24 and 48 hours. The concentration of MfEO able of inhibiting 50% of cell viability (IC_{50}) was determined as described for mammalian cells. To investigate the effects of MfEO on the intracellular amastigote forms of *L. amazonensis*, J774A.1 macrophages (1×10^6 cells/mL) were seeded in 24-well plates containing a coverslip on the bottom. The cells were cultivated in RPMI medium, supplemented with 10% iFBS. Adhered macrophages were infected with *L. amazonensis* promastigotes (20:1 promastigotes/macrophage) at 37 °C for 14 hours. The non-internalized parasites were removed by washing in RPMI and the samples were incubated in the absence or presence of MfEO for 24 hours, at concentrations ranging from $^1/_8$ IC_{50} to IC_{50} values, obtained for promastigote forms. Infected cells treated with the reference drugs (Meglumine antimoniate or Amphoterin B) were used as positive controls. After treatment, the cells were washed in PBS, and submitted to panoptical fast staining kit (Laborclin, São Paulo, Brazil). The percentage of infected cells was determined by counting of 150 randomly chosen macrophages in duplicate. The survival index was determined by multiplying the percentage of infected macrophages by the average number of parasites per infected cell. The IC_{50} for amastigote forms was determined by regression analysis as described above. The selectivity index (SeI) of MfEO for promastigotes

and amastigotes was determined by the ratio of CC_{50} macrophages to IC_{50} of these developmental stages of *L. amazonensis*.

2.5 Ultrastructural assay **-** To verify the effects of MfEO on the ultrastructure of *L. amazonensis*, promastigote forms were treated with 1x and 2x IC_{50} value of MfEO for 48 hours. Untreated promastigotes were used as a control. After incubation with this essential oil, the parasites were fixed for 2 hours at room temperature in a solution containing 2.5% glutaraldehyde/ 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2. After washing in the same buffer, the cells were post-fixed for 1 hour with 1% osmium tetroxide/0.8% potassium ferrocyanide/5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. The parasites were then washed in 0.1 M cacodylate buffer and allowed to adhere to coverslips, previously coated with poly-L-lysine. The samples were dehydrated in ethanol, dried at a critical point HCP-2 (Hitachi, Tokyo, Japan), coated with a 20 nm thick gold layer and observed in a JEOL T-200 Scanning Electron Microscope.

2.6 Molecular Docking **-** The X-ray crystallographic structures of sterol 14-alpha demethylase) from *Leishmania infantum* (Lepesheva et al., 2011), obtained from protein databank (PDB), was used in our docking analysis. To verify the homology of *Li*CYP51 from *L. infantum* and hypothetical proteins of *L. amazonensis*, the FASTA sequence of *Li*CYP51, (PDB: 3L4D) was used for local alignment of the protein sequence using BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul, 2005). The molecular docking of δ-cadinene and γ-cadinene onto *Li*CYP51 was carried out using AutoDock 4.2 software. The smiles from chemical structure of cadinenes were applied to obtain the compounds in PDB format, using the Open Babel GUI program (https://openbabel.org/docs/dev/GUI/GUI.html). Water molecules were removed, hydrogens, Kollman, and Gasteiger charge were added. The protein and ligand structures were saved in the PDBQT file format**.** For each ligand-protein complex 10-docking poses were generated using Lamarckian Genetic Algorithm (López-Camacho et al., 2015). The pose with the lowest binding energy was selected as the final docking result. The interactions of the target-ligand complex were analyzed and rendered using Discovery Studio 2020 (Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016).

2.7 Statistical analysis**-** Linear regression analysis was performed using SPSS 8.0 software (IBM Co., New York, USA). Nonparametric data were analyzed using one-way analysis of

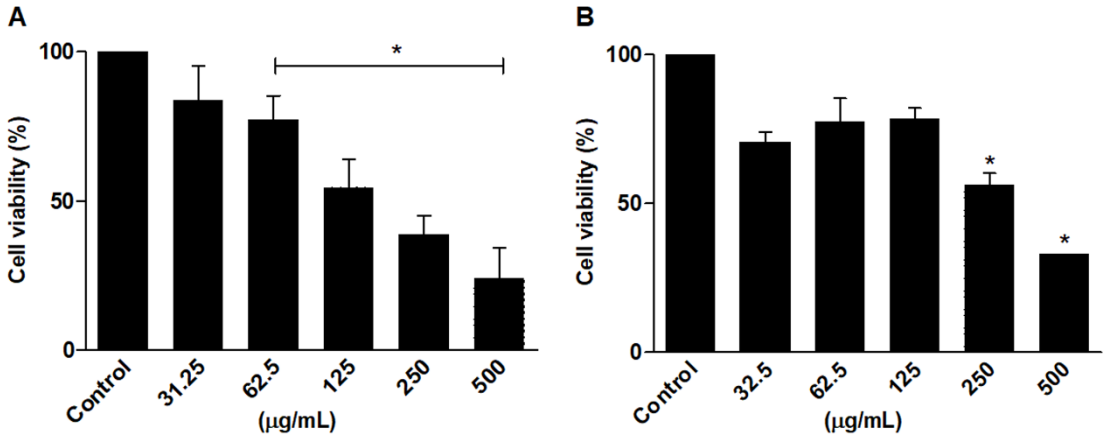
variance (ANOVA) followed by Bonferroni test. Statistical analysis was performed using the Prism 5.0 software (GraphPad, San Diego, CA, USA). All statistical analyzes were performed at the level of significance $p < 0.05$.

3. Results and Discussion

Essential oils are a mixture of compounds rich in active molecules, which present interesting biological characteristics for the development of new drugs against neglected diseases (Prado et al., 2018; Oliveira et al., 2020). In this study we exploited the *in vitro* potential of the essential oil from peel of *M. floribunda* fruits (MfEO) on *Leishmania amazonensis*, the main etiological agent of CL in Brazil.

One of the main desirable characteristics for a promising leishmanicidal compound is its selective toxicity towards the parasite without causing damage to the host cells (Santos et al., 2008). In this regard, the cytotoxic potential of MfEO on mammalian cells was evaluated in Vero cells and J774A.1 macrophages, using the MTT methodology. Our results showed that MfEO inhibited significantly the viability of both cell models (Figure 12), with a CC_{50} of 260.9 and 332.9 µg/mL, for Vero cell and macrophages, respectively (Table 4). According to Ríos et al. (2008), EOs having CC_{50} between 100 and 500 µg/mL are considered moderately toxic. Our results showed that macrophages were more resistant to the treatment with MfEO compared to the Vero cells. These results are particularly important because macrophages are the main cell target of *Leishmania* spp. infection. The higher resistance of macrophages to MfEO could be attributed to its well-known ability to neutralize potentially toxic compounds (Chowdhary, Chowdhary & Kashaw 2016; Wu & Lu, 2020).

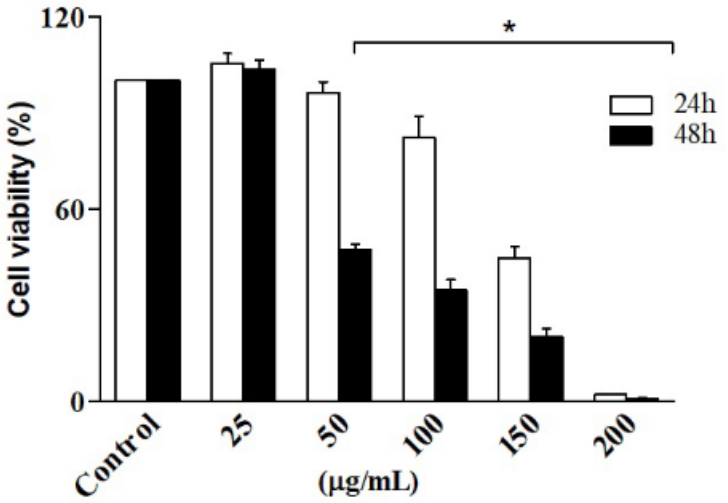
Figure 12. Effects of MfEO on mammalian cells after 48 hours of treatment. (A) Vero cell, (B) Macrophage. The bars represent the mean ± SD of three independent experiments in triplicate. * Significant difference compared to the control group ($p<0.05$).



Because MfEO presented moderate cytotoxicity to mammalian cells, we further investigated the effects of this essential oil on promastigote viability by measuring the ATP production by these cells. The quantification of the amount of ATP present is considered as a molecular indicator of metabolically active cells. The CellTiter-Glo® assay generated a "glow-type" luminescent signal produced by luciferase that was proportional to the percentage of living cells (El-Obeid et al., 2020). Our results showed that the treatment with MfEO inhibited significantly the parasite viability. Although there is no statistic difference compared to the control cells, at 25 µg/mL the viability of treated cells, mainly at 24 hours of cultivation, was slightly higher than untreated cells. This effected could be due to a shift on cellular metabolism in an attempt to circumvent the stress induced by treatment with EO (Monzote et al., 2018). After 24 hours of treatment, a significant decrease in the viability of promastigote forms was observed for concentration up to 100 µg/mL, whereas in cells treated for 48 hours, this inhibitory effect is earlier observed, for concentrations up to 50 µg/mL (Figure 13). The estimated IC_{50}/48 hours for promastigote forms was 55.84 µg (Table 4).

When the cytotoxicity of MfEO for mammalian cells was compared with the leishmanicidal effect on promastigote we found that this essential oil was almost 6 and 5 times more toxic for the parasite than to the macrophages and Vero cells, respectively. It is interesting to note that at the concentration of 200 µg/mL, the parasite viability was almost completely abolished (Figure 13).

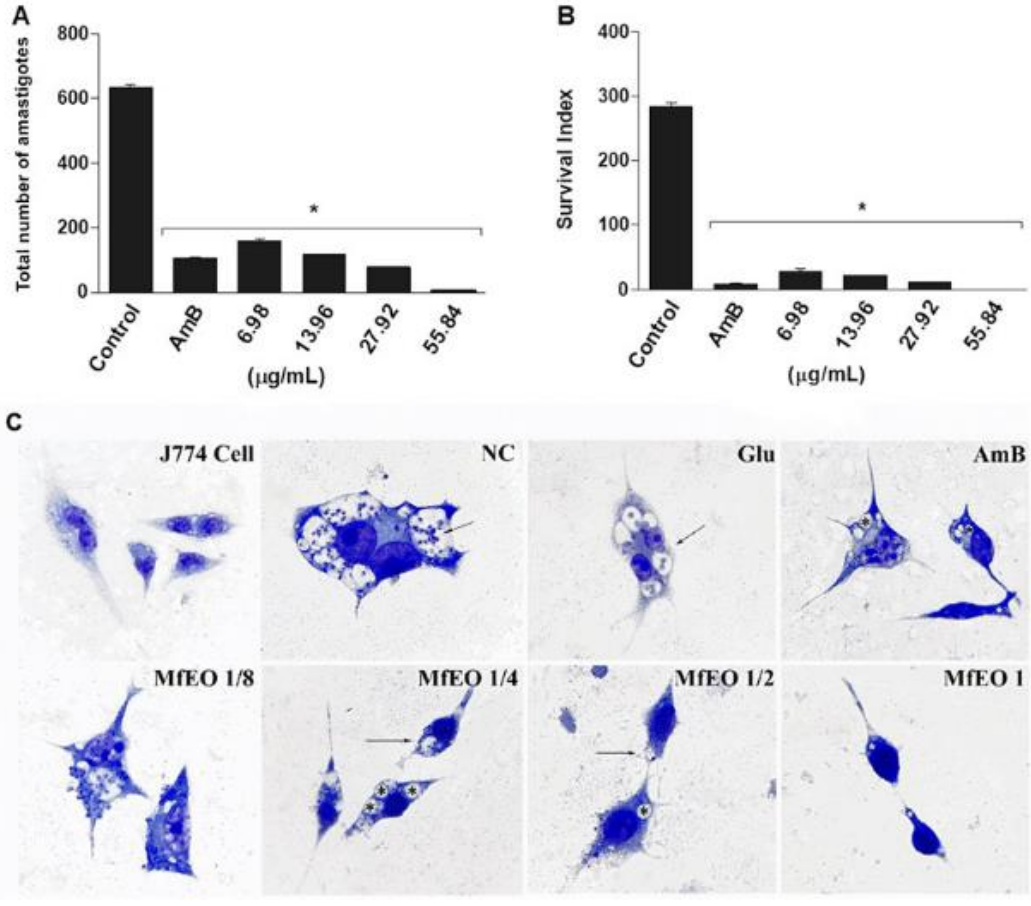
Figure 13. The effects of MfEO on promastigote forms of *Leishmania amazonensis* after 24 and 48 hours of treatment. The bars represented the mean ± SE of three independent experiments in triplicate. * Significant difference when compared to the control group (p<0.05).



In the view of our promising results of MfEO on the promastigote forms of *L. amazonensis*, we further investigated the action of this essential oil on the most relevant intracellular amastigote forms. Because amastigotes are responsible for the clinical manifestation of leishmaniasis in mammalian host, the evaluation of drug candidates on this developmental stage of parasite is the most effective way to relate the anti-leishmania activity *in vitro* and *in vivo* (Russel & Talamas-Rohana, 1989). The treatment of infected-macrophages with both MfEO and Amb significantly reduced the number of amastigotes inside these cells, in a dose-dependent way, when compared to untreated-infected ones (Figure 14A). At lower concentration of MfEO (6.98 µg/mL), the inhibitory effect on amastigotes was comparable to those found for amphotericin B (the reference drug) at a concentration of 9.75 µg/mL (Figure 14A-B). The inhibitory effect of MfEO on the macrophage infection by *L. amazonensis* is consistent with the decrease of survival index of amastigote inside these cells (Figure 14B). The lower values of Survival Index at higher concentration of MfEO (near to zero, at 55.84 µg/mL of MfEO) indicated that this essential oil was able, not only to significantly decrease the number of intracellular amastigotes, but also to reduce the number of infected cells. The effects of MfEO on macrophage infection could be better visualized by light microscopy (Figure 14C). The analysis of untreated-infected cells showed a high rate of infection with numerous internalized amastigotes inside large parasitophorous vacuoles (PVs). In some cells the number of amastigotes/PV was higher than 10 (Figure 14C). As expected, the treatment of infected cells with MfEO or the reference drugs Glucantime (Glu) and Amphotericin B (Amb), induced a

significant decrease in the number of amastigote/parasitophorous vacuole and consequently, in the rate of infection. At higher tested concentration of MfEO, the morphology of macrophages was compared with non-infected cells (Figure 14C) and no detectable PVs could be observed. The effects of MfEO on macrophage infection could be related to the direct action of essential oil components on the amastigote. However, we cannot rule out the possibility of MfEO is also acting on macrophages, making them more competent to fight the parasite (Ueda-Nakamura et al., 2006; Jihene et al., 2020).

Figure 14. Effect of MfEO on intracellular amastigotes forms of *L. amazonensis.* (A) Inhibitory effect of MfEO on the total number of amastigotes within infected-macrophages in 150 cells; (B) Survival index of amastigotes inside J774A.1 macrophages. Each bar represents the mean ± SD of three independent experiments performed in duplicate. (*) Significant difference compared to the control group ($p<0.05$). (C) Light microscopy or macrophage-infected cultures, stained with Giemsa treated or not (negative control, NC) with MfEO, Glucantime (Glu) or Amphotericin B (AmB). The arrows indicate the intracellular amastigote inside PVs. Note the presence of empty PVs in treated cells (black asterisk).



Our results showed that the MfEO was more selective for amastigotes than to macrophages, with SeI of 44.1 (Table 4). This value was considerably higher than those reported for reference drugs, which are very toxic to mammalian cells. Previous studies have shown that meglumine antimoniate (Glucantime) and Amphotericin B have low selectivity for *L. amazonensis* with SeI values of 2.46 and 0.15 respectively (Carvalho et al., 2017). The intracellular amastigote forms were also more susceptible to the treatment with MfEO than free-promastigotes, with an IC_{50} value of 7.54 µg/mL which was about 7.4 times lower than the IC_{50} value for promastigote.

It is usually assumed that the effect of EOs on trypanosomatids is due to the isolated or combined action of their major compounds. In a previous work we identified the sesquiterpenes δ-cadinene (26.8%), γ-cadinene (15.69%), γ-muurolene (6.21%), α-selinene (6.11 %), α-muurolene (6.11 %) and (E)-caryophyllene (5.54%) as the main constituents of MfEO (Barbosa et al., 2020). These compounds were also present in other EOs with leishmanicidal activity (Santos et al., 2008; Andrade et al., 2016; Macedo et al., 2020). The effect of MfEO on promastigotes and amastigotes may be related to the lipophilic nature its constituents, facilitating cell penetration and interaction with intracellular targets (Cortes et al., 2020). In our previous study, we used *in silico* approaches to predict the ADMET properties of the six most abundant constituents of MfEO. Our results showed that these molecules presented high lipophilicity with ClogP > 4.0 (Barbosa et al., 2020). This finding was particularly interesting because, to exert their effects on amastigotes, which live and proliferate intracellularly, the MfEO constituents must have to cross host cells membranes (plasma membrane and PVs) (Muylder et al., 2011).

Table 4. Leishmanicidal and cytotoxic effects of essentials oil of *M. floribunda.*

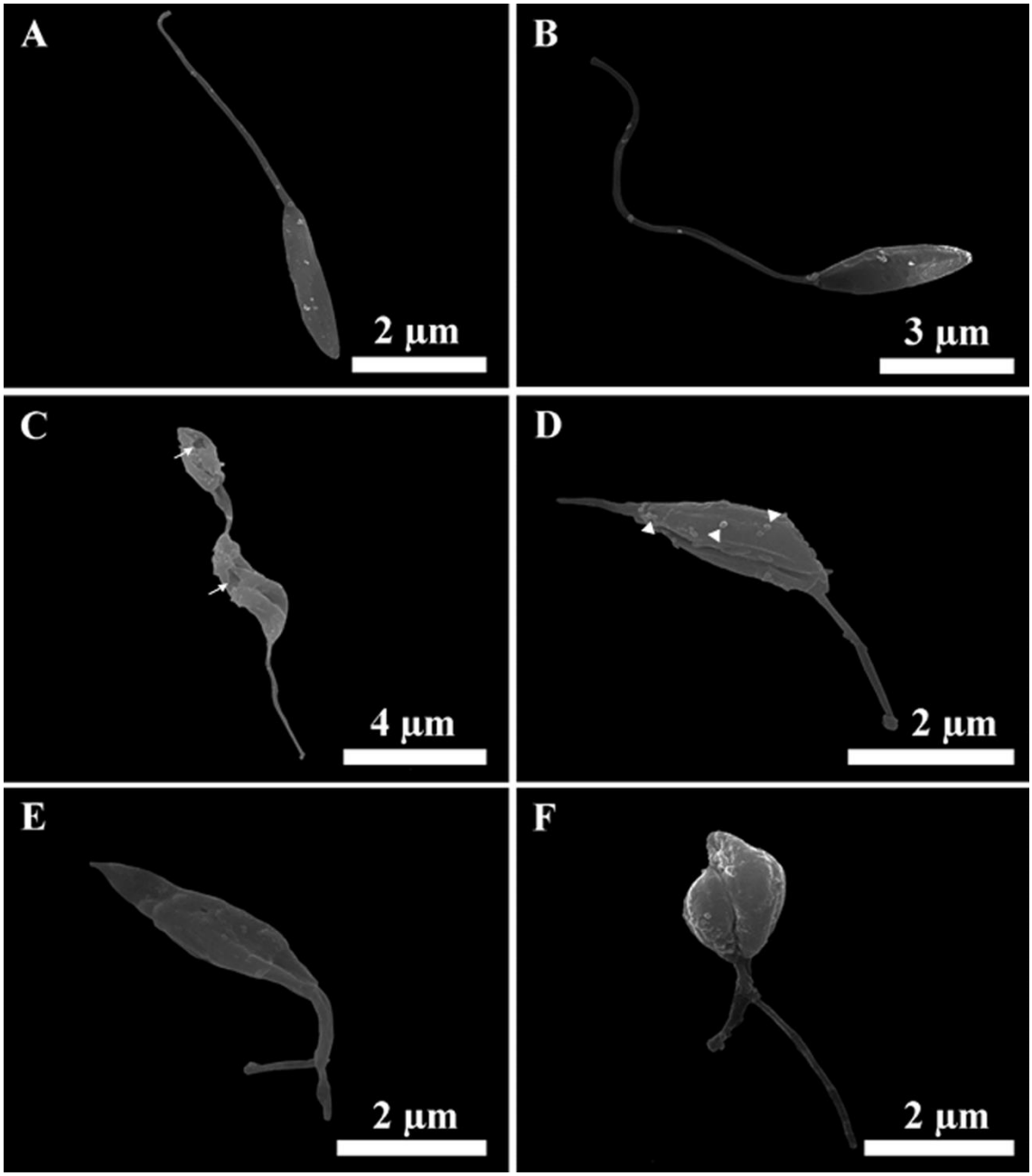
Cell Type	MfEO (µg/mL)				
	CC_{50}	IC_{50}		SeI	
		Pro	Ama	Pro	Ama
Vero	260.9 ± 6.3	-	-	4.67	n.d
J774	332.9 ± 13.1	-	-	5.96	44.15
L. braziliensis	-	55.84 ± 1.1	7.54 ± 0.6		

CC_{50} - concentration required to reduce cell viability by 50%; IC_{50} - Inhibitory concentration for 50% of parasites; SeI – Selectivity Index; Pro – promastigote; Ama - amastigotes; n.d. not

determined. The Data represent the mean ± SD of three independent experiments performed in triplicate.

It has been shown that terpenes, present in essential oils, are able to interact with liposaccharides, fatty acids, phospholipids of *Leishmania*, impairing key metabolic pathways for parasite survival (Díaz et al., 2019). In other to investigate the effects of MfEO on plasma membrane and overall ultrastructure of *L. amazonensis* promastigotes, scanning electron microscopy analysis was performed (Figure 15). Control cells showed typical morphology, with a spindle-shaped cell body, a preserved elongated free-flagellum and a smooth plasma membrane (Figure 15A-B). On the other hand, the treatment of with MfEO induced significant ultrastructural changes such as: the wrinkling of the plasma membrane, increased cell volume, with a shortening of both the cell body and flagellum (Figure 15C-F). In some drastically affected cells, it was possible to observe the presence of membrane perforations (Figure 15 C, arrow), indicative of damaged plasma membrane. Atypical dividing cells were also usually found in samples treated with MfEO (Figure 15E-4F), which is suggestive of arresting of cell cycle in cytokinesis stage (Silva et al., 2015). The injury in the plasma membrane can ultimately lead to loss of cell viability and parasite death (Tiuman et al., 2005; Silva et al., 2018). Treated cells presented higher exocytic activity when compared to untreated control cells (Figure 15C). The release of vesicles from the membrane can be stimulated by stress conditions. It has been reported that *L. amazonensis* promastigotes and *T. cruzi* epimastigostes budding blebs after treatment with enzyme inhibitors involved in sterol biosynthesis (Adade & Souto-Padrón, 2010).

Figure 15. The effects of MfEO on *Leishmania amazonensis* ultrastructure as observed by scanning electron microscopy. (A-B) Untreated promastigotes showing typical spindle-shaped morphology and elongated flagellum (C-D). Promastigotes treated with 55.84 μg/mL MfEO. (C) Note the presence of perforations on the plasma membrane (arrow). (D) Detail of exocytic vesicles budding from cell surface (arrowhead). (E-F) Atypical dividing promastigotes treated with 111.68 μg/mL MfEO.



Based on our ultrastructural results, we hypothesized that the major constituents of MfEO could be acting on key enzymes or other molecules that are essential for survival and proliferation of *L. amazonensis*. In the last decades, the identification and validation of biochemical pathways present in trypanosomatids but absent or divergent from their host has been provide promissory targets for the rational design of novel drugs against *Leishmania* spp. (Romero and López, 2017). Among these validated targets, the enzyme sterol 14-alpha demethylase (CYP51) have a pivotal role in the physiology of *Leishmania* spp. as well as other trypanosomatids (Mukherjee et al., 2020**)**. It has been shown that disruption of CYP51 results

in changes in the ultrastructure of several organelles, decline of endogenous sterols in the parasites, and an accumulation of various 14-alpha-methyl sterols with major cytostatic and cytotoxic consequences (Chen et al., 2010). Thus, in the search of putative inhibitor of this key enzyme, we decided to carry out a theorical molecular docking of δ- and γ-cadinene, the major compounds of MfEO, over CYP51. Because the crystal structure of CYP51 of *L. amazonensis* was not available on the protein data bank (PDB) we used the CYP51 of *L. infantum* (*Li*CYP51 protein data bank entry: 3L4D) as a template for molecular docking assays. Our BLAST analysis showed that this protein presented 97% of amino acid sequence similarity with two hypothetical orthologue protein of *L. amazonensis* (GenBank: BAE79802.1) (data not shown). Molecular docking analysis revealed that both isomers δ- and γ-cadinene presented higher binding energy with *Li*CYP51 of -7.17 e -7.31 kcal/mol, respectively when compared to fluconazole (-6.28 kcal/mol), a standard inhibitor of this enzyme (Table 5).

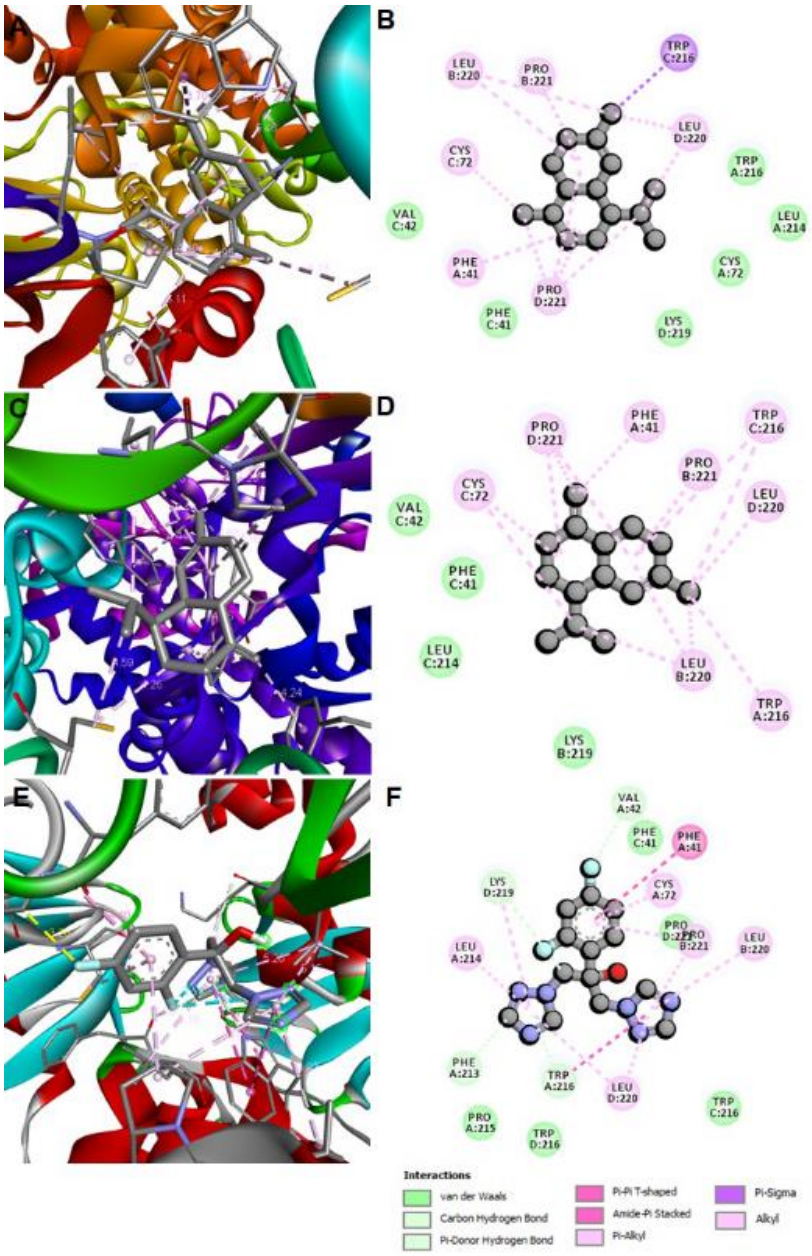
Table. 5. Interaction profile MfEO major constituents, δ-Cadinene (δ-Cad) and γ-Cadinene (γ-Cad) with the enzyme sterol 14-alpha demethylase (*Li*CYP51).

	CYP51		
	δ-Cad	γ -Cad	Fluconazole
BE (kcal/mol)	-7.17	-7.31	-6.28
LE	-0.48	-0.49	-0.29
IC (μM)	5.59	4.38	24.72
IE	-7.46	-7.61	-8.07
VDW-Hb (DE)	-7.46	-7.61	-8.04
EE	0.0	0.0	-0.03
TI	-0.31	-0.28	-1.70
TE	0.3	0.3	1.79
UnE	-0.31	-0.28	-1.70
Rseed 1/2	None	None	None

BE: binding energy; LE: ligand efficiency; IC: inhibition constant; IE: intermolecular energy; VDW-Hb (DE): van der Waals-Hydrogen bond (dissolve energy); EE: electrostatic energy; TI: total Internal; TE: torsional-energy; UnE: unbound-energy; Rseed: seeds of specific random numbers used for fitting the current conformation.

CYP51 is key enzyme of sterol biosynthesis in trypanosomatids and therefore, plays an important role in the maintenance of membrane structure, growth and development process. Consistently, our ultrastructural analysis showed defects on cell division and damage of plasma membrane which are also observed in *Leishmania* treated with sterol inhibitors (Macedo-Silva et al., 2015). CYP51 is a cytochrome P450 monooxynenase (CYP51 gene family), the heme-thiolate protein that catalyses a unique three-step reaction of oxidative removal of the 14 α-methyl group from the sterol core (Lepesheva & Waterman, 2011). The chemical interaction between both cadinene isomers and *Li*CYP51 is due to interactions of these compounds with hydrophobic amino acids residues of *Li*CYP51, mainly by van der Waals (VAL 42, PHE 41, LEU 214 and TRP 216) and alkyl (PRO 221, LEU 220, PHE 41 and TRP 216) bounds. Our molecular docking analysis revealed that the interaction profile between cadinenes and *Li*CYP51 was similar to those observed for fluconazole, a standard inhibitor of this enzyme. Both cadinenes isomers interacts with 15 amino acids in the active site of *Li*CYP51, 13 of which were found to also bind to Fluconazole. These results suggested that both δ-Cad and γ-Cad bind to the same active site of CYP41 which is inhibited by fluconazole (Figure 16).

Figure 16. Molecular docking of δ-Cadinene, γ-Cadinene and Fluconazole over to the *Li*CYP51 (A, C and E), respectively. Interactions of cadinenes and fluconazole with the amino acid residues of the enzyme (B, D and F).



4. Conclusion

Essential oil from fruit peel of *Myrciaria floribunda* was evaluated for antiparasitic potential against *L. amazonensis* and for cytotoxicity in Vero cells and macrophages. Our results showed for the first time that MfEO was effective against promastigote and amastigote forms, with high selectivity against the intracellular forms of parasite (SeI=44.15) and moderate

cytotoxicity towards mammalian cells. The treatment with MfEO induced a significant decrease in promastigotes viability and morphological changes compatible with damage to plasma membrane. It is important to mention that MfEO reduced the number of both infected cells and intracellular amastigotes. Our molecular docking analysis showed that the main compounds of MfEO, δ-cadinene and its isomer γ-cadinene, interact with the enzyme sterol 14-alpha demethylase (CYP51), which are essential for the survival and proliferation of *Leishmania* spp. Because CYP51 plays an important role in sterol biosynthetic pathway in this parasite, it is possible that δ-cadinene and γ-cadinene, caused a disturbance in the cell membrane which ultimately, can lead the parasite to death. Further studies are necessary to determine the exact mechanism of action of these compounds alone or in association. Our results points to the possibility of the use of cadinene as a scaffold for the development of novel chemotherapeutic agent for the treatment of cutaneous leishmaniasis.

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5 CONCLUSÃO

A utilização da casca do fruto de *M. floribunda* como possibilidade de aplicação biotecnológica é inovadora e compreende um campo aberto para estudo nas áreas alimentícia, cosmética e farmacêutica. O MfEO apresentou propriedades biológicas promissoras como inibidor de acetilcolinesterase e atividade anti-leishmania, sendo um potencial bioativo para o desenvolvimento de novas formulações farmacológicas mais efizaces, menos tóxicas e de baixo custo, que atenda as limitações dos fármacos disponíveis para o tratamento de doenças neurodegenerativas e negligenciadas. Este estudo foi pioneiro na descrição da composição química e aplicações biológicas para óleo essencial da casca do fruto de *M. floribunda*

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APÊNDICE A – ARTIGO

Chemical composition and acetylcholinesterase inhibitory potential, *in silico*, of *Myrciaria floribunda* (H. West ex Willd.) O. Berg fruit peel essential oil

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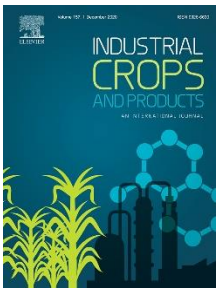

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Chemical composition and acetylcholinesterase inhibitory potential, *in silico*, of *Myrciaria floribunda* (H. West ex Willd.) O. Berg fruit peel essential oil

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ABSTRACT

Essential oils (EOs) are volatile compounds obtained from aromatic plants that can act as modulators of neurological systems. Several neurodegenerative diseases have been correlated with changes in the cholinergic system. *Myrciaria floribunda* (H. West ex Willd.) O. Berg (Myrtaceae) was chosen to investigate its biological potential for treating neurodegenerative diseases. *M. floribunda* fruit peel essential oil (MfEO) was collected for analysis. In this study, the chemical composition and *in silico* acetylcholinesterase inhibitory activities were determined. The results revealed the presence of 26 (96.86%) compounds obtained for GC/MS, of which the major compounds were the following: δ-Cadinene (26.8%), γ-Cadinene (15.69%), γ-Muurolene (6.21%), α-Selinene, α-Muurolene (6.11%) and (*E*)-Caryophyllene (5.54%). The ADMET analysis showed that MfEO compounds have a predicted lethal dose 628 times higher than neostigmine. The molecular docking results between the major components of MfEO and the enzyme acetylcholinesterase resulted in an average energy minimization of −6.6 Kcal/mol when the two structures coupled. In the assay of acetylcholinesterase activity, *M. floribunda* fruit peel oil presented an IC_{50} of 0.08 μg/ml and 23 μg/ml for commercial AChE of *E. electricus* and that extracted from *C. rhizophorae*, respectively. MfEO showed enzymatic inhibition and other relevant information of bioactive compounds. This study provides the first report of the chemical composition of the fruit peel essential oil of *M. floribunda* and highlights the promising results obtained through *in silico* analysis and the inhibitory potential of AChE through an *in vitro* assay.

1. Introduction

Essential oils (EOs) are volatile substances of natural origin, including a variety of compounds and can be obtained from aromatic plants (Bakkali et al., 2008). Recently, the biological potential of EOs has been investigated, revealing broad potential for biomedical applications with emphasis on antimicrobial and antibiofilm (Kuhn et al., 2019), larvicidal (Pandiyan et al., 2019), leishmanicidal (Silva et al., 2018), trypanocidal (Azeredo and Soares, 2013; Costa et al., 2018) and fungicidal activities (Santos et al., 2018), as well as activities against autoimmune diseases (Cock and Cheesman, 2019) and inflammation (Chou et al., 2018) and as a modulator of the neurological system (Arruda et al., 2012; Zarrad et al., 2015; Bhavya et al., 2018). Among the groups of botanical species that produce EO the Myrtaceae family stands out, which comprises 121 genera, is abundant in tropical regions and frequently produces edible fruit (Stefanello et al., 2011).

The variety of plant species in Brazil makes the country an essential center of genetic diversity for fruit species worldwide and has been the subject of studies over the years (Pereira et al., 2012; Costa et al., 2017). Among the most important families of the Brazilian flora, Myrtaceae occur throughout the country with more than one thousand species reported (Koch, 2014; Silva and Mazine, 2016). *Myrciaria floribunda* H. West ex Willd.) O. Berg (Myrtaceae) is native to Brazil and popularly known as *camboim* or *jabuticabinha*; it is characteristic of

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coastal sandy areas (*restingas*) but has a wide geographical distribution (Amaral et al., 2013; Oliveira et al., 2018).

The fruits of *M. floribunda* are important sources of β-cryptoxanthin (pro-vitamin A), are acidic and can be consumed fresh or in processed form (Oliveira et al., 2018; Teixeira et al., 2019). Just like other species of the family, *M. floribunda* has investigated for its biological potential through the use of various plant parts (Franceschinelli et al., 2007; Vaz et al., 2012), However, the use of fruit peel remains poorly explored. Studies using the EO of *M. floribunda* leaves, stems and flowers have revealed the potential for treating neurodegenerative diseases (Tietbohl et al., 2014).

Neurodegeneration plays an important role in various brain diseases, leading to the loss of cerebral neurons; further progression can spread and affect specific neurons such as those in the cholinergic system (Pepeu et al., 2015; Renard and Ludovic, 2017). Several neurodegenerative diseases have been correlated with changes in the cholinergic system, among which the most commonly cited are Alzheimer's disease (Ovsepian, et al., 2016; Hampel et al., 2018), Parkinson's disease (Bohnen and Albin, 2011; Bohnen et al., 2019), Huntington's disease (D'Souza and Waldvogel, 2016), amyotrophic lateral sclerosis (Campanari et al., 2016), myasthenia gravis (Brenner et al., 2008; Han et al., 2017) and glaucoma (Faiq et al., 2019). Thus, it is extremely important to develop new treatments for neurological and psychiatric syndromes. In these syndromes, cholinergic system dysfunction may occur and as consequences cognitive deficits, including memory loss, emotional state modulations, mood changes and dementia (Mu and Huang, 2019; Sharma, 2019; Morris et al., 2019). The development of drugs for treatment is based, among other factors, on decreasing the acetylcholine (ACh) deficit, which is directly linked to the progression and development of neurodegenerative diseases (García-Font et al., 2016).

ACh is a neurotransmitter responsible for propagating an electrical impulse from a pre-synaptic neuron to a post-synaptic neuron (Sirin et al., 2012). ACh is synthesized in vesicles and during the neurotransmission process, the vesicle fuses with the neuron's plasma membrane and releases ACh into the synaptic cleft, which binds to receptors in the postsynaptic neuron, thereby conducting the electrical impulse (Tougu, 2001; Colovic et al., 2013). Acetylcholinesterase (AChE) is a serine hydrolase responsible for terminating nerve impulse transmission by rapidly catalyzing ACh into acetate and choline in cholinergic pathways of the central and peripheral nervous systems (Quinn, 1987; Mercey et al., 2012).

Acetylcholinesterase (IAChE) inhibitors prevent the enzyme from breaking down the neurotransmitter, increasing the level and duration of ACh in the synaptic cleft, and have been targeted for the development of drugs against neurodegenerative diseases. Restoration of neurotransmitter levels may provide a beneficial alternative treatment, reducing cognitive and functional symptoms with increased synaptic availability of ACh (Sumit and Kumar, 2019). One of the first AChE inhibitor drugs was Tacrina, a compound belonging to the acridine class with substitution by an amino group at position 9. This compound was withdrawn from clinical use due to hepatotoxicity caused during treatment, as evidenced by increased transaminases in users (Sameem et al., 2017). Like tacrine, neostigmine is another AChE inhibitor, which can also cause severe side effects such as cardiac bradyarrhythmias, bronchoconstriction, nausea, increased peristalsis and potential adverse effects in pregnant women (Neely and Kohli, 2018).

In view of the above, it is important to search for AChE inhibitor compounds that present a lower capacity of systemic toxicity as a tool to fight neurological diseases. In this context, we investigated for the first time the potential of *M. floribunda* fruit peel essential oil (MfEO) in the *in vitro* inhibition of AChE and evaluated the pharmacokinetic and toxicological properties *in silico* of its major compounds.

2. Material and methods

2.1. Chemicals and reagents

Acetylcholinesterases extracted from *Crassostrea rhizophorae* and commercial AChE *Electrophorus electricus*, acetylcholine substrate (ASCh) obtained from Sigma-Aldrich (St. Louis, MO, USA). 5,5′-dithiobis-2-nitrobenzoic acid (DNTB); neostigmine; dimethyl sulfoxide; 96-well microplate.

2.2. Plant material and extraction of essential oil and chemical composition

The fruits of *M. floribunda* were collected in the city of Exu (Latitude: 7.355809029182327 °S; Longitude: 39.887568331218304 °W), state of Pernambuco, Brazil, in 2018. The species was collected and a specimen was herborized and deposited at the Herbarium of the Agronomic Institute of Pernambuco (IPA), voucher number 92,722. The extraction of essential oil from *M. floribunda* fruit peels was performed by the hydrodistillation method (clevenger apparatus). To obtain the oil, 200 g of the fruit rind were mixed with 2.5 l distilled water and subjected to hydrodistillation for 3 h. After extraction, the oil was stored at 5 °C and protected from light.

2.2.1. Gas chromatography-mass spectrometry analysis (GC/MS)

The analysis of the essential oil was performed using a Thermo Trace GC Ultra gas chromatograph (Thermo Scientific, Milan, Italy), coupled with a flame ionization detector (FID). A fused silica capillary column VB-5 5 (ValcoBond 30 m × 0.25 mm × 0.25 mm) was used for the separation of constituents (Valco Instruments Company Inc., Houston, TX, USA) and nitrogen was used as a carrier gas at a flow rate of 1 l/min and inlet pressure of 30 psi. The initial temperature gradient was 40 °C for 2 min and increased at 4 °C/min to 230 °C, where it was maintained for 5 min. Injector and detector temperatures were set to 250 °C and 280 °C, respectively. The sample (1 µl) was injected without separation and the relative amount of each component estimated by the corresponding peak and expressed as a percentage of the total chromatogram area. Mass spectra were performed at 70 eV (in EI mode) with a speed of 1.0 scans of *m/z* 35-350. Identification of the individual components was performed compared to the retention index values of n-alkane series according the literature (Van den Dool and Kratz, 1963), data available in GC–MS system mass spectral library (MassFinder 4, Hamburg, Germany); Spectral Mass Library NIST08 (ChemSW Inc. Fairfield, CA, USA) and published mass spectral data (Adams, 2007).

2.3. In silico ADMET

For predicting the pharmacokinetic and druglike properties of the major compounds of MfEO the following online platforms were used: Swiss ADME, Swiss Target Prediction (SIB., Lausanne, Switzerland) PROTOX-II Server (Charité University, Berlin, Germany), Molinspiration (Molinspiration Chemin formatics, Nova Ulica, Slovakia), Osiris Property Explorer, Osiris Data Warrior (Actelion Pharmaceuticals Ltd., Allschwil, Switzerland) and pKCSM (Pires et al., 2015). Reference drug information (Neostigmine PuChem CID: 4456) for analysis was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

2.4. Molecular docking

The docking approach used in this study aimed to identify binding to the active site capable of modulating the enzymatic activity of acetylcholinesterase (PDB code: 4PQE). The analysis of interactions between oil compounds and target proteins was performed through molecular anchoring using Autodock 4.2 and iGemDock 2.1 software. The corresponding protein structures were obtained from the Protein

Data Bank (Berman et al., 2003). The water molecules were removed and the binding site was established previously. The analysis was performed on a 60 × 60 × 60 grid with a spacing of 0.375 Å, centered on the active site of acetylcholinesterase, with enzymatic residues within 8Å in all cases. Results were analyzed using the Lamarckian genetic algorithm (López-Camacho et al., 2015) with a population size of 300, 80 generations and 10 different solutions. The functional empirical score of docking tests consists of the sum of Van der Walls-type bonds, hydrogen bridges and electrostatic energy.

2.5. Evaluation of acetylcholinesterase inhibitory activity (IAChE)

Evaluation of the enzymatic inhibition of acetylcholinesterase was determined in a microplate by the spectrophotometric method (Assis et al., 2012; Souza et al., 2018). The enzymes used for the assays were acetylcholinesterase extracted from *C. rhizophorae* gills and commercial AChE from the electric organ of *E. electricus* (Sigma). The commercial enzyme is an excellent *in vitro* model for anti-acetylcholinesterase evaluation of compounds due to its great similarity with the human enzyme. Incubation was performed by adding 10 µl of AChE to 10 µl of oil at different concentrations (0.0001 a 1 mg/ml), considering the absence of oil (0.0 mg/mL) as a negative control (100% activity). DMSO + enzyme buffer (0.5 M Tris−HCl pH 8.0) was used as a blank and neostigmine as a positive control (standard inhibitor). The plate was incubated for 1 h at 25 °C. After the incubation period, 200 µl of the chromogenic reagent DNTB (0.25 mM) and 20 µl of 62 mM acetylthiocholine (ASCh) substrate were added and read at 405 nm at 0 and 180 s. The percentage of enzyme inhibition was determined based on the controls. The test was performed in quadruplicate and the calculations were made using Microsoft Excel and Microcal Origin.

3. Results and discussion

The wide spectrum of biological activities present in EOs has driven the development of increasingly promising research in the field of pharmacology and in the production of therapeutic alternatives. The Myrtaceae family is known for its essential oils which offer good yields and important biological activities, especially the species *M. floribunda* (Apel et al., 2006a; Tietbohl et al., 2014). In this study, we obtained MfEO through the hydrodistillation method. The potential of the fruit peel of this species has been little explored, but the fruit is of great economic and food importance (Santos et al., 2017). Ripe fruits can be consumed *in natura* and are traditionally collected and used whole or macerated for the production of liqueurs (alcoholic solution produced with the fruit and cachaça) by several Brazilian communities (Souza and Morim, 2008; Lima and Barbosa, 2012). These properties, added to the *M. floribunda* biotechnological potential, constitute a field for research in the area of biosciences (Oliveira et al., 2018).

Aromatic plants of the Myrtaceae family show variations in the yields of essential oils, in this study MfEO presented a yield of 0.6% (200 g). In a study on the extraction of essential oils from species of this family from the Cerrado biome, Silva et al. (2018) presented values of 0.05% (*Plinia cauliflora*) and 0.51% (*Eugenia uniflora*), demonstrating the occurrence of variability. Extraction of essential oil from *M. floribunda* has been described in the literature for leaf, stem and flower portions, yielding 0.37% (1100 g), 0.02% (600 g) and 0.64% (186 g), respectively. Considering these data, the fruit peel of *M. floribunda* presents a good EO yield, being similar to the yield already described for flower oil. The chemical characterization of the components of MfEO is essential for understanding the mechanisms of action correlated with the biological potential and possibility of isolation, purification or synthesis of analogs that present the biological activity desired.

Table 1
Identified chemical constituents of MfEO[a] obtained by GC/MS[b].

Compounds	I.R.[c] calculated	I.R. read	A[d] (%)	S. D.[e]
(E)-β-Ocimene	1050	1044	0.19	0.01
α-Cubebene	1352	1348	0.01	0.00
α-Ylangene	1374	1373	0.34	0.01
α-Copaene	1378	1374	5.02	0.28
Sativene	1392	1390	0.22	0.01
Sibirene	1398	1400	0.23	0.01
α-Gurjunene	1412	1409	1.25	0.04
(E)-Caryophyllene	1422	1417	5.54	0.22
β-Copaene	1432	1430	1.89	0.08
Aromadendrene	1442	1439	3.95	0.18
α-Humulene	1457	1452	1.81	0.03
allo-Aromadendrene	1464	1458	1.47	0.03
trans-Cadina-1(6),4-diene	1477	1475	0.01	0.00
γ-Muurolene	1480	1478	6.21	0.24
β-Selinene	1489	1489	4.21	0.07
δ-Selinene	1494	1492	0.18	0.10
α-Selinene	1498	1498	6.11	0.29
α-Muurolene	1503	1500	6.11	0.29
γ-Cadinene	1518	1513	15.69	0.38
δ-Cadinene	1527	1522	26.84	0.50
trans-Cadina-1,4-diene	1536	1533	0.12	0.00
α-Cadinene	1541	1537	1.96	0.05
α-Calacorene	1547	1544	2.31	0.10
Germacrene B	1561	1559	0.31	0.08
β-Calacorene	1567	1564	0.31	0.06
α-Cadinol	1646	1652	4.57	0.91
		Identified	96.86	

[a] MfEO: Essential oil of *Myrciaria floribunda* peel fruit.
[b] GC/MS: Gasose Cromatography/Mass Spectre.
[c] I.R.: Retexion Index.
[d] A: Area.
[e] S.D.: Standart Deviation.

3.1. Essential oil composition

The chemical characterization of MfEO (Table 1) revealed the presence of 26 compounds that together correspond to 96.86% of the total essential oil, as identified by GC/MS. Most compounds are sesquiterpenes, including six of the major compounds: *δ*-Cadinene (26.8%), γ-Cadinene (15.69%), γ-Muurolene (6.21%), α-Selinene, α-Muurolene (6.11%) and (*E*)-Caryophyllene (5.54%) (Fig. 1). In another study, *M. floribunda* leaf and flower essential oil presented distinct characterization, most of them being monoterpenes, while trunk oil presented 72.2% sesquiterpenes, a result similar to this study. Medicinal plants have molecules with therapeutic potential and represent an important source for compound identification and production of new drugs (Boudjedjou et al., 2019).

MfEO presented two principal components, the Cadinene isomers γ-cadinene and *δ*-cadinene (Fig. 2) which together account for 42.43% of the total composition. Cadinene is a sesquiterpene present in essential oils, with reported insecticidal (Govindarajan et al., 2016), acaricidal (Guo et al., 2017) and antiprotozoal (Ghazouani et al., 2017) activities and described in the composition of the fruit essential oil of *Myrciaria jaboticaba* (Vell.) O. Berg (Plagemann et al., 2012). Cadinene has not been frequently reported in the literature as a major component of essential oils, where in the family Myrtaceae, α-pineno, α-caryophyllene, (Plagemann et al., 2012), d-limonene, β-caryophyllene (Franco and Shibamoto, 2000) and β-Myrcene (Fortes et al., 2011) are the most common. The compounds α-Silenene and α-Muurolene were identified as major components of the essential oil of the fruits of *Myrciaria jaboticaba* (Vell.) O. Berg, while γ-Muurolene was described in the composition of *P. trunciflora* (Apel et al., 2006b). Variation in the composition of essential oils of species of the same genus or family occurs due

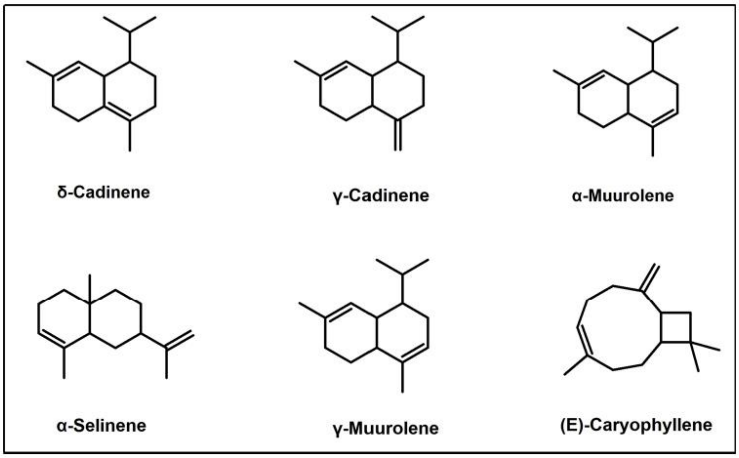

Fig. 1. Chemical structure of the major components of the essential oil of *M. floribunda*.

to factors such as environmental conditions and seasonal variation. Importantly, the composition of the fruit peel oil of *M. floribunda* described in this study has not yet been reported in the literature (Silva et al., 2019).

3.2. In silico ADMET

The association of folk medicine with medicinal chemistry has become central to developing new medicines. Pharmacokinetic and toxicological analyses of natural compounds are important steps in the classification of molecules based on absorption, distribution, metabolism, excretion and toxicity (ADMET), enabling better targeting in the formulation of medicines for the treatment of human diseases (Abdulfatai et al., 2017; Daina et al., 2017). For the development of safer and more effective chemotherapy in the treatment of neurodegenerative diseases, new compounds must have characteristics compatible with orally administered pharmaceutical formulations in order to facilitate treatment, patient compliance and avoid discomfort to users (Gleeson and McCartney, 2019). The lipid nature of EO gives these substances an additional advantage for oral capsule formulation due to increased physical absorption and minimization of oxidation susceptibility (Hauss, 2007).

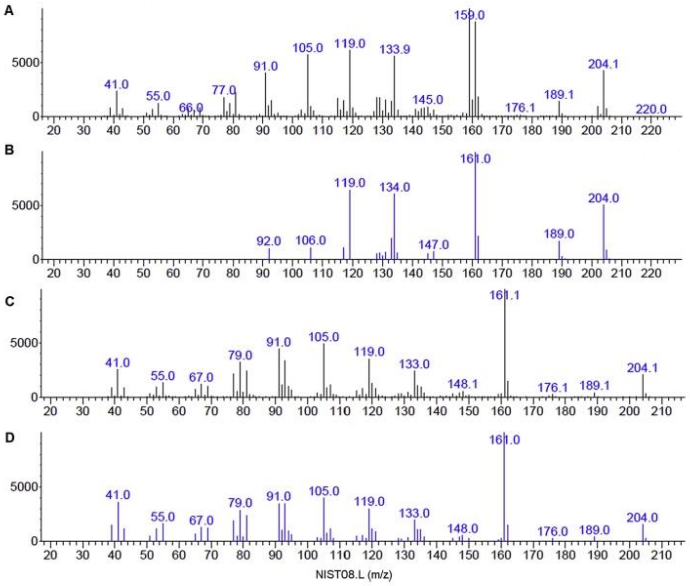

Fig. 2. Mass spectrometry of δ-Cadinene in the essential oil (A), reference standard of δ-Cadinene (B), γ-Cadinene in the essential oil (C), reference standard γ-Cadinene (D).

Table 2
In silico ADMET[a] analysis of major constituents of MfEO[b] and neostigmine.

Property	δ-Cad[c]	γ-Cad[d]	α-Mu[e]	α-Sel[f]	γ-Mu[g]	Car[h]	Neo[i]
PHYSICOCHEMICAL PROPERTIES							
HBA[j]	0	0	0	0	0	0	3
HBD[k]	0	0	0	0	0	0	0
ClogP	4.14	4.18	4.08	4.40	4.18	4.24	0.40
MW[l] (g/mol)	204.35	204.35	204.35	204.35	204.35	204.35	223,29
n-ROTB[m]	1	1	1	1	1	0	4
ABSORPTION							
BBB[n]	No	No	No	No	No	No	Yes
HIA[o]	Low	Low	Low	Low	Low	Low	High
P-GPs[p]	No	No	No	No	No	No	Yes
Log Kp (cm/s)[q]	−4.85	−4.49	−4.65	−3.85	−4.49	−4.44	−6,51
METABOLISM							
CYP450[r] 2C9 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No
CYP450 2D6 inhibitor	No	No	No	No	No	No	No
CYP450 2C19 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No
CYP450 3A4 inhibitor	No	No	No	No	No	No	No
CYP450 1A2 inhibitor	No	No	No	No	No	No	No
TOXICITY							
MUT[s]	No	No	No	No	No	No	Little
TUM[t]	No	No	No	No	No	No	–
IRR[u]	No	No	No	No	No	No	–
HEP[v]	No	No	No	No	No	No	Litte
LD_{50}[w] mg/Kg	4400	4400	4400	5000	4400	5000	7

[a] ADMET: absorption, distribution, metabolism, excretion and toxicity.
[b] MfEO: Essential oil of *Myrciaria floribunda* peel fruit.
[c] *δ*-Cad: delta- Cadinene.
[d] γ-Cad: gama-Cadinene.
[e] α-Mu:alfa-Muurolene.
[f] α-Sel: alfa-Selinene.
[g] γ-Mu:gama- Muurolene.
[h] Car: (*E*)-Caryophyllene.
[i] Neo: neostigmine.
[j] HBA: Number of hydrogen force acceptors.
[k] HBD: Number of hydrogen donors.
[l] MW: Molecular Weight.
[m] n-ROTB: Number of rotary connections.
[n] BBB: blood-brain barrier.
[o] HIA: gastrointestinal absorption.
[p] P-GPs: permeability glycoprotein.
[q] Log Kp (cm/s): Skin permeation.
[r] CYP450: Cytochrome P450 Enzyme.
[s] MUT: mutagenic.
[t] TUM: tumorigenic.
[u] IRR: irritant.
[v] HEP: hepatotoxic.
[w] LD_{50}: lethal dose at 50%.

The potential of a compound for oral treatment is related to important factors to ensure adequate absorption including solubility, permeability to biological membranes, dissolution under gastrointestinal conditions, transit time in the digestive tract, among other aspects. In recent years, the development of *in silico* analysis platforms has enabled more accurate information on the absorption of new drug candidate compounds even before being subjected to *in vivo* testing (Daina and Zoete, 2016). Suitable physicochemical properties for a molecule to be absorbed by the gastro-intestinal tract include a molecular weight of 150 to 500 g/mol, a number of hydrogen bond acceptors less than 10, TPSA 20 to 130, (cLogP) partition coefficient logarithm between octanol (lipophilicity) and water from 0 to 5 and finally a number of hydrogen donors below 5 (Daina and Zoete, 2016; Khalid et al., 2018). Thus, we performed an ADMET analysis of the major compounds of MfEO (Table 2).

The major MfEO compounds had similar pharmacokinetic and toxicological potential. Thus, none of the six major compounds had mutagenic, irritant, tumorigenic or hepatotoxic potential, whereas the reference drug used, neostigmine, had little mutagenic and hepatotoxic potential. The analysis showed that MfEO compounds have a predicted lethal dose 628 times higher than neostigmine, thus highlighting the high toxic potential of the reference drug when compared to the major compounds that make up *M. floribunda* essential oil. The high toxicity of neostigmine has been reported in the literature, thus confirming the data highlighted in the ADMET analysis of this study (Ream, 1963; Adiamah et al., 2017). Besides toxicity, neostigmine has a cLogP of 0.40, characterizing it as very hydrophilic. Compounds that have high affinity for water tend to have a shorter half-life due easy excretion. This feature is unattractive in oral chemotherapeutic agents because in these cases there is need for multiple-dose administration over a short time span (Van de Waterbeemd and Gifford, 2003; Newby, et al., 2015). The six major EOMf compounds have cLogP values above 4, they are compounds with a higher degree of lipophilicity and may have a longer half-life than the reference drug (Newby et al., 2015). These characteristics lead us to believe that, in the *in vivo* treatment model, the EOMf could be administered with longer periods between doses,

Table 3
Interaction profile between major oil compounds with acetylcholinesterase enzyme.

Comp.	BE[a] (Kcal/mol)	KiC[b] (uM)	FE[c] (Kcal/mol)	EE[d] (Kcal/mol)	TIE[e]	IS[f]
δ-Cad[g]	−6.77	10.99	−75.1	−0.01	−7.06	653.724
γ-Cad[h]	−7.35	4.10	71.7	0.00	−7.65	661.2
α-Mu[i]	−5.89	17.11	−71.4	−0.01	−6.32	591.832
α-Sel[j]	−6.37	6.33	−73.3	−0.00	−6.98	761.412
γ-Mu[k]	−6.23	8.91	−71.4	−0.02	−7.06	661.21
Car[l]	−6.99	12.33	−72.4	−0.00	−7.32	721.654
Neo[m]	−5.09	184.65	−74.13	−0.03	−5.77	657.542

[a] BE: Binding energy.
[b] KiC: Ki Constant.
[c] FE: Free Energy.
[d] EE: Electrostatic Energy.
[e] TIE: Total intermolecular energy.
[f] IS: Interaction Surface.
[g] δ-Cad: delta-Cadinene.
[h] γ-Cad: gama-Cadinene.
[i] α-Mu: alfa-Muurolene.
[j] α-Sel: alfa-Selinene.
[k] γ-Mu: gama- Muurolene.
[l] Car: (*E*)-Caryophyllene.
[m] Neo: neostigmine.

reinforcing its pharmacological potential with low chances of toxicity.

3.3. Molecular docking: in silico screening of acetylcholinesterase inhibitors in MfEO

Based on the interaction data of the docking test (Table 3), it can be observed that the major constituents interact with acetylcholinesterase with a total free energy of approximately -75 kcal/mol, most of which are Van der Waals interactions.

Molecular docking results between the major components of MfEO and the enzyme acetylcholinesterase resulted in an average energy minimization of –6.6 Kcal/mol when the two structures coupled, as shown in Fig. 3. This result implies that the coupling between molecules is energetically favorable (exergonic bond: dissipates energy) and is located between the Van der Waals bond energy level (< -1 Kcal/mol) and the covalent chemical bond level (< -100 Kcal/mol) (Ferreira et al., 2015). Thus, such binding may be considered exergonically favorable, reversible (because it is non-covalent), may be competitive (the relationship between concentrations or bioavailability of the molecules involved). A link between molecules with these characteristics—exergonic, reversible and competitive—as well as the result of energy minimization obtained, allows the action of other substances (drugs, substances naturally found in the body) either in favor of molecular docking or in its impediment.

In relation to the most expressive major compounds, δ and γ cadinene, they establish connections with low binding energy, but with moderate entropy (total energy). These bonds may occur randomly and establish a continuous interaction between the major compound and the enzyme, which corroborates the inhibitory activity (Silva et al., 2019). As bicyclic sesquiterpenes, they establish hydrophobic and Van der Walls interactions with the amino acids TRP86, TYR124, PHE295, TYR337, TRP286 and VAL294, respectively. Thus, they integrate a type of intermolecular interaction in which nonpolar compounds of these amino acids suffer consequences of the dynamic actions of the polar compounds of the oil, besides establishing communications between the electronic clouds at the dipole-dipole and dipole-permanent levels (Bhuvanendran et al., 2019). The interactions dominated in the region of His447 and Trp286 amino acid residues due to the existence of the pi-alkyl interaction at the catalytic anionic site and the hydrogen bonds with Tyr124 and Phe295 at the edge of the peripheral site region (Fig. 3).

The compounds δ-cadinene and γ-cadinene against the Alzheimer's Disease target protein AChE showed a higher binding affinity docking score as indicated by a docking score of -7.35 and -6.77, respectively, and forms π -alkyl bonds of length 2.1 Å to the polar aliphatic residues, that is, His447 and Trp286. The other site A2 and A3 form bonds of length 1.7 and 2.1 Å to the hydrophobic residues Phe295 and Tyr124. Docking results also exposed π–π stacking, the site interacted with the receptor and formed Van der Walls stacking with two amino acid residues, Tyr341 and Tyr337. Thus, the functionalities such as positively charged groups at P6 and aromatic rings R8 and R7 were identified to be important for AChE opening activity (Ali et al., 2018). Even though site H5 did not interact with the receptor but due to its presence in the dataset, it can serve as a target for the inhibition process. As with the currently obtained data, computational methods have become essential to biological investigations. Here we have used the computational approach to further understand the mechanism of interactions and binding affinity between AChE and these molecules. Moreover, our findings corroborate the data observed in recent studies of major components of essential oils, including structural similarities (Silva et al., 2019; Byler and Setzer, 2018).

3.4. MfEO is effective in in vitro inhibition of acetylcholinesterase in a dose-dependent manner

In silico tests are important tools for directing and estimating drug release and predicting aspects of *in vivo* toxicity (Karkossa and Klein, 2019). The promising results obtained in this study through ADMET and molecular docking analyses led us to investigate the *in vitro* effect of *M. floribunda* essential oil on AChE enzymatic activity. *In vitro* enzyme tests can be designed to explain the ability of enzymes to catalyze a specific type of chemical reaction and provide information mechanism studies (Küchler et al., 2016).

In the assay of acetylcholinesterase activity, *M. floribunda* fruit peel oil presented a median inhibitory concentration (IC_{50}) of 0.08 µg/ml and 23 µg/ml for commercial AChE of *E. electricus* and that extracted from *C. rhizophorae*, respectively. While neostigmine (the standard used) had an IC_{50} of 23.3 µg/ml and 6.2 µg/ml, respectively, for the same enzymes (Fig. 4). The IAChE action may be correlated to the presence of δ and γ cadinene compounds present in the oil, which are part of the sequisterpene group.

The differences between the IC_{50} values for the commercial enzyme (*E. electricus*) and *C. rhizophorae* extracts may be related to the degree of purity, sensitivity or small structural distinctions between the AChEs of the species under study. The difference in the binding affinity of inhibitors to the enzyme catalytic site has been reported as variable in relation to the effect exerted by the same compound on terrestrial vertebrates and fish AChEs and between fish AChEs (Assis et al., 2012; Freitas et al., 2016).

The results obtained with MfEO acetylcholinesterase inhibition were higher than those found for the essential oil of flowers (IC_{50} 1583 µg / ml) and leaves (IC_{50} 681 µg/ ml) of the same species (Tietbohl et al., 2012). In that study, the essential oil of *M. floribunda* stems showed no enzymatic inhibition, having (2E, 6E)-farnesyl acetate and (2E, 6Z)-farnesol as major components, which belong to the same chemical class as cadinenes (δ and γ). However, there are significant differences in a chemical structure that may influence interaction and enzyme inhibition. Furthermore, *in silico* studies have contributed to understanding how the chemical structure and enzyme binding affinity behave by extracting relevant information from bioactive compounds (Rodrigues et al., 2016; Scotti and Scotti, 2018).

Cyclic compounds such as 1,8-cineole (Petrachaianan et al., 2019; Kahkeshani et al., 2018; Abdelgaleil et al., 2019), α-pinene (Karakaya et al., 2019; Shahriari et al., 2018) and cariophylene (Owokotomo et al., 2015; Xiang et al., 2017), found in essential oils, have been reported to have AChE inhibitory activity. According to Miyazawa et al.

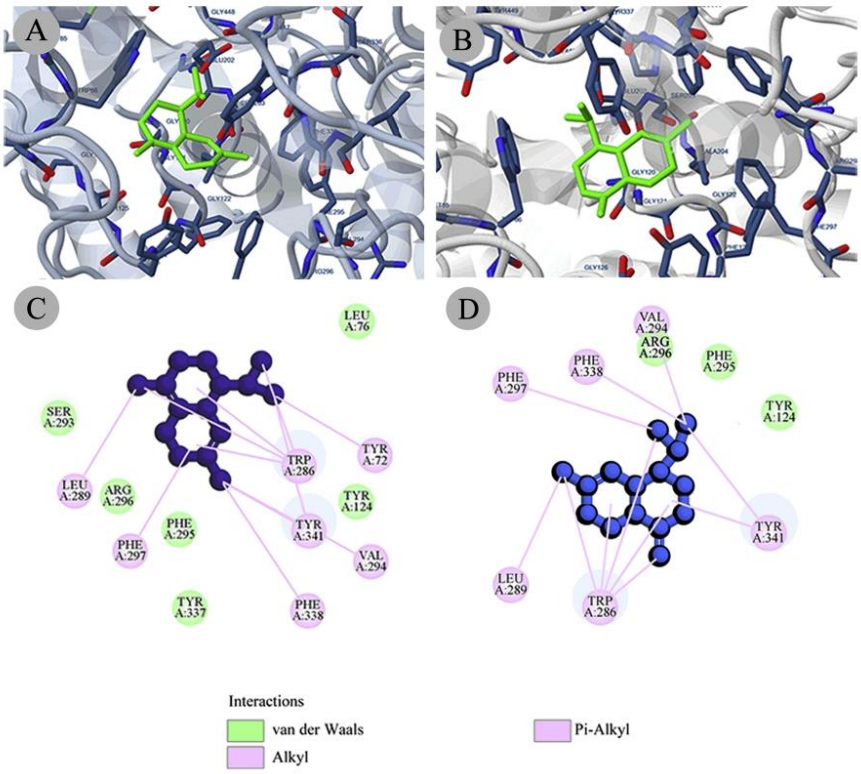

Fig. 3. Interaction of δ (A) and γ (B) cadinene with human AChE[a]. Binding and related amino acid residue in molecular docking (C, D).
AChE: Acetylcholinesterase.

(2016), essential oils made mostly of sesquiterpenes have a greater inhibitory effect than those made of monoterpenes. The synergistic association of these sesquiterpenes may be responsible for their inhibitory action. Thus, the essential oil of *M. floribunda* described in this study may serve as an active principle for the development of new drugs against neurodegenerative diseases.

4. Conclusion

Taken together, our results showed that the essential oil of the fruit peel of *M. floribunda* inhibited the acetylcholinesterase of *Crassostrea rhizophorae* and *Electrophorus electricus* (commercial) and in the latter showed a superior effect to neostigmine, a commercial drug with an anticholinesterase effect. Our *in silico* analysis demonstrated good pharmacokinetic activity for the major compounds δ-Cadinene, γ-Cadinene, γ-Muurolene, α-Selinene, α-Muurolene, (*E*)-Caryophyllene and low potential for toxicity. Molecular docking analyses, associated with *in vitro* enzyme inhibition assays for acetylcholinesterase, indicate that *M. floribunda* essential oil is promising for the development of new formulations for the treatment of neurodegenerative diseases. The exploitation of the pharmacological potential of MfEO is worthy of mention due to the wide variety of organic compounds that compose this plant material. The study of the chemical characteristics of the major compounds of the essential oil of the fruit peel of *M. floribunda* and their interactions with biomacromolecules, such as acetylcholinesterase, provides support for the development of alternative therapeutic measures and the design of new bioactive compounds based on constituents of the oil.

CRediT authorship contribution statement

Deyzi Caroline da Silva Barbosa: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing. **Vanderlan Nogueira Holanda:** Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing. **Caio Rodrigo Dias de Assis:** Methodology, Validation, Resources, Investigation, Writing - original draft. **Júlio César Ribeiro de Oliveira Farias de Aguiar:** Methodology, Validation, Resources, Investigation. **Pedro Henrique doNascimento:** Methodology, Validation, Resources, Investigation, Software, Writing - original draft. **Welson Vicente da Silva:** Methodology, Validation, Resources, Investigation, Software, Writing - original draft. **Daniela Maria do Amaral Ferraz Navarro:** Methodology, Validation, Investigation, Supervision, Project administration. **Márcia Vanusa da Silva:** Investigation, Supervision, Project administration. **Vera Lúcia de Menezes Lima:** Investigation, Supervision, Project administration. **Maria Tereza dos Santos Correia:** Investigation, Supervision, Writing - review & editing, Project administration.

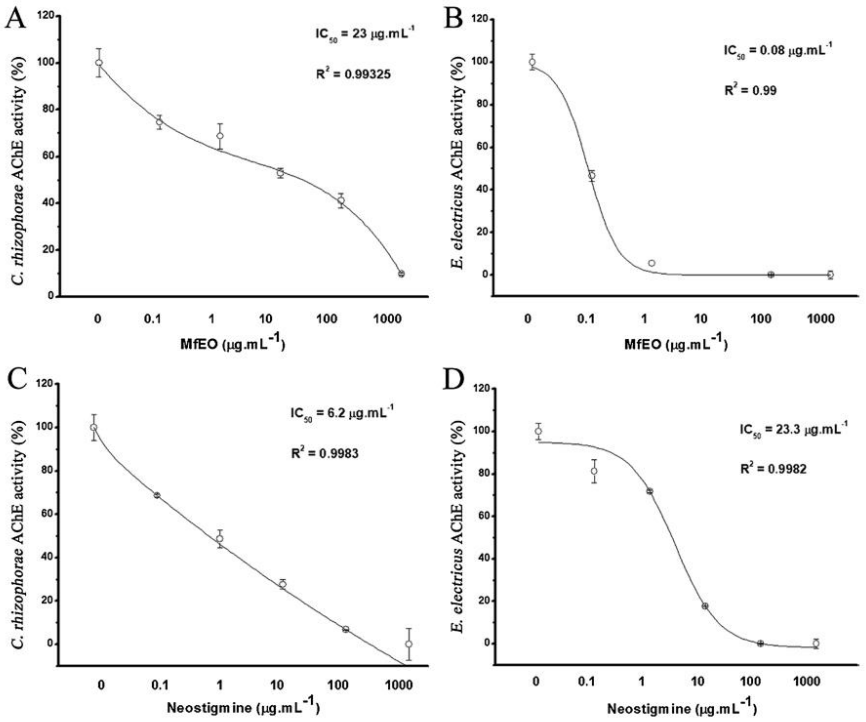

Fig. 4. Determination of inhibition activity (IC_{50}[a]) of MfEO[b] (A, B) and neostigmine (C, D) on *Crassostrea rhizophorae* and *Electrophorus electricus* AChE[c] respectively.
IC_{50}: median inhibitory concentration.
MfEO: Essential oil of *Myrciaria floribunda* peel fruit.
AChE: Acetylcholinesterase.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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