



UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

VANDERLAN NOGUEIRA HOLANDA

**SÍNTESE E AVALIAÇÃO DO POTENCIAL DE HÍBRIDOS DERIVADOS DE
FTALIMIDAS E TRIAZÓIS NO DESENVOLVIMENTO DE NOVOS
FÁRMACOS CONTRA A LEISHMANIOSE**

Recife
2022

VANDERLAN NOGUEIRA HOLANDA

**SÍNTESE E AVALIAÇÃO DO POTENCIAL DE HÍBRIDOS DERIVADOS DE
FTALIMIDAS E TRIAZÓIS NO DESENVOLVIMENTO DE NOVOS
FÁRMACOS CONTRA A LEISHMANIOSE**

Tese apresentada ao Programa de Pós-Graduação Bioquímica e Fisiologia da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de doutor em Bioquímica e Fisiologia.

Área de Concentração: Bioquímica e Fisiologia.

Orientadora: Profa. Dra. Vera Lúcia de Menezes Lima

Coorientadores: Profa. Dra. Regina Célia Bressan de Figueiredo

Prof. Dr. César Augusto da Silva

Recife

2022

Catálogo na Fonte:
Bibliotecária Natália Nascimento, CRB4/1743

Holanda, Vanderlan Nogueira.

Síntese e avaliação do potencial de híbridos derivados de ftalimidas e triazóis no desenvolvimento de novos fármacos contra a leishmaniose. / Vanderlan Nogueira Holanda. – 2022.

307 f. : il., fig.; tab.

Orientadora: Vera Lúcia de Menezes Lima.

Coorientadora: Regina Célia Bressan de Figueiredo.

Coorientador: César Augusto da Silva.

Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-graduação em Bioquímica e Fisiologia, Recife, 2022.

Inclui referências.

1. Leishmaniose. 2. Quimioterapia. 3. 1,2,3-triazol. 4. Ftalimidas. 5. Click chemistry. I. Lima, Vera Lúcia de Menezes. (orient.). II. Figueiredo, Regina Célia Bressan de. III. Silva, César Augusto da. IV. Título.

587

CDD (22.ed.)

UFPE/CB – 2023-053

VANDERLAN NOGUEIRA HOLANDA

**SÍNTESE E AVALIAÇÃO DO POTENCIAL DE HÍBRIDOS DERIVADOS DE
FTALIMIDAS E TRIAZÓIS NO DESENVOLVIMENTO DE NOVOS
FÁRMACOS CONTRA A LEISHMANIOSE**

Tese apresentada ao Programa de Pós-Graduação Bioquímica e Fisiologia da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de doutor em Bioquímica e Fisiologia.

Área de Concentração: Bioquímica e Fisiologia.

Aprovado em: 16/05/2022.

BANCA EXAMINADORA

Prof.^a Dra. Maria Carolina Accioly Brelaz de Castro (Examinador Externo)
Centro Acadêmico de Vitória
Universidade Federal de Pernambuco

Prof.^a Dra. Regina Celia Bressan Queiroz de Figueiredo (Examinador Externo)
Instituto Aggeu Magalhães/Fundação Oswaldo Cruz

Prof. Dr. César Augusto da Silva (Examinador Externo)
Colegiado de Medicina
Universidade Federal do Vale do São Francisco

Prof. Dr. Ronaldo Nascimento de Oliveira (Examinador Externo)
Departamento de Química
Universidade Federal Rural de Pernambuco

Prof.^a Dra. Vera Lúcia de Menezes Lima (Orientadora)
Departamento de Bioquímica
Universidade Federal de Pernambuco

À minha família, por me proporcionar acesso à educação, me apoiar nas decisões difíceis e por sempre motivar-me a lutar pelos meus sonhos.

Aos meus amigos, que sempre acreditaram em mim e estiveram do meu lado quando eu mais precisei.

Dedico.

AGRADECIMENTOS

Agradeço a minha família pelo constante apoio em todas as decisões que tomei e ao longo da minha vida, especialmente no âmbito acadêmico. Ingressar no Doutorado em Bioquímica e Fisiologia foi a concretização do sonho de tornar-me pesquisador e colaborar com a saúde e a ciência. Obrigado às minhas mães, Raimunda Amorim e Vanuza da Silva, e à Maria grande amiga, por todo apoio e por sempre acreditarem em mim.

À Professora Dra. Vera Lúcia de Menezes Lima, orientadora e amiga. Obrigado pela oportunidade que me destes e por enxergar potencial em mim. Ao longo da caminhada na pós-graduação, tive a honra de ser orientado pela senhora, uma professora tão cuidadosa, amável e presente. Serei eternamente grato por me acolher em 2016 e me proporcionar uma excelente formação científica, técnica, crítica e ética, possibilitando me tornar o pesquisador que sou hoje. A senhora sempre será uma grande referência na minha vida.

À Professora Dra. Regina Celia Bressan Queiroz de Figueiredo, amiga e orientadora. Sou muito grato por todo o apoio e incentivo desde o início da nossa colaboração, ainda no mestrado. Ao longo destes últimos anos tive o prazer de trabalhar ao seu lado, uma pesquisadora tão competente, exigente e excelente no que faz. Muito obrigado por me ajudar a enxergar mais de mim e acreditar que é possível realizar nossos sonhos. Serei eternamente grato por tudo o que me ensinastes. Você sempre será referência na minha vida e poderá sempre contar comigo.

Ao Professor Dr. Ronaldo Nascimento de Oliveira, pesquisador com quem tive a oportunidade de colaborar, aprender e a gostar ainda mais da Química Orgânica. Muito obrigado pela acolhida no Laboratório de Síntese de Compostos Bioativos-UFRPE, por sempre estar disponível e acreditar no meu trabalho. O senhor é uma grande referência de profissionalismo, ética e dedicação. Sou muito grato pela oportunidade de colaborarmos e por tudo que tenho aprendido. Sem dúvida alguma, todos os ensinamentos e experiência que o senhor me proporcionou foram determinantes para a realização desta conquista em sua totalidade. Serei eternamente grato.

Ao Professor César Augusto da Silva, muito obrigado pela disposição em orientar-me e colaborar comigo para elaboração desta tese. Desde o início do

doutorado o senhor tem sido muito gentil e disponível para contribuir sempre que necessário. Estarei sempre disponível para colaborarmos. Sou grato por todo apoio.

Ao meu companheiro e parceiro de todos os momentos, Elton Marlon de Araújo Lima, obrigado por dividir a vida comigo e estar sempre do meu lado, mesmo nos momentos mais difíceis. Você esteve presente sempre que eu precisei e nunca hesitou em me apoiar no que fosse preciso. Esse apoio foi fundamental para que eu pudesse me sentir seguro e amado durante uma jornada tão difícil que é o doutorado. Aprendi com você a me tornar uma pessoa melhor e a me reinventar sempre que necessário. Você estará sempre no meu coração. Te amo.

Aos meus amigos-família, joias preciosas que tive a honra de conhecer e conviver quando cheguei ao Recife: Romário Correia e Ricardo Nagô. Obrigado por sempre serem meu apoio e por se tornarem minha família. Sou grato por ter cada um de vocês dois, verdadeiros irmãos, cúmplices e companheiros de jornada. Ao meu grande amigo, Magérbio Torres, por sempre me incentivar a seguir a diante e me apoiar desde o primeiro momento, muito obrigado por se fazer sempre presente da graduação ao doutorado. Tenho muito orgulho de vocês três e sou feliz por tê-los. Jamais os esquecerei.

Aos meus alunos de iniciação científica: Welson Vicente e Pedro Henrique. Muito obrigado por me oportunizarem a experiência docente durante o doutorado. Vocês me fizeram lembrar de um dos grandes propósitos que me levou à pós-graduação, a vontade de ser professor. Ensinar e aprender foi um processo contínuo desde o primeiro dia que começamos a trabalhar juntos. Agradeço especialmente a Welson Vicente, que para além da relação orientador-aluno, tornou-se um grande amigo. Welson, muito obrigado pela sua dedicação e importantíssima contribuição a esta tese. Você foi (e é) fundamental para o sucesso dos nossos trabalhos. Muito obrigado pela confiança e apoio. Conte sempre comigo.

Às equipes de Laboratórios: obrigado aos membros do Laboratório de Lipídios e Aplicação de Biomoléculas em Doenças Prevalentes e Negligenciadas, em especial a Rebeca Xavier, Ana Paula Sant'Ana e João Ricardhis. Muito obrigado por sempre estarem disponíveis e me receberem tão bem. O Lab. Lipídios sempre será um lugar de boas lembranças. Aos membros

do Laboratório de Síntese de Substâncias Bioativas da UFRPE, em especial à Kethully, Bruna e Carla, pela excelente colaboração na etapa de síntese das moléculas orgânicas, pela paciência e atenção em me ensinar e por sempre me receberem de forma tão gentil. Aos amigos do Laboratório de Biologia Celular de Patógenos – Fiocruz/PE, em especial a Duda e a Deyzi, duas grandes pesquisadoras e amigas que nunca mediram esforços para me ajudar. Agradeço aos professores e pesquisadores Dr. Rafael Maia, Dr. Sérgio Rushi e Dra. Maria Carolina Accioly pela brilhante colaboração com esta tese e sobretudo, por estarem sempre disponíveis a ensinar de uma forma muito acolhedora e gentil. Serei eternamente grato.

Agradeço aos amigos que fiz durante a realização do doutorado sanduíche na Universidad de León, Espanha: Janayna Marcelo, Daniel Robles Garcia, Bruna Duarte, Beatriz Duarte Silva, Bruna Granjeiro e José María Vázquez. Muito obrigado pela acolhida, escuta e aventuras vividas nos últimos seis meses. Lembrar de tudo o que vivemos é perceber que a distância Brasil-Europa foi encurtada devido à nossa amizade.

Agradeço ao Prof. Dr. Rafael Balaña Fouce e a todos que fazem o grupo ENTROPiA do Departamento Ciências Biomédicas da Universidad de León, em especial a Profa. Dra. Rosa María Reguera Torres, Prof. Dr. Carlos García, Maria Cambra, Estela, Yolanda, Bárbara, Irene Pilar, Ruben González, Maria Álvarez e Nerea García. Muito obrigado por todos os ensinamentos, convivência agradável e troca de experiências durante a minha estância de intercambio na Espanha. Obrigado Prof. Rafael por me acolher como um amigo e possibilitar a realização de mais um sonho. Serei eternamente grato.

Às agências FACEPE, CAPES e CNPq, pelo apoio ao desenvolvimento desta pesquisa. Às Universidades Federal de Pernambuco (UFPE) e Federal Rural de Pernambuco (UFRPE), Instituto Aggeu Magalhães (IAM/FIOCRUZ) e à Universidad de León (ULE), pela infraestrutura e apoio técnico-científico. E por último, a todos que acreditaram em mim, muito obrigado.

I wanna be defined by the things that I love
Not the things I hate
Not the things that I'm afraid of,
Not the things that haunt me in the middle of the night,
I just think that
You are what you love
(SWIFT, 2019).

RESUMO

Moléculas orgânicas formadas pela união de dois ou mais grupos farmacofóricos têm sido alvo de estudo para o desenvolvimento de novos agentes leishmanicidas e nesse contexto, os derivados do triazol, ftalimida e naftoquinona têm ganhado destaque. Dessa forma, a presente tese de doutorado teve por objetivo investigar o potencial leishmanicida de sete derivados triazólicos ligados a ftalimida ou naftoquinona obtidos por *click chemistry*, sobre as formas evolutivas de *Leishmania amazonensis* e *Leishmania braziliensis*. Para isso, foi avaliado o perfil farmacológico, citotoxicidade e potencial leishmanicida. Os compostos foram ranqueados quanto a seletividade, a partir dos dados obtidos na citotoxicidade e atividade leishmanicida. Nesse sentido, duas moléculas foram escolhidas como compostos líderes e tiveram o mecanismo de ação investigado através da análise da fisiologia mitocondrial e dos compartimentos ácidos em promastigotas de *Leishmania*. Também foi investigado o potencial inibitório dos compostos líderes sobre as enzimas esterol 14 alfa-demetilase e tripanotiona redutase, alvos moleculares comumente associados a ação de drogas. O ensaio de citotoxicidade sobre macrófagos e fibroblastos demonstrou que os compostos contendo naftoquinona (FT6 e FT7) foram mais tóxicos, com valores de CC₅₀ que variaram de 20.8 a 314.2 µM, enquanto os derivados com a ftalimida foram os menos tóxicos (CC₅₀ > 2000 µM). Os compostos FT1 e FT2 não apresentaram atividade hemolítica e foram efetivos na redução do índice de sobrevivência de amastigotas axênicas e intracelulares, apresentado IC₅₀ média de 50 µM. O tratamento FT1 e FT2 resultou em alterações drásticas na ultraestrutura de promastigotas como o enrugamento da membrana plasmática, diminuição e inchaço do corpo celular. Também foi observado alteração do potencial de membrana mitocondrial, aumento das espécies reativas de oxigênio e dos compartimentos ácidos. Em conjunto, estes resultados apontam as moléculas FT1 e FT2 como potentes agentes leishmanicidas promissores para uso, de forma isolada ou combinada no tratamento da leishmaniose.

Palavras-chave: Leishmaniose; Quimioterapia; 1,2,3-triazol; Ftalimidas; Click chemistry.

ABSTRACT

Organic molecules formed by the union of two or more pharmacophoric groups have been studied for the development of new leishmanicidal agents and in this context, the triazole, phthalimide and naphthoquinone derivatives have gained prominence. Thus, this doctoral thesis aimed to investigate the leishmanicidal potential of seven triazole derivatives linked to phthalimide or naphthoquinone obtained by click chemistry, on the evolutionary forms of *Leishmania amazonensis* and *Leishmania braziliensis*. For this, the pharmacological profile, cytotoxicity and leishmanicidal potential were evaluated. The compounds were ranked for selectivity, based on data obtained on cytotoxicity and leishmanicidal activity. In this sense, two molecules were chosen as lead compounds and had their mechanism of action investigated through the analysis of mitochondrial physiology and acid compartments in *Leishmania* promastigotes. The inhibitory potential of the lead-compounds on the enzymes sterol 14 alpha-demethylase and trypanothione reductase, molecular targets commonly associated with drug action, was also investigated. The cytotoxicity assay on macrophages and fibroblasts demonstrated that the compounds containing naphthoquinone (FT6 and FT7) were more toxic, with CC₅₀ values ranging from 20.8 to 314.2 µM, while derivatives with phthalimide were the least toxic (CC₅₀ > 2000 µM). All compounds showed leishmanicidal activity against the promastigote forms of *L. amazonensis* and *L. braziliensis*, the highest selectivity indexes were found for the FT1 and FT2 derivatives (SI > 20). The compounds FT1 and FT2 did not show hemolytic activity and were effective in reducing the survival rate of axenic and intracellular amastigotes, with an average IC₅₀ of 50 µM. The FT1 and FT2 treatment resulted in drastic changes in the ultrastructure of promastigotes such as the wrinkling of the membrane, shrinkage and swelling of the cell body. Changes in mitochondrial membrane potential, increase in reactive oxygen species and acid compartments were also observed. Taken together, these results point to FT1 and FT2 molecules as potent leishmanicidal agents that are promising for use, alone or in combination, in the treatment of leishmaniasis.

Keywords: Leishmaniasis. Chemotherapy. 1,2,3-triazole. Phthalimides. Click chemistry.

LISTA DE ILUSTRAÇÕES

Figura 1 -	Períodos históricos da leishmaniose no Brasil.....	25
Figura 2 -	Ciclo de vida de protozoários do gênero <i>Leishmania</i>	26
Figura 3 -	Ultraestrutura do protozoário <i>Leishmania</i> sp. Formas promastigota (A) e amastigota (B).....	27
Quadro 1 -	Espécies de <i>Leishmania</i> e manifestações clínicas associadas.....	28
Figura 4 -	Leishmaniose cutânea localizada (LCL). Lesão ulcerosa com borda eritematosa antes do tratamento (A). Lesão ulcerosa durante o tratamento com anfotericina B lipossomal.....	29
Figura 5 -	Resposta imunológica inicial à infecção por <i>Leishmania</i> sp. EROs: Espécies Reativas de Oxigênio; NETs: <i>neutrophil extracellular traps</i>	30
Figura 6 -	Modulação da resposta inflamatória na leishmaniose cutânea por citocinas.....	31
Figura 7 -	Leishmaniose Cutânea Difusa (LCD). Lesão ulcerada em membro superior (A), infiltração auricular e nódulos eritematosos disseminados (B) e lesões difusas por todo o corpo após recidiva (C, D e E).....	33
Figura 8 -	Leishmaniose Mucocutânea (LMC). Placas infiltrativas, eritematosas e ulceradas cobrindo o couro cabeludo e testa (A) e a parte esquerda da face (B).....	35
Figura 9 -	Aspectos gerais da resposta imunológica mediada por células e citocinas, associada ao desenvolvimento de Leishmaniose Visceral.....	37
Figura 10 -	Leishmaniose Visceral. Criança de dois anos com acentuado quadro de hepatoesplenomegalia (A), homem de dezesseis anos com hepatoesplenomegalia e evidente perda de peso (B), achado pós-morte de esplenomegalia maciça e hepatomegalia (C).....	39
Figura 11 -	Estratificação de risco da LV por município brasileiro durante o período de 2017-2019.....	42
Figura 12 -	Estratificação de risco da LC por município brasileiro durante o período de 2017-2019.....	43
Figura 13 -	Distribuição do número de casos de LV por macrorregiões de saúde do Estado de Pernambuco.....	44
Figura 14 -	Distribuição do número de casos de LC por macrorregiões de saúde do Estado de Pernambuco.....	45
Figura 15 -	Formas amastigotas de <i>Leishmania</i> sp. no interior de macrófagos. Parasitos representativos estão demonstrados nas setas pretas. Vacúolo parasitóforo ocupando quase todo o espaço intracelular do macrófago (a).....	48
Figura 16 -	Estrutura química do tártaro de antimônio e potássio.....	52
Figura 17 -	Estrutura química dos medicamentos de primeira linha contra as leishmanioses. Antimoniato de meglumina e Estibogluconato de sódio.....	53

Figura 18 -	Mecanismos de ação propostos para o efeito leishmanicida dos antimoniais pentavalentes.....	55
Figura 19 -	Estrutura química da anfotericina B.....	59
Figura 20 -	Estrutura química da miltefosina.....	61
Figura 21 -	Estruturas químicas da pentamidina e paromicina.....	62
Figura 22 -	Etapas do processo de pesquisa e desenvolvimento de novos fármacos.....	63
Figura 23 -	Heterocíclicos de cinco membros contendo 1 ou 2 heteroátomos e seus respectivos homólogos saturados. Compostos: 1 - pirrol; 2 - furano; 3 - tiofeno; 4 - pirazol; 5 - imidazol; 6 - oxazol; 7 - isoxazol; 8 - tiazol; 9 - isotiazol; 10 - pirrolidina; 11 - tetrahydrofurano; 12 - tetrahidrotiofeno; 13 - pirazolidina; 14 - imidazolidina; 15 - oxazolidina; 16 - isoxazolidina; 17 - tiazolidina; 18 - isotiazolidina; 19 - benzotiofeno.....	65
Figura 24 -	Reação de <i>Click Chemistry</i>	66
Figura 25 -	Estruturas químicas dos isômeros triazólicos: 1,2,3-triazol e 1,2,4-triazol.....	67
Figura 26 -	Derivados triazólicos com ação leishmanicida produzidos a partir da modificação estrutural de imidazóis (composto 1) e pela incorporação de derivados epóxido (composto 2).....	68
Figura 27 -	Derivados de lapachol-1,2,3-triazol produzidos por <i>click chemistry</i> com efeito leishmanicida <i>in vitro</i>	70
Figura 28 -	Derivados triazólicos bioisósteros da miltefosina (11) e 1,4 substituídos (12) com potente efeito leishmanicida sobre <i>L. amazonensis</i>	71
Figura 29 -	Estrutura química da ftalimida (1,3-isoindolindiona).....	73
Figura 30 -	Moléculas híbridas derivadas de ftalimida e 1,2,3-triazol com efeito inibitório sobre as enzimas tirosinase (13 e 14) e α -glicosidase (15). Vermelho: ftalimida; azul: 1,2,3-triazol....	73
Figura 31 -	Derivados de ftalimida e 1,2,3-triazol com atividade anticâncer (16) e anti- <i>Mycobacterium</i> (17 e 18). Vermelho: ftalimida; azul: 1,2,3-triazol.....	74
Figura 32 -	Estrutura química e mecanismo de ação do derivado PT4 sobre as formas evolutivas de <i>L. amazonensis</i> e <i>L. braziliensis</i> . Vermelho: ftalimida; azul: 1,2,3-triazol.....	75

LISTA DE TABELAS

Tabela 1 –	Incidência média anual de LV durante 2007-2017 no Nordeste do Brasil, de acordo com o estado e município com maior número de casos.....	41
Tabela 2 –	Medidas profiláticas atuais para combater vetores da Leishmaniose canina.....	51
Tabela 3 –	Distribuição dos estudos que abordam a ineficácia, toxicidade ou desfecho fatal em usuários de antimoníato de meglumina (AM) ou estibogluconato de sódio (ES) durante o tratamento das leishmanioses.....	57

LISTA DE ABREVIATURAS E SIGLAS

ADMET	Propriedades Físico-químicas, farmacocinéticas e toxicológicas
AM	Antimoniato de Meglumina
AmB	Anfotericina B
BBB	Barreira hematoencefálica
C14DM	Esterol 14-alfa-demetilase
CC ₅₀	Concentração citotóxica em células de mamíferos
CEELVA	Comissão Encarregada do Estado da Leishmaniose Visceral Americana
CEP	Comitê de Ética em Pesquisa
CEUA	Comissão de Ética no Uso de Animais
CONEP	Comissão Nacional de Ética em Pesquisa
COVID-19	Doença do Novo Coronavírus 2019
DL ₅₀	Dose letal para 50% de uma determinada população
DMEM	<i>Dulbecco's Modified Eagle Medium</i>
DMSO	Dimetilsulfóxido
DTNs	Doenças Tropicais Negligenciadas
EROs	Espécies Reativas de Oxigênio
ES	Estibogluconato de Sódio
FT	Derivados de Ftalimida e 1,2,3-triazol
FT1	Etil-1-[2-(1,3-dioxo-1,3-di-hidro-2 <i>H</i> -isoindol-2-il)etil]-1 <i>H</i> -1,2,3-triazol-4-carboxilato
FT2	Etil-1-[3-(1,3-dioxo-1,3-di-hidro-2 <i>H</i> -isoindol-2-il)propil]-1 <i>H</i> -1,2,3-triazol-4-carboxilato
FT3	2-[[4-(2-hidroxiopropan-2-il)-1 <i>H</i> -1,2,3-triazol-1-il]metil]-1 <i>H</i> -isoindol-1,3(2 <i>H</i>)-diona
FT4	2-[[1-(3-nitrofenil)-1 <i>H</i> -1,2,3-triazol-4-il]metil]-1 <i>H</i> -isoindol-1,3(2 <i>H</i>)-diona
FT5	[1-(3-nitrofenil)-1 <i>H</i> -1,2,3-triazol-4-il]metanol
FT6	2-[4-(3-hidroxiopropil)-1 <i>H</i> -1,2,3-triazol-1-il]naftaleno-1,4-diona
FT7	Etil 1-(1,4-dioxo-1,4-dihidronaftalen-2-il)-1 <i>H</i> -1,2,3-triazol-4-carboxylato

GERES	Gerências Regionais de Saúde
GIA	Absorção gastrointestinal
HBA	Aceptores de ligação de hidrogênio
HBD	Doadores de Ligação Hidrogênio
hERG	Gene humano <i>ether-a-go-go</i>
HIV	Vírus da Imunodeficiência Humana
IC ₅₀	Constante de inibição de 50% da cultura de parasitos
IL-10	Interleucina-10
IL-12	Interleucina-12
IL-13	Interleucina-13
IL-17	Interleucina-17
IL-4	Interleucina-4
INF- γ	Interferon Gamma
IS	Índice de Seletividade
K _i	constante de inibição enzimática
LC	Leishmaniose Cutânea
LCD	Leishmaniose Cutânea Difusa
LCL	Leishmaniose Cutânea Localizada
LMC	Leishmaniose Mucocutânea
LogP	Coeficiente de partição octanol-água
LV	Leishmaniose Visceral
MAA	Meio amastigota-axênica
MeOH	Metanol
MØ	Macrófagos
mPEC	Macrófagos de exsudato peritoneal
MTT	3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio brometo)
MW	Massa molecular
NETs	<i>Neutrophil Extracellular Traps</i>
NK	Células Natural <i>killer</i>
NO	Óxido Nítrico
n-ROTB	Número de ligações rotativas
OCT2	Organic Cation Transporter
OMS	Organização Mundial de Saúde
OPAS	Organização Pan-Americana de Saúde

PALS	Bainha Periarteriolar de Linfócitos
PBMC	Células Mononucleares do Sangue Periférico
PCR	Reação da Polimerase em Cadeia
PDB	<i>Protein Data Bank</i>
P-Gp	Glicoproteína P
PT4	4-fenil-1-[2-(ftalimido-2-il)etil]-1 <i>H</i> -1,2,3-triazol
PTR1	Pteridina redutase 1
RM	Região Metropolitana
RMSD	Root-mean-square deviation of atomic positions
SANAR	Programa de Enfretamento às Doenças Negligenciadas
Sb ³⁺	Tártaro Emético
SBF	Soro Fetal Bovino
Sb ^V	Antimoniais Pentavalentes
SINAN	Sistema Nacional de Informações de Agravos de Notificação
TGF- β	Fato de Crescimento Transformador <i>beta</i>
TNF- α	Fator de Necrose Tumoral
TPSA	Polar surface área
TryR	Tripanotiona Redutase

SUMÁRIO

1	INTRODUÇÃO.....	19
2	OBJETIVOS.....	22
2.1	OBJETIVO GERAL.....	22
2.2	OBJETIVOS ESPECÍFICOS.....	22
3	FUNDAMENTAÇÃO TEÓRICA.....	23
3.1	LEISHMANIOSES: COMPLEXO DE DOENÇAS HISTORICAMENTE NEGLIGENCIADAS E UM DOS PRINCIPAIS DESAFIOS DA SAÚDE PÚBLICA MUNDIAL.....	24
3.1.1	Leishmaniose Cutânea.....	29
3.1.2	Leishmaniose Cutânea Difusa.....	32
3.1.3	Leishmaniose Mucocutânea.....	34
3.1.4	Leishmaniose Visceral.....	36
3.2	EPIDEMIOLOGIA DAS LEISHMANIOSES	40
3.3	MEDIDAS DE CONTROLE DAS LEISHMANIOSES: DIAGNÓSTICO, CONTROLE DO VETOR E FORMAS DE TRATAMENTO.....	46
3.3.1	Diagnóstico das Leishmanioses.....	47
3.3.2	Entre o Amor ao Animal e à Saúde Pública: Medidas Sanitárias Controversas de Controle das Leishmanioses no Brasil.....	49
3.4	TRATAMENTO FARMACOLÓGICO DAS LEISHMANIOSES.....	52
3.4.1	Antimoniais Pentavalentes.....	52
3.4.2	Anfotericina B.....	58
3.4.3	Miltefosina, Paromicina e Pentamidina.....	60
3.5	PESQUISA E DESENVOLVIMENTO DE NOVAS DROGAS CONTRA AS LEISHMANIOSES.....	62
3.5.1	Compostos Heterocíclicos: Grupos Funcionais Privilegiados na Descoberta de Novos Medicamentos.....	64
3.5.2	Potencial Leishmanicida de Compostos Bioativos Derivados do 1,2,3-Triazol: Do Planejamento Estrutural à Compreensão dos Mecanismos de Ação.....	67
3.5.3	Potencial Terapêutico e Atributos Medicinais de Compostos Bioativos Derivados de Ftalimida e 1,2,3-Triazol.....	72
4	MATERIAIS E MÉTODOS.....	76
4.1	SÍNTESE DOS DERIVADOS DE 1,2,3-TRIAZOL E FTALIMIDA..	76
4.2	ANÁLISE IN SILICO DO PERFIL FÍSICO-QUÍMICO, FARMACOCINÉTICO E TOXICOLÓGICO.....	79
4.3	ENSAIO DE CITOTOXICIDADE EM CÉLULAS DE MAMÍFEROS.....	79
4.4	AVALIAÇÃO DO EFEITO DOS DERIVADOS DE FTALIMIDA E 1,2,3-TRIAZOL SOBRE FORMAS PROMASTIGOTAS DE <i>Leishmania amazonensis</i> E <i>Leishmania braziliensis</i>	80
4.5	INVESTIGAÇÃO DO POTENCIAL HEMOLÍTICO DOS DERIVADOS FTALIMÍDICOS-TRIAZÓLICOS DE MAIOR SELETIVIDADE CONTRA <i>Leishmania</i> sp.....	81

4.6	AVALIAÇÃO DO EFEITO LEISHMANCIDA DOS DERIVADOS FT1 E FT2 SOBRE FORMAS AMASTIGOTAS DE <i>Leishmania amazonensis</i> E <i>Leishmania braziliensis</i>	81
4.7	INVESTIGAÇÃO DO EFEITO DOS DERIVADOS FT1 E FT2 NA PRODUÇÃO DE ÓXIDO NÍTRIO EM MACRÓFAGO DE EXSUDATO PERITONEAL.....	83
4.8	ANÁLISE DO EFEITO DOS DERIVADOS FT1 E FT2 SOBRE A ULTRAESTRUTURA DE PROMASTIGOTAS DE <i>Leishmania amazonensis</i> E <i>Leishmania braziliensis</i>	84
4.9	AVALIAÇÃO DO POTENCIAL DE MEMBRANA MITOCONDRIAL, PRODUÇÃO DE ESPÉCIES REATIVAS DE OXIGÊNIO E COMPARTIMENTOS ÁCIDOS EM PROMASTIGOTAS TRATADAS COM OS DERIVADOS FT1 E FT2.....	84
4.10	ANCORAGEM MOLECULAR E ESTUDO DAS INTERAÇÕES QUÍMICAS ENTRE OS DERIVADOS FT1 E FT2 SOBRE OS PRINCIPAIS ALVOS FARMACOLÓGICOS EM <i>Leishmania</i> sp....	85
5	RESULTADOS.....	86
5.1	ARTIGO 1.....	86
5.2	ARTIGO 2.....	148
5.3	PATENTE.....	159
5.4	ARTIGO 3.....	201
5.5	ARTIGO 4.....	208
5.6	ARTIGO 5.....	228
5.7	ARTIGO 6.....	238
5.8	ARTIGO 7.....	250
6	CONCLUSÃO.....	251
	REFERÊNCIAS.....	252
	APÊNDICE A - ARTIGO PUBLICADO NA REVISTA MATERIALS SCIENCE AND ENGINEERING: C (FATOR DE IMPACTO: 7.32).....	277
	APÊNDICE B - ARTIGO PUBLICADO NA REVISTA JOURNAL OF APPLIED MICROBIOLOGY (FATOR DE IMPACTO: 3.77)...	288
	APÊNDICE C - ARTIGO PUBLICADO NA REVISTA DA ASSOCIAÇÃO MÉDICA BRASILEIRA (FATOR DE IMPACTO: 1.20).....	298
	ANEXO A – CERTIFICADO DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS.....	304
	ANEXO B - CERTIFICADO DE APROVAÇÃO DA COMISSÃO ÉTICA NO USO DE ANIMAIS – CEUA.....	307

1 INTRODUÇÃO

As doenças tropicais negligenciadas constituem um dos principais grupos de enfermidades que afetam as populações mais pobres ao redor do mundo (Hotez, 2017). Neste grupo estão as leishmanioses, um complexo de doenças causadas por protozoários do gênero *Leishmania* sp. com amplo espectro clínico, das quais se destacam formas cutânea, mucocutânea e visceral, acometendo animais e seres humanos (Hotez *et al.*, 2020). Devido aos impactos econômicos, sociais e ao grande potencial dessas doenças causarem deformidades físicas, funcionais e metabólicas, as medidas de combate às Leishmanioses são consideradas componentes cruciais para o desenvolvimento sustentável e à cobertura mundial da saúde (Molyneux *et al.*, 2017).

As leishmanioses estão dentre as doenças parasitárias emergentes transmitidas por vetores mais importantes no contexto da saúde pública. No Brasil, a leishmaniose cutânea (LC) e a leishmaniose mucocutânea (LMC) são comumente causadas pelas espécies *Leishmania amazonensis* e *Leishmania braziliensis*, enquanto a leishmaniose visceral (LV) pode estar associada à infecção por *Leishmania infantum* (Brasil, 2017a; b). Devido à falta de vacinas, as principais medidas de controle para essas doenças consistem em intervenções que visam a eliminação dos vetores e, principalmente, a realização de um tratamento adequado (Kevric *et al.*, 2015).

O tratamento das leishmanioses é realizado através da administração parenteral dos fármacos antimoniatos de meglumina (Glucantime®) ou estibogluconato de sódio (Pentostam®), dos quais somente o primeiro está disponível para uso no serviço de saúde pública brasileiro (Brasil, 2017a). Estas drogas são utilizadas desde o início do século passado, quando foi descoberta a eficácia do antimônio (Sb^{3+}) no tratamento da LMC (Vianna, 1912) e posteriormente da LV (Cristina e Caronia, 1915). O uso clínico do Sb^{3+} foi descontinuado devido aos graves efeitos colaterais observados nos pacientes (Marsden, 1985). Embora essas drogas apresentem certa eficácia, seu uso tem apresentado cada vez mais limitações relacionadas ao alto índice de toxicidade e efeitos adversos, incluindo a morte dos usuários (Sampaio *et al.*, 1988; Oliveira *et al.*, 2005). Quando o tratamento com antimoniais não é eficiente, fármacos como a anfotericina B e pentamidina são recomendados como alternativas terapêuticas (Comandolli-Wyrepkowski *et al.*, 2017; Shirzadi, 2019).

A pentamidina e a anfotericina B, inicialmente recomendadas para o tratamento de infecções fúngicas são utilizadas como drogas de segunda escolha para as leishmanioses, entretanto, também apresentam limitações para uso na terapia antiparasitária (Goa e Campoli-Richards, 1987; Gallis *et al.*, 1990). A ocorrência de espécies de *Leishmania* resistentes a estes fármacos tem sido relatada como um dos principais desafios atuais na terapêutica contra esses protozoários (Pountain *et al.*, 2019). Adicionalmente, a toxicidade e o alto custo na aquisição das drogas de segunda linha pelo sistema público de saúde acentuam a necessidade pela busca de novos fármacos para o tratamento das leishmanioses (Zhou e Ipp, 1989; Laniado-Laborín e Cabrales-Vargas, 2009).

A pesquisa e o desenvolvimento de novas moléculas anti-*Leishmania* é objetivo de diversos grupos de pesquisa ao redor do mundo (Mantravadi *et al.*, 2020). Nesse contexto, dois grupos farmacofóricos se destacam nos campos da síntese orgânica, bioquímica e química medicinal: o heterocíclico 1,2,3-triazol e a ftalimida (Holanda *et al.*, 2020). O núcleo 1,2,3-triazol é o principal produto da reação de *click chemistry* utilizado para hibridação molecular de duas ou mais moléculas (Agalave *et al.*, 2011). Nos últimos anos, foi observado que o 1,2,3-triazol possui atividade biológica própria, característica de fármacos (Bozorov *et al.*, 2019). Diversos estudos comprovaram o potencial biológico do anel 1,2,3-triazólico para aplicações como: atividade anti-inflamatória (Sarigol *et al.*, 2015), antimicrobiana (Aly *et al.*, 2015), antibacteriana (Fichtali *et al.*, 2016), antiviral (Lourdes *et al.*, 2014) e antiparasitária (Campo *et al.*, 2012).

Assim como o 1,2,3-triazol, a ftalimida também tem sido amplamente explorada no que diz respeito ao seu potencial farmacológico (Tehrani *et al.*, 2019). A ftalimida pertence à classe das imidas cíclicas e pode ser obtida por síntese orgânica, a partir do anidrido ftálico (Bhat *et al.*, 2020). No contexto biológico, as ftalimidas apresentam uma variedade de atividades comprovadas, das quais se destacam as ações hipolipemiante (Assis *et al.*, 2014), analgésica (Silva *et al.*, 2017), anti-inflamatória (Assis *et al.*, 2012), antimicrobiana (Singh *et al.*, 2015) e antiparasitária (Aliança *et al.*, 2017). Estudos recentes apontam a união desses dois grupos químicos como método promissor no desenvolvimento de moléculas mais robustas, com duas ou mais atividades biológicas ou efeito farmacológico potencializado (Holanda *et al.*, 2021).

O desenvolvimento de novos compostos bioativos com efeito leishmanicida se constitui de um campo aberto, multidisciplinar e promissor no contexto atual de

combate às doenças negligenciadas (Yang *et al.*, 2020). Os derivados do 1,2,3-triazol e da ftalimida possuem características importantes no que diz respeito à produção, bem como quanto às propriedades biológicas e potencial biomédico (Sadat-Ebrahimi *et al.*, 2020). Nesse contexto, a presente tese de doutorado teve como objetivo o desenvolvimento de novos compostos bioativos derivados da ftalimida e do 1,2,3-triazol como alternativas terapêuticas para o tratamento das leishmanioses.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito de derivados híbridos de ftalimidas e 1,2,3-triazóis sobre as formas evolutivas de *Leishmania amazonensis* e *Leishmania infantum*

2.2 OBJETIVOS ESPECÍFICOS

- Sintetizar novos híbridos derivados de ftalimida e 1,2,3-triazol (FT);
- Determinar os parâmetros físico-químicos, farmacocinéticos e toxicológicos *in silico* dos FTs;
- Avaliar o potencial inibitório *in silico* dos FTs sobre alvos moleculares de *Leishmania* sp.;
- Avaliar a citotoxicidade *in vitro* dos derivados FTs sobre as linhagens de macrófagos J774 e fibroblastos;
- Determinar o potencial hemolítico dos FTs nos diferentes fenótipos sanguíneos: A, B, AB e O;
- Analisar o efeito do tratamento *in vitro* dos FTs sobre a produção de óxido nítrico em macrófagos J774;
- Investigar o efeito do tratamento *in vitro* dos FTs sobre o crescimento e viabilidade de formas promastigotas de *L. amazonensis* e *L. infantum*;
- Investigar o efeito do tratamento *in vitro* dos FTs sobre formas amastigotas axênicas e intracelulares de *L. amazonensis* e *L. infantum*;
- Classificar os compostos de acordo com o índice de seletividade para formas promastigotas e amastigotas de *L. amazonensis* e *L. infantum*;
- Avaliar o efeito do tratamento dos FTs sobre a ultraestrutura de promastigotas de *L. amazonensis* e *L. infantum*;
- Verificar a função mitocondrial de promastigotas de *L. amazonensis* e *L. infantum* tratadas com os FTs;

3 FUNDAMENTAÇÃO TEÓRICA

O combate às doenças tropicais negligenciadas (DTNs) tem sido uma das principais metas no campo da saúde pública internacional ao longo dos últimos anos (Unicef e Organization, 2010). A Organização Mundial da Saúde classifica as DTNs como as enfermidades da pobreza, que afetam populações com pouca visibilidade e pouca voz política, resultando em discriminação e estigma com forte impacto na morbidade e mortalidade (OMS, 2010). No cenário mundial, o Brasil está incluído no grupo dos países afetados pelas DTNs e o efeito dessas doenças na economia, saúde pública e no desenvolvimento psicossocial é potencializado devido ao alto índice de desigualdade social (Bardosh, 2014; Landmann-Szwarcwald e Macinko, 2016).

Diante da urgente necessidade de estabelecer medidas efetivas no combate às DTNs, o Governo Federal investiu mais de R\$ 7 milhões em medicamentos no período que compreendeu os anos de 2004 a 2008, nos componentes básico, estratégico e especializado da assistência farmacêutica (Garcia *et al.*, 2011). Em 2013, o Governo de Pernambuco instituiu, através do Decreto nº 39.497 de 11 de junho, o Programa de Enfrentamento às Doenças Negligenciadas (SANAR), incluído na forma Superintendência da Secretaria Estadual de Saúde e definido como medida prioritária no enfrentamento às DTNs para o quadriênio 2015-2018 (Pernambuco, 2015). O SANAR foi elaborado dois anos antes da sua instituição por meio de decreto oficial, o que fez de Pernambuco o primeiro Estado brasileiro a desenvolver um programa específico de combate às doenças negligenciadas (Pernambuco, 2015).

Dentre as enfermidades tropicais classificadas como doenças negligenciadas que merecem atenção e intervenção das políticas de saúde, está a Leishmaniose (ou Leishmanioses, quando se refere ao espectro clínico que acomete diferentes órgãos e sistemas como pele, mucosas, fígado e baço), endêmica no Brasil e amplamente distribuída no Estado de Pernambuco (Leite *et al.*, 2020). Em consonância com as políticas da Organização Mundial de Saúde (OMS), foi estabelecido um plano de ação para fortalecimento da vigilância e controle das leishmanioses nas Américas que destaca o desenvolvimento de medidas terapêuticas alternativas como uma das principais medidas de controle das leishmanioses (OPAS, 2017).

3.1 LEISHMANOSES: COMPLEXO DE DOENÇAS HISTORICAMENTE NEGLIGENCIADAS E UM DOS PRINCIPAIS DESAFIOS DA SAÚDE PÚBLICA MUNDIAL

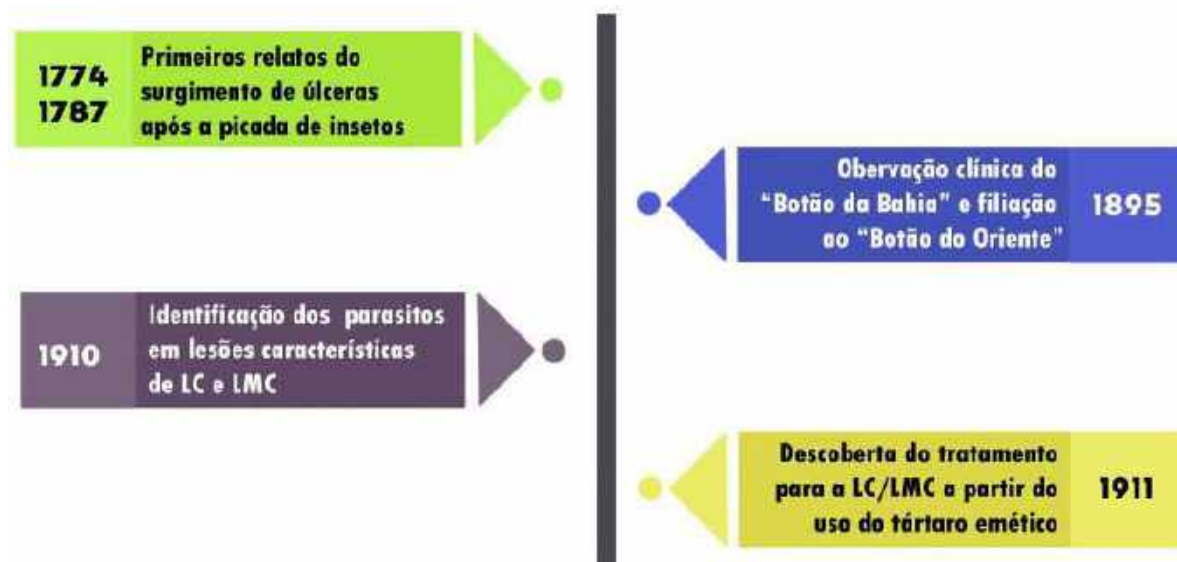
As Leishmanioses são doenças crônicas causadas por protozoários tripanossomatídeos do gênero *Leishmania* e apresentam distribuição mundial, com maioria dos casos notificados na América do Sul, bacia do mediterrâneo e algumas áreas da Ásia e da África (Abadías-Granado *et al.*, 2021). Os protozoários causadores da desse complexo de doenças são parasitos intracelulares obrigatórios transmitidos por insetos vetores e, durante a infecção em hospedeiros vertebrados, se desenvolvem no interior de macrófagos (Mirzaei *et al.*, 2020). A infecção por *Leishmania* pode resultar na ocorrência de três formas distintas da doença, a Leishmaniose Cutânea (LC), Leishmaniose Mucocutânea (LMC) e Leishmaniose Visceral (LV), todas com amplo potencial de causar danos irreversíveis ou levar os indivíduos à morte, quando não tratadas (Showler e Boggild, 2017).

O acometimento humano pelos diferentes tipos de leishmaniose tem sido relatado desde a antiguidade, através da constatação de mutilações de lábios e narizes em vasos antropomórficos pré-colombianos (400 a 900 anos d.C.), característicos de *espundia*, uma denominação antiga utilizada para se referir à LMC (Basano e Camargo, 2004). No Brasil, apesar da ausência de elementos que permitam assegurar as origens da doença, dados de 1774-1787 relativos às opiniões de cronistas e viajantes citam as descrições de um médico que residia no Amazonas sobre uma praga de mosquitos que suas picadas davam por vezes lugar a úlceras (Costa, 1992).

Devido a facilidade de ser reconhecida por causar sinais e sintomas na pele, a LC e LMC são descritas em diversos estudos que abordam o surgimento da leishmaniose (Cerutti *et al.*, 2017). Em 1985, pela primeira vez, a lesão até então denominada “botão da Bahia” foi relacionada ao “botão do Oriente”, sugerindo que a doença poderia ter sido importada, por meio de incursões sírias às Américas em tempos anteriores (Vale e Furtado, 2005). No início do século XX, em meio à grande epidemia de úlceras e lesões mucosas em São Paulo, denominadas de “úlceras de Bauru” durante a construção da Estrada de Ferro Noroeste, o agente etiológico da leishmaniose tegumentar foi então identificado (Araujo Filho, 1981). Em 1911, Gaspar

Vianna descobre a eficácia do antimoniato trivalente no tratamento para a LC e LMC (Rath *et al.*, 2003).

Figura 1 – Períodos históricos da leishmaniose no Brasil



Fonte: O autor (2022).

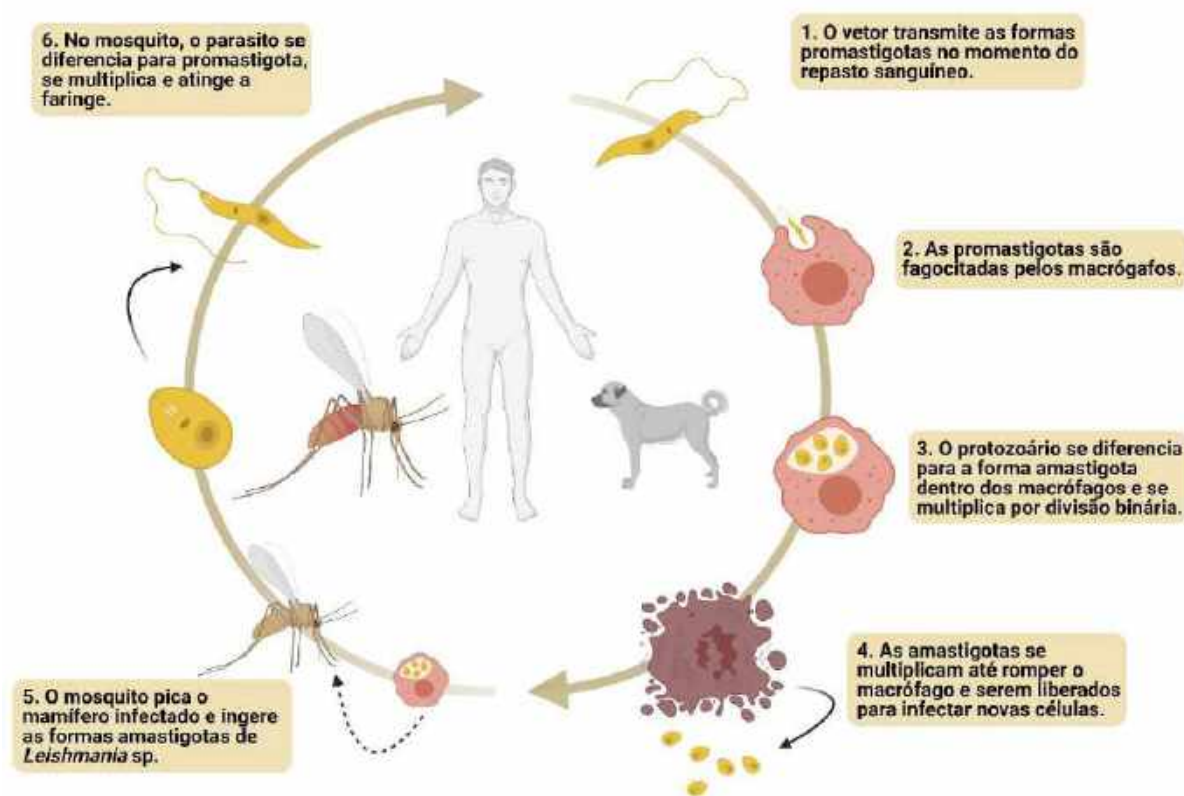
A LV emergiu como um problema de saúde pública no Brasil somente em 1934, durante as pesquisas de rotina para o diagnóstico de outras doenças. Neste período, o Serviço Cooperativo de Febre Amarela, com laboratório sediado na Bahia, identificou protozoários do gênero *Leishmania* em amostras de fígado provenientes de 41 pacientes do Norte e Nordeste, que haviam falecido de causas suspeitas (Benchimol, 2019). Em junho de 1936 foi criada a Comissão Encarregada do Estado da Leishmaniose Visceral Americana (CEELVA), chefiada por Evandro Chagas que ao longo dos anos se dedicou ao estudo da LV e consolidou a valiosa contribuição da CEELVA a partir de diversas publicações ao longo da década de 1930 sobre vários aspectos da LV (Benchimol *et al.*, 2019).

Após 20 anos da identificação das formas parasitárias em amostras de fígado humano, ocorreu a notificação do primeiro surto de LV popularmente conhecida com calazar, em Sobral, interior do Estado do Ceará (Deane, 1956). Anos posteriores a LV avançou com rapidez para todo o Brasil e deixou de se concentrar em zonas rurais e interioranas, alcançando grandes centros urbanos com predominância na região Nordeste (Gontijo e Melo, 2004). A década de 90 foi marcada pelo aumento significativo de casos de calazar nos Estados do Pará, Tocantins, Mato Grosso do Sul,

Minas Gerais e São Paulo, possivelmente devido ao aumento da exploração das matas e ampla distribuição do vetor (Carmo, 2003).

Os países tropicais são ambientes naturais de diversas espécies de animais, vegetais e insetos, alguns destes últimos são responsáveis pela transmissão de doenças como dengue, zika, malária, febre amarela e leishmaniose (Andriamifidy *et al.*, 2019). Um dos principais vetores das leishmanioses no Brasil é o flebotomíneo *Lutzomyia longipalpis* inicialmente com maior prevalência no ambiente silvestre e rural, característica esta que também se estendia à leishmaniose (Rangel e Vilela, 2008). As alterações ambientais drásticas resultantes da ação humana mudaram a ecologia de algumas espécies de flebotomíneos e, conseqüentemente, a epidemiologia das leishmanioses. Nesse contexto, o ser humano deixou de ser um hospedeiro acidental e passou a viver em estreita e frequente associação à cadeia epidemiológica da doença (Rodrigues *et al.*, 2017). O ciclo de vida da *Leishmania* envolve dois hospedeiros, o mosquito vetor e mamíferos, incluindo o cão doméstico (figura 2).

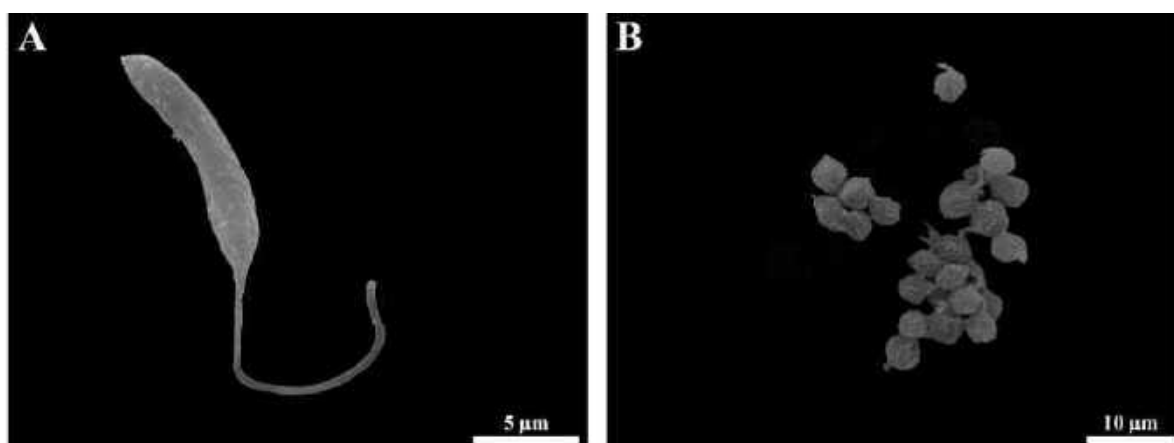
Figura 2 – Ciclo de vida de protozoários do gênero *Leishmania*



Fonte: O autor (2022).

A infecção por *Leishmania* se inicia no momento em que os mosquitos vetores infectados se alimentam de sangue de mamíferos silvestres como raposas, gambás, bichos-preguiça ou animais urbanos, no qual se destaca o cão doméstico (Roque e Jansen, 2014). A *Leishmania* tem duas morfologias principais no ciclo de vida: a amastigota intracelular no hospedeiro mamífero e a promastigota no vetor (Silva e Cano, 2017). A forma promastigota é caracterizada pelo formato fusiforme e a existência de um flagelo alongado, enquanto que a amastigota apresenta tamanho menor, flagelo internalizado e forma ovoide (figura 3). Após a inoculação do parasito pelo mosquito, uma resposta imunológica é desencadeada resultando no recrutamento de células do sistema imune, em especial macrófagos que estão próximos ou no local da infecção (Loría-Cervera e Andrade-Narvaez, 2020).

Figura 3 - Ultraestrutura do protozoário *Leishmania* sp. Formas promastigota (A) e amastigota (B)



Fonte: Holanda *et al.* (2019).

Os macrófagos são as principais células-alvo da *Leishmania* e atuam na fagocitose das promastigotas. No interior desses fagócitos, as promastigotas se diferenciam em amastigotas, se multiplicam intensamente e causam a ruptura da célula hospedeira resultando na liberação destes parasitos nos tecidos ou na corrente sanguínea, que continuam a infectar outros macrófagos (Duque *et al.*, 2019). O ciclo de vida do protozoário é completado quando outro mosquito realiza o repasto sanguíneo e ingere as amastigotas, que se diferenciam em promastigotas para uma nova infecção (Teixeira *et al.*, 2013). Associado a ampla distribuição do vetor, existem mais de 20 espécies de *Leishmania* capazes de causar as diferentes formas de leishmanioses em seres humanos (quadro 1). Os fatores associados às formas

clínicas da doença envolvem, além da espécie do protozoário, aspectos genéticos e imunológicos que podem determinar a gravidade e síndrome clínica seja na LC, incluindo a variações Leishmaniose Cutânea Localizada (LCL) e Leishmaniose Cutânea Difusa (LCD); LMC ou LV (Dayakar *et al.*, 2019).

Quadro 1 – Espécies de *Leishmania* e manifestações clínicas associadas

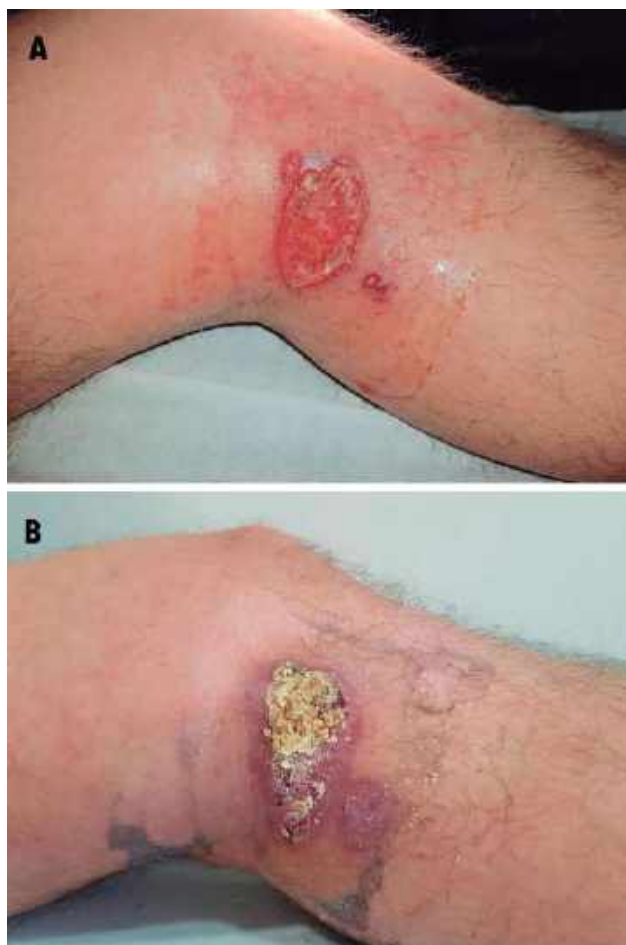
Subgênero	Espécie	Manifestação Clínica	Distribuição
<i>Leishmania</i>	<i>L. acthiopica</i>	LCL e LCD	Etiópia e Kenya
	<i>L. amazonensis</i>	LCL, LCD e LMC	Brasil, Venezuela e Bolívia
	<i>L. donovani</i>	LV	África Central, Índia e China
	<i>L. infantum</i>	LV, LC	Mediterrâneo, Europa, Ásia e Américas Central e dos Sul
	<i>L. major</i>	LC	Norte e Centro da África, Oriente Médio e Ásia Central
	<i>L. mexicana</i>	LCL e LCD	EUA, Equador, Venezuela e Peru
	<i>L. tropica</i>	LCL e LV	Índia, Ásia Central, Oriente Médio e Centro-Sul da África
	<i>L. venezuelensis</i>	LCL	Norte da América do Sul e Venezuela
	<i>L. waltoni</i>	LCD	República Dominicana
<i>Viannia</i>	<i>L. braziliensis</i>	LCL e LMC	Bacia Amazônica Central, Brasil, Bolívia e Venezuela
	<i>L. guyanensis</i>	LCL e LMC	Guiana Francesa, Suriname, Brasil e Bolívia
	<i>L. lainsoni</i>	LCL	Brasil, Bolívia e Peru
	<i>L. lindenbergi</i>	LCL	Brasil
	<i>L. naiffi</i>	LCL	Brasil e Guiana Francesa
	<i>L. panamensis</i>	LCL e LMC	Panamá, Colômbia, Venezuela e Brasil
	<i>L. peruviana</i>	LCL e LMC	Peru, Bolívia
	<i>L. shawi</i>	LCL	Brasil

Fonte: Adaptado de Steverding (2017).

3.1.1 Leishmaniose Cutânea

A LC é a forma clínica caracterizada pelo desenvolvimento de lesões ulcerativas no local da picada do mosquito vetor (Reithinger *et al.*, 2007). Normalmente a LC não oferece risco à vida, porém é uma das principais causas de estigma e segregação social (Scorza *et al.*, 2017). Apesar de muitos indivíduos não apresentarem lesões clássicas, o primeiro sinal clínico da LC consiste na formação de um eritema que se desenvolve em pápula, seguido de um nódulo que ulcera gradativamente até assumir o padrão anatomopatológico da doença (Van-Henten *et al.*, 2018). Inicialmente as lesões ocorrem de forma localizada e restritas ao local no qual os parasitos foram inoculados (figura 4), sendo definida como LCL (Sechi *et al.*, 2021).

Figura 4 – Leishmaniose cutânea localizada (LCL). Lesão ulcerosa com borda eritematosa antes do tratamento (A). Lesão ulcerosa durante o tratamento com anfotericina B lipossomal.

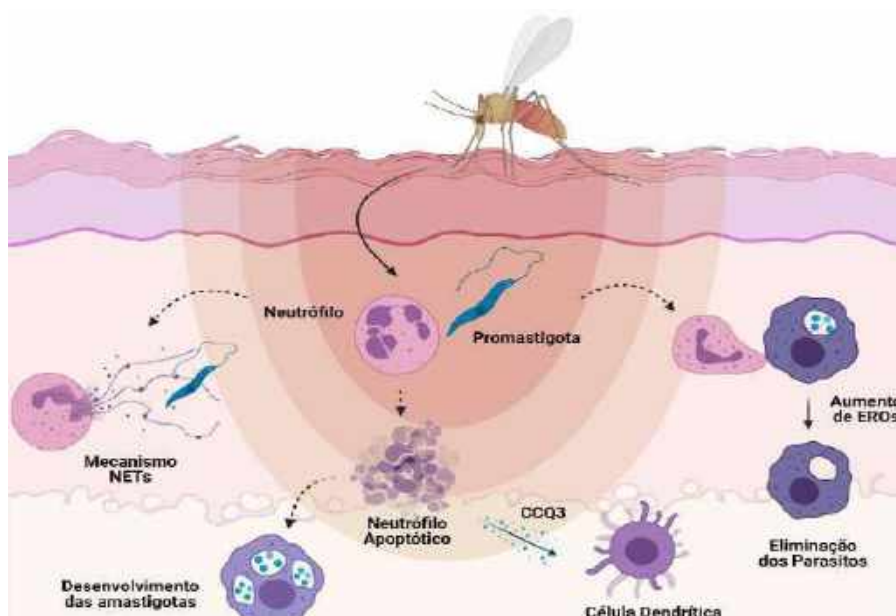


Fonte: Navarrete-Dechent *et al.* (2018).

A extensão e a gravidade da lesão estão associadas à intensa resposta imune do hospedeiro contra a infecção por *Leishmania* sp. (Kaye e Beattie, 2016). À medida que as células do sistema imunológico são recrutadas para o local da infecção, um infiltrado inflamatório se instala e resulta no surgimento de edema, e dano tecidual (Wijnant *et al.*, 2018). Os neutrófilos compõem o grupo de células inflamatórias que, assim como os macrófagos, atuam na resposta imunológica celular à infecção. Estas células combatem os parasitos a partir de armadilhas extracelulares de neutrófilos, em inglês “*neutrophil extracellular traps (NETs)*” (Rochaël *et al.*, 2015) ou pela interação com macrófagos infectados, induzindo o aumento da produção de espécies reativas de oxigênio (Conceição *et al.*, 2016).

Além do papel na ativação de macrófagos, os neutrófilos também atuam no recrutamento de células dendríticas para o local da infecção, através da produção e liberação de CC-Quimiocina 3 (CCQ3) e normalmente, entram em apoptose ainda no tecido cutâneo (Charmoy *et al.*, 2010). Em alguns casos, esses neutrófilos apoptóticos podem ser captados por macrófagos e células dendríticas, limitando a ação dessas células e favorecendo o desenvolvimento intracelular do parasito (Afonso *et al.*, 2008), conforme representado na figura 5. A ação dos neutrófilos pode ser modulada pela espécie do parasito envolvido na infecção e características genéticas do hospedeiro (Ribeiro-Gomes e Sacks, 2012).

Figura 5 – Resposta imunológica inicial à infecção por *Leishmania* sp. EROs: Espécies Reativas de Oxigênio; NETs: *neutrophil extracellular traps*;

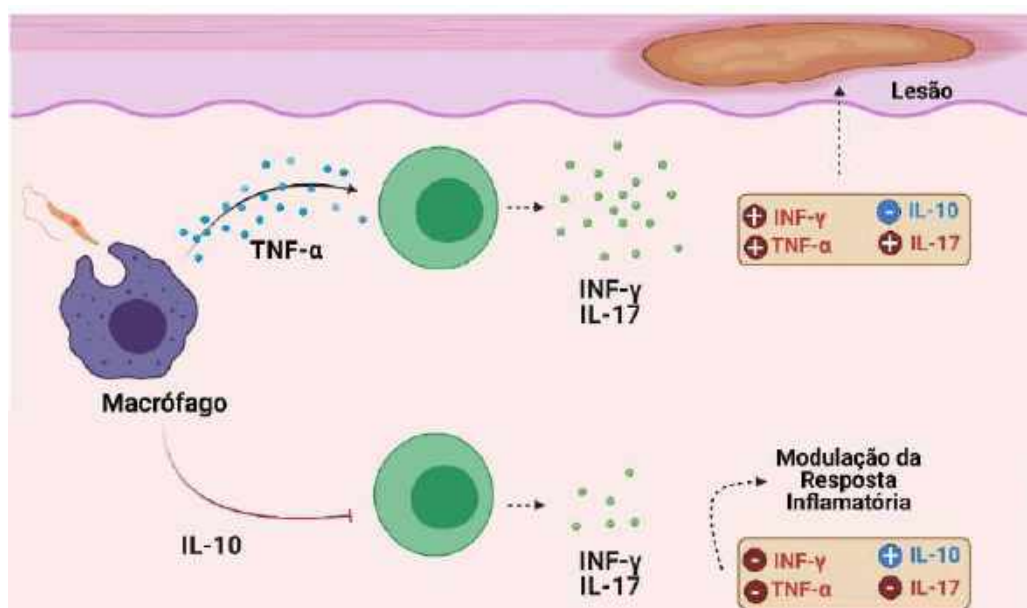


Fonte: O autor (2022).

Outro fator importante no desenvolvimento da lesão cutânea é a liberação de citocinas pró-inflamatórias pelas células do sistema imune (Scorza *et al.*, 2017). Nesse contexto, o interferon Gamma (INF- γ), secretado principalmente por linfócitos TCD4⁺, TCD8⁺ e células natural *killer* (NK), e o fator de necrose tumoral (TNF- α), produzido principalmente por macrófagos, são duas citocinas envolvidas na imunopatologia da LC (Maspi *et al.*, 2016). Essas citocinas atuam na estimulação da produção de óxido nítrico (NO), um mediador bioquímico importante no mecanismo de eliminação das formas amastigotas em macrófagos (Mukbel *et al.*, 2007). No entanto, diferentes estudos apontam que a suprarregulação das citocinas pró-inflamatórias, incluindo o INF- γ e TNF- α , está associada ao aumento do dano tecidual no local da infecção (Bacellar *et al.*, 2002).

Além dos altos índices de INF- γ e TNF- α , a modulação da resposta inflamatória em pacientes com LC também está associada a diminuição na produção de citocinas regulatórias como a interleucina-10 (IL-10) ou do seu receptor (Oliveira *et al.*, 2011). A IL-10 apresenta propriedades anti-inflamatórias e tem sido observado que essa citocina pode suprimir a produção de TNF- α e de interleucina-17 (IL-17), agente quimiotático de neutrófilos, presente no local das lesões e indutor da produção de vários mediadores pró-inflamatórios como interleucina-1, interleucina-6 e a própria TNF- α (Oliveira *et al.*, 2014). O mecanismo de regulação da resposta imune associada a lesão na LC está representado na figura 6.

Figura 6 – Modulação da resposta inflamatória na leishmaniose cutânea por citocinas



Fonte: O autor (2022).

As lesões dérmicas observadas na LC constituem um fator de risco para o acometimento por infecções bacterianas ou fúngicas associadas (Agudelo *et al.*, 1999). A coinfeção da úlcera causada por *Leishmania* sp. com *Staphylococcus aureus* pode aumentar a gravidade da lesão em até duas vezes, quando comparada àquelas causadas exclusivamente pelo protozoário (Borbón *et al.*, 2019). Outras manifestações clínicas também são comuns à LC como surgimento de placas eritematosas, lesões verrucóides e lesões descamativas, normalmente precedidos por linfadenopatia, febre e dor no corpo (Cavalcante *et al.*, 2019; Holanda *et al.*, 2019).

Mesmo com baixa mortalidade, as cicatrizes das lesões podem ser facilmente reconhecidas e associadas ao espectro da LC, interferindo na autoimagem do indivíduo e causando impacto negativo na qualidade de vida (Silva Junior *et al.*, 2020). Em casos de ineficácia do tratamento ou redução da competência imunológica do hospedeiro, as lesões podem cicatrizar, mas ainda haver recidivas (Gontijo e Carvalho, 2003), possibilitando a disseminação dos protozoários por todo o tegumento e desenvolvimento da forma cutânea difusa (Silveira, 2009).

3.1.2 Leishmaniose Cutânea Difusa

A LCD, também conhecida por leishmaniose cutânea anérgica difusa ou leishmaniose anérgica hansenóide foi inicialmente descrita no Estado do Pará como uma forma rara de LC, característica pela extensão das lesões dérmicas em todo o corpo, incluindo mãos e pés (Silveira, 2009). Assim como nos casos anteriores, evolução clínica e progressiva da LCD tem sido relacionada às características do parasito e aspectos imunogenéticos do hospedeiro (Costa *et al.*, 2009).

Essa forma de acometimento cutâneo é geralmente causada por espécies de *Leishmania* associadas ao acometimento tegumentar, com destaque para a *Leishmania amazonensis* (Christensen *et al.*, 2019). Nesta doença, ocorre a disseminação hematogênica dos parasitos, resultando no surgimento de lesões com aspecto eritematoso, nodular ou até mesmo tumoral com baixa resposta ao tratamento quimioterápico (Scorza *et al.*, 2017). Na fase avançada, quando ocorre o acometimento de múltiplas regiões do tegumento, os indivíduos acometidos apresentam prurido intenso, lesões de aspecto granulomatoso ou verrucoso, com infiltração inflamatória, histiocítica e linfocítica (Soares *et al.*, 2020). Devido à ausência de características patognomônicas que facilitem a identificação da LCD (figura 7) e

com a possibilidade de dessa doença ser agravada em casos de imunossupressão, o diagnóstico é composto pelas abordagens clínica, epidemiológica e laboratorial (Meireles *et al.*, 2017).

Figura 7 – Leishmaniose Cutânea Difusa (LCD). Lesão ulcerada em membro superior (A), infiltração auricular e nódulos eritematosos disseminados (B) e lesões difusas por todo o corpo após recidiva (C, D e E).



Fonte: Soares *et al.* (2020).

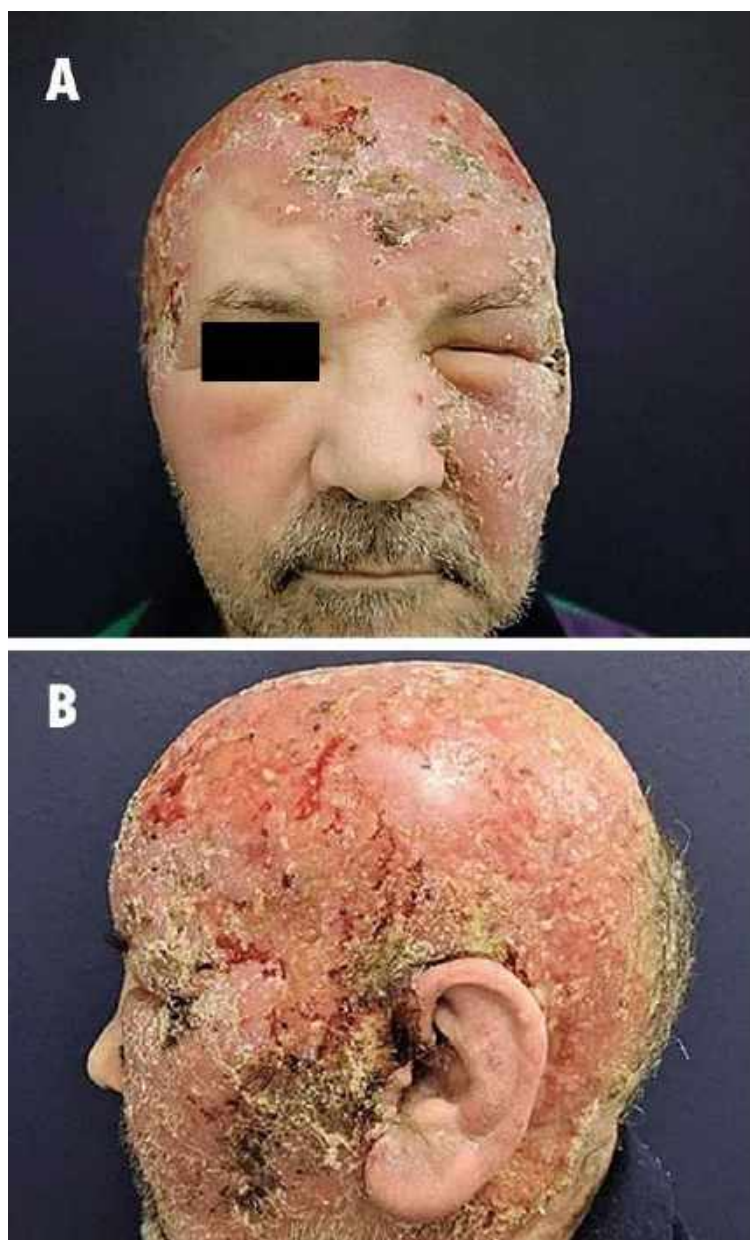
A associação entre LCD e a infecção pelo vírus da imunodeficiência humana (HIV) tem sido amplamente discutida como uma questão global emergente, sobretudo pelo fato de a leishmaniose ser a terceira infecção mais comum em pacientes com HIV, depois da toxoplasmose e criptosporidiose (Kassardjian *et al.*, 2021). Enquanto que na LC as células mononucleares do sangue periférico produzem citocinas relacionadas à resposta Th1 em direção à ativação dos macrófagos e inflamação, na LCD ocorre uma falha na produção dessas citocinas e isso pode estar relacionado à disseminação dos parasitos (Castellano *et al.*, 2009). Mesmo após anos de cicatrização das lesões cutâneas, a leishmaniose tegumentar pode apresentar-se novamente através de feridas em regiões de cartilagens e mucosas como nariz, faringe, boca e orelhas e se estabelecer como um novo quadro de leishmaniose mucocutânea (Handler *et al.*, 2015).

3.1.3 Leishmaniose Mucocutânea

A LMC ocorre após o desaparecimento dos sinais clínicos da LC, ainda que a existência de lesões simultâneas na pele e em mucosas seja consideravelmente possível (Daneshbod *et al.*, 2011). O desenvolvimento da forma mucosa pode acontecer em um período variado de tempo, levando de 2 a 30 anos para ressurgir em indivíduos que já apresentaram algum sintoma mais brando de LC (Handler *et al.*, 2015). Dentre as espécies relacionadas aos casos de LMC, a *Leishmania braziliensis* tem sido apontada como o principal agente etiológico, seguida de outras espécies como *L. panamensis*, *L. guyanensis* e *L. amazonensis* (Strazzulla *et al.*, 2013).

Clinicamente a LMC acomete as regiões das cavidades nasal e oral, podendo se estender ainda para a orofaringe e traqueia (Lella *et al.*, 2006). Os sintomas iniciais são inespecíficos e podem incluir congestão e coceira no nariz que progride para a formação de crostas e sangramento (figura 8). A principal característica observada é a presença de infiltrado inflamatório com linfócitos, plasmócitos e macrófagos que evolui desde o surgimento inicial de um edema para granuloma e necrose (Marra *et al.*, 2014; Handler *et al.*, 2015). Assim como observado na LC, estudos no campo da imunologia destacam o papel da IL-10 e, adicionalmente, do fato de crescimento transformador beta (TGF- β) na modulação da resposta imune em pacientes com LCM (Oliveira *et al.*, 2014).

Figura 8 – Leishmaniose Mucocutânea (LMC). Placas infiltrativas, eritematosas e ulceradas cobrindo o couro cabeludo e testa (A) e a parte esquerda da face (B).



Fonte: Vanlier *et al.* (2021)

Apesar de rara evolução do acometimento cutâneo para o óbito, a LC (incluído as suas variações como LCL ou LCM) possui um alto potencial estigmatizante como consequência das lesões (Bedoya *et al.*, 2017). Doenças que afetam a pele, em especial através da formação de úlceras, podem ser o ponto de partida para sofrimento psíquico e importantes consequências psicológicas como baixa autoestima, ansiedade, depressão e suicídio (Costa *et al.*, 1987). A segregação social de indivíduos com LC pode dificultar a procura de assistência médica e realização de

um diagnóstico precoce, favorecendo assim a evolução do quadro clínico que pode acentuar o dano tecidual ao tegumento ou apresentar uma falsa cura com posterior recidiva (Murback *et al.*, 2011; Holanda *et al.*, 2019). Fatores moleculares, bioquímicos e genéticos dos parasitos e hospedeiros, e a falta ou realização de um tratamento inadequado podem resultar na migração dos protozoários para órgãos viscerais e então ocorrer o desenvolvimento visceral (Zhang *et al.*, 2003).

3.1.4 Leishmaniose Visceral

A LV é a forma clínica mais grave das leishmanioses e afeta principalmente órgãos internos como fígado e baço, podendo ainda ultrapassar a barreira hematoencefálica e causar danos ao sistema nervoso central (Márquez *et al.*, 2013). Quando não tratada, a LV pode causar a morte dos indivíduos acometidos o que torna esse tipo de doença a forma com maior letalidade dentre o espectro clínico das leishmanioses (Abuzaid *et al.*, 2020).

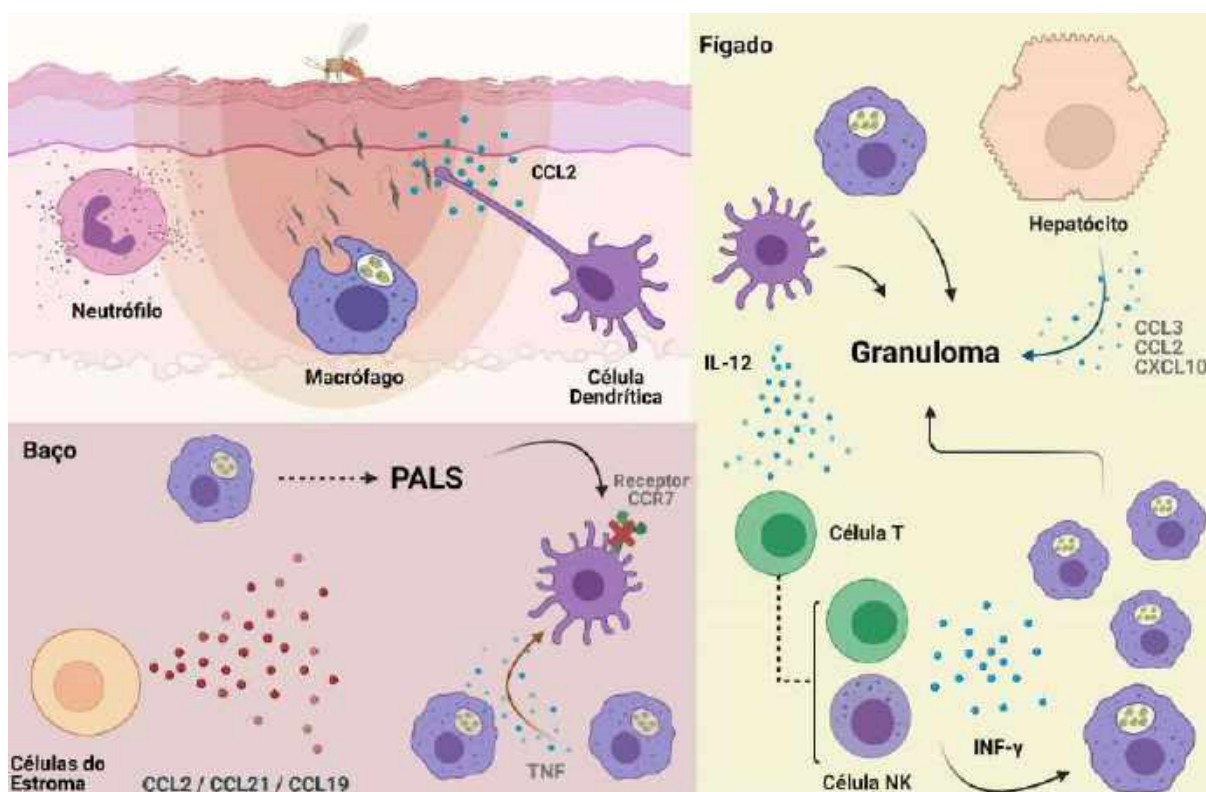
Popularmente a LV é conhecida como calazar e os sintomas clínicos comuns incluem febre, perda de peso, hepatomegalia, esplenomegalia, anemia e linfadenopatia (Thakur *et al.*, 2018). Mundialmente, a LV apresenta uma incidência anual de meio milhão de casos, com aproximadamente 50 mil mortes por ano (Bi *et al.*, 2018). Diferente das formas cutâneas, não são observados sinais e sintomas externos nas etapas iniciais da doença, o que dificulta a realização do diagnóstico precoce e, conseqüentemente, do tratamento e acompanhamento médico (Al-Salem *et al.*, 2016).

Além da forma clássica de transmissão da doença pelo vetor, a transfusão de sangue, transplante de órgãos e acidentes congênitos e laboratoriais constituem possibilidades de transmissão da LV (Van-Griensven e Diro, 2019). Após a infecção pelo protozoário, o indivíduo pode levar de 2 a 6 meses para o desenvolvimento de sintomas e mesmo quando o tratamento é aparentemente bem sucedido a LV pode ressurgir, principalmente diante de quadros de imunossupressão (Van-Griensven *et al.*, 2014).

Os mecanismos fisiológicos relacionados ao fracasso da defesa do organismo e ao crescimento e disseminação sistêmica dos parasitos na LV envolvem diversos aspectos da resposta imune a esta doença (Araújo *et al.*, 2020). A ausência da resposta Th1 específica do antígeno nas células mononucleares do sangue periférico

na LV, assim como a índices elevados de INF- γ nos tecidos lesionados e aumento das citocinas pró-inflamatórias têm sido associados à progressão da doença (Nylén e Sacks, 2007). A produção de interleucina-12 (IL-12) pelas células apresentadoras de antígeno e INF- γ por células T é considerada necessária para o controle da infecção, enquanto que as formas não curáveis e visceralizantes da doença estão relacionadas a produção precoce e prolongada de interleucina-4 (IL-4) e/ou interleucina-13 (IL-13) (Babaloo *et al.*, 2001). Os mecanismos celulares e moleculares do envolvimento visceral na leishmaniose estão representados na figura 9.

Figura 9 – Aspectos gerais da resposta imunológica mediada por células e citocinas, associada ao desenvolvimento de Leishmaniose Visceral.



Fonte: O autor (2022).

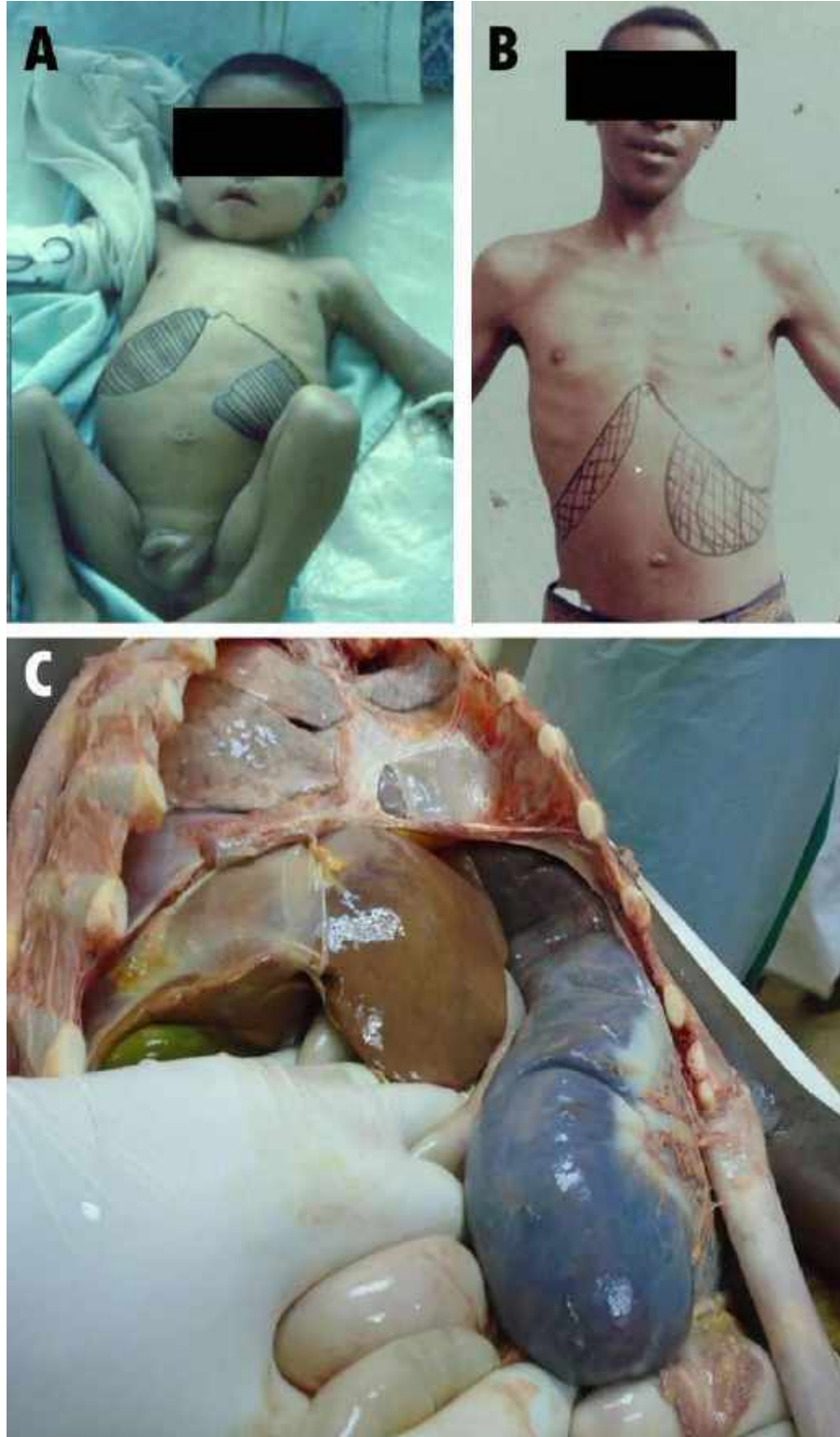
Após serem inoculados pelo vetor, os protozoários são fagocitados na derme e carregados pelas células do sistema imunológico numa resposta celular que envolve macrófagos, neutrófilos e células dendríticas, conforme já discutido no âmbito da LC (figura 5). Os macrófagos e células dendríticas infectados deixam o local da infecção e disseminam os parasitos para outros órgãos como baço e fígado, através do estímulo e resposta mediada por quimiocinas características no quadro da LV como

CCL3, CCL2 e CXCL10, que contribuem para a formação de granuloma hepático (Singh e Sundar, 2017). A CXCL10 atua na mobilização de células T e recruta células produtoras de INF- γ , ativando macrófagos para a eliminação do agente etiológico (Palomino e Marti, 2015).

As quimiocinas CCL2 e CCL3 promovem o controle na multiplicação do parasito, principalmente induzindo o aumento da produção de óxido nítrico nos macrófagos (Giudice *et al.*, 2012). No baço, a infecção por *Leishmania* promove uma redução no número de células estromais e aumento da bainha periarteriolar de linfócitos (PALS), inibindo a expressão do receptor CCR7, relacionado ao endereçamento de células dendríticas ao linfonodo (Ato *et al.*, 2002). Em conjunto, esse processo contribui para a elevação das quimiocinas de endereçamento de células T e dendríticas aos linfonodos (CCL19 e CCL21) que, somando a redução de CCR7, resultam numa acentuada imunossupressão e persistência dos protozoários no baço (Engwerda *et al.*, 2004). O desenvolvimento e multiplicação das formas amastigotas no interior de macrófagos dos tecidos hepático e esplênico culmina no quadro de hepatoesplenomegalia, forma clássica da LV em humanos (Henke *et al.*, 2021).

Os sintomas mais comuns descritos em pacientes com LV consistem em febre, anorexia, astenia, perda de peso, icterícia, diarreia, sangramento, edema e como descrito, hepatomegalia e esplenomegalia (Henn *et al.*, 2018). Quando ocorre a coinfeção de *Leishmania* e o vírus HIV, o quadro pode ser acentuado, elevando o risco de morte (Nair *et al.*, 2020). Nos casos em que não há tratamento ou de falha terapêutica, a LV pode evoluir para a forma crônica e comprometer outros tecidos e sistemas como a medula óssea e a hematopoese (Abidin *et al.*, 2017). As alterações físicas mais marcantes em indivíduos com LV consistem na expressiva perda de peso e no aumento da região abdominal (figura 10 A e B). Em alguns casos, órgãos como fígado e baço podem ocupar quase toda a cavidade abdominal devido a elevação no tamanho (figura 10 C).

Figura 10 – Leishmaniose Visceral. Criança de dois anos com acentuado quadro de hepatoesplenomegalia (A), homem de dezesseis anos com hepatoesplenomegalia e evidente perda de peso (B), achado pós-morte de esplenomegalia maciça e hepatomegalia (C).



Fonte: Mollinedo *et al.* (2020) e Henke *et al.* (2021)

A ocorrência de LV associada às outras infecções, como tuberculose, HIV etc. tem sido encarada de forma preocupante por pesquisadores e profissionais de saúde (Van-Griensven *et al.*, 2018). Recentemente a população global foi fortemente afetada por uma das maiores pandemias causada por uma doença infecciosa dos tempos modernos, a doença do novo coronavírus (COVID-19) (Liu *et al.*, 2020). Nesse contexto, a intersecção entre a endemicidade da leishmaniose visceral e a pandemia de COVID-19 tem sido considerada (Carvalho *et al.*, 2020). Pouco mais de um ano após o início da pandemia no Brasil, a COVID-19 permanece com considerável taxa de transmissão, atingindo comunidades pobres e sobrepondo vulnerabilidades já associadas às leishmanioses como condições precárias de moradia, saneamento básico e desnutrição (Ehrenberg *et al.*, 2020; Schellekens e Sourrouille, 2020).

A literatura científica sobre o acometimento múltiplo de LV e COVID-19 é escassa e um caso de associação entre LV e COVID-19 foi reportado na Itália, em um homem de 79 anos que apresentava astenia, falta de ar, perda de peso e pancitopenia. Após a realização da biópsia de medula óssea, o paciente recebeu o diagnóstico de LV e teve quadro clínico piorado pela COVID-19, seguido da morte (Miotti *et al.*, 2020). A infecção causada pelo novo coronavírus e LV induzida por *L. infantum* não compartilham características clínicas, mas devido às diferenças observadas na patogenia dessas doenças, os indivíduos com LV podem apresentar vulnerabilidade imunológica e na resposta ao tratamento por antivirais (Carvalho *et al.*, 2020). Nesse contexto, o tratamento precoce e adequado dos casos de leishmaniose se torna imprescindível, sobretudo observando os fatores de risco de associação entre as diferentes formas de leishmaniose e outras infecções (Park *et al.*, 2020; Talbi *et al.*, 2020).

3.2 EPIDEMIOLOGIA DAS LEISHMANIOSES

As leishmanioses são doenças endêmicas em quase 100 países ao redor do mundo, afetando entre 700 mil e 1 milhão de indivíduos por ano (Estumano *et al.*, 2020). Nas Américas, essas doenças ocorrem desde o sul dos Estados Unidos até o norte da Argentina, onde mais de 90% dos casos acontecem no Peru, Bolívia e Brasil (Abraão *et al.*, 2020). De 2001 a 2019 mais de 1 milhão de casos de LC/LCM foram notificados pela Organização Pan-Americana de Saúde (OPAS), resultando em uma média de 54.108 casos por ano (OPAS, 2020). Especialmente no caso do Brasil, o

maior e mais populoso país da América do Sul, as leishmanioses apresentam notificação em todas as regiões (Brasil, 2017a; Rocha e Petroni, 2017).

Os casos de leishmaniose são classificados como notificação compulsória no Brasil e preenchidos no Sistema Nacional de Informações de Agravos de Notificação (SINAN) pelas secretarias de saúde dos estados e municípios (Laguardia *et al.*, 2004). Até a década de 1990, mais de 90% dos casos de LV notificados no Brasil eram na região Nordeste e isso dava ao fato de que essa doença está fortemente ligada à pobreza (Lindoso e Lindoso, 2009). De 2007 a 2017, foram notificados 41.204 casos de LV no Brasil, dos quais 52,7% ainda correspondeu ao Nordeste. O ano de 2014 concentrou o maior número de casos (2.439), resultando numa incidência de 4,3 por 100.000 habitantes (Machado *et al.*, 2020). De 2007 a 2017 o estado de Pernambuco apresentou a sétima maior incidência de LV com destaque para o município de Carnaubeira da Penha na região do Sertão do Submédio São Francisco com 26.8 casos/100 mil habitantes (tabela 1).

Tabela 1 – Incidência média anual de LV durante 2007-2017 no Nordeste do Brasil, de acordo com o estado e município com maior número de casos.

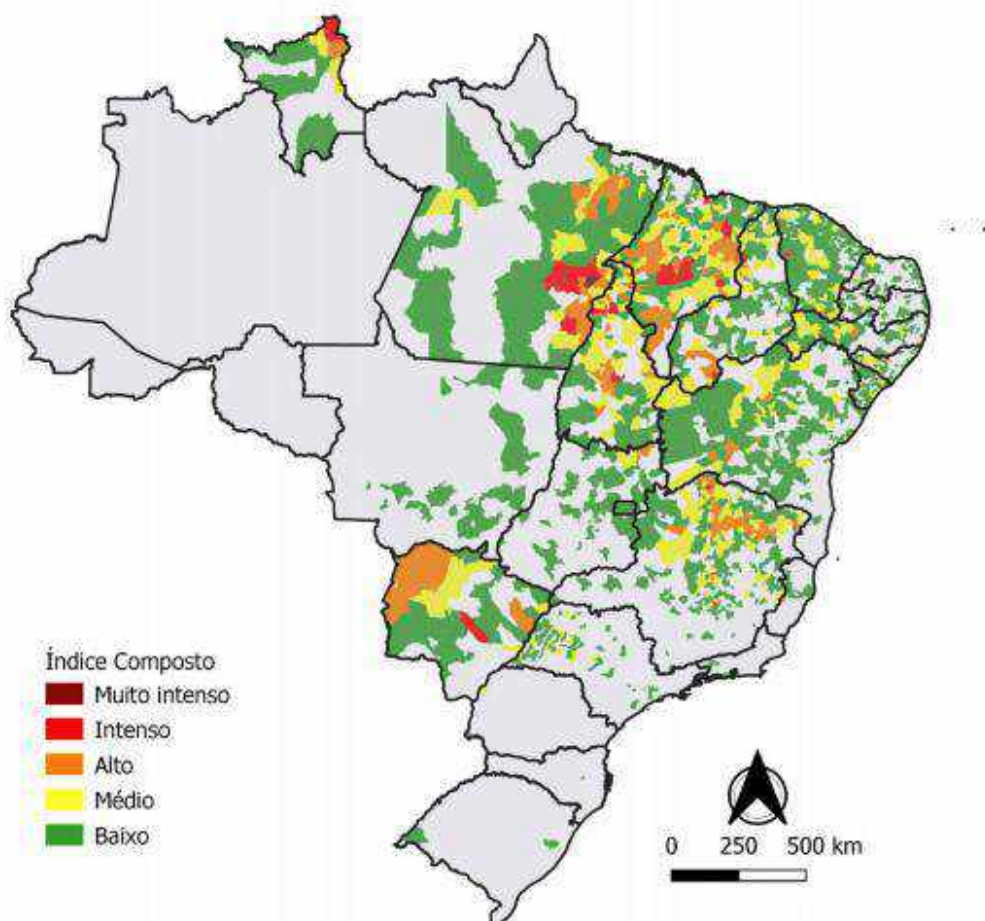
Estado	Incidência Estadual (por 100 mil habitantes)	Município com maior incidência	Incidência Municipal (por 100 mil habitantes)
Maranhão	8.3	Coelho Neto	37.5
Piauí	7.0	Pavussu	29.1
Ceará	6.0	Barbalha	22.8
Sergipe	2.8	Itaporanga D'ajuda	7.4
Rio Grande do Norte	2.8	Riacho da Cruz	28.2
Bahia	2.3	Salinas da Margarida	63.1
Pernambuco	1.2	Carnaubeira da Penha	26.8
Alagoas	1.1	Japaratinga	10.2
Paraíba	1.0	Catolé do Rocha	9.0

Fonte: Machado *et al.* (2020)

A partir do ano de 2017 o Governo Federal adotou a metodologia de classificação epidemiológica da LV de acordo com a OPAS, que consiste no índice composto de Leishmaniose Visceral para a classificação das áreas de transmissão em cinco extratos: muito intenso, intenso, alto, médio e baixo (OPAS, 2016). Durante o período de 2017 a 2019, o município de Eldorado dos Carajás (PA) foi classificado com transmissão muito intensa para a LV e outros oito municípios, dos quais três estão

localizados nas regiões Norte e Nordeste receberam a classificação de transmissão intensa (Figura 11).

Figura 11 – Estratificação de risco da LV por município brasileiro durante o período de 2017-2019.



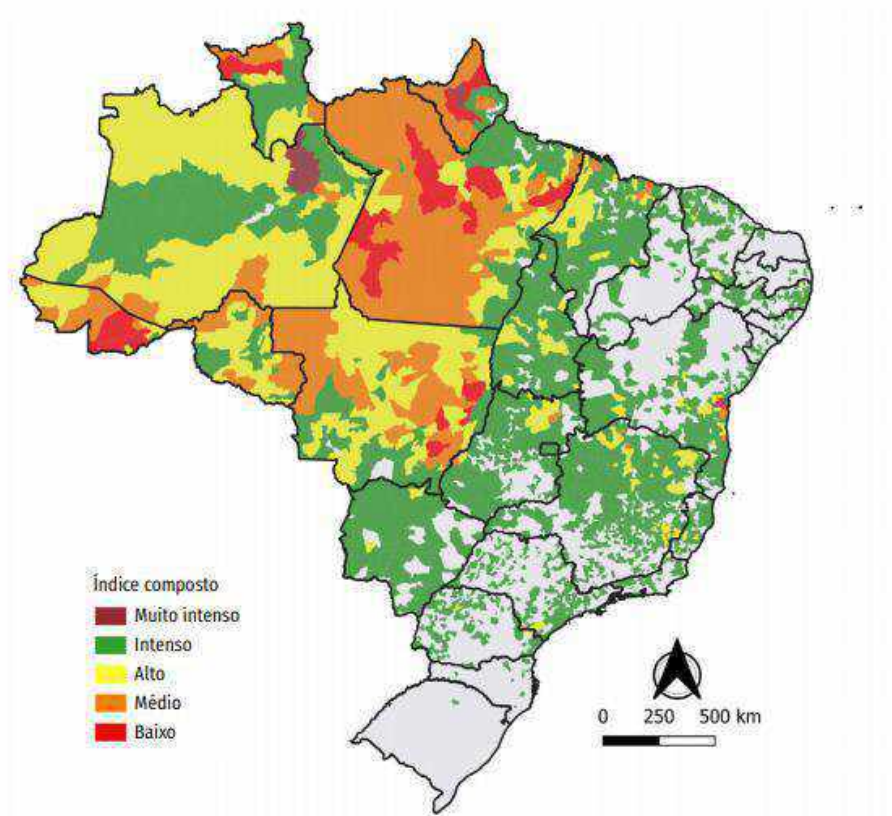
Fonte: SINAN/Ministério da Saúde (2021).

Em 2019 os casos da LV foram confirmados nas 27 Unidades Federativas do Brasil, totalizando 2.529 casos dos quais a região Nordeste correspondeu ao maior registro do País, com cerca de 49,1% (Brasil, 2021). A forma visceral da leishmaniose acomete adultos jovens, com faixa etária entre 20 e 49 nos principalmente do sexo masculino e no referido ano, a doença apresentou uma taxa de letalidade de 9%, a mais elevada da última década (Brasil, 2021). Mediante a estes dados, o Ministério da Saúde elencou cinco principais metas de combate à LV, das quais se destacam: reduzir a taxa de letalidade em 50% no Brasil e reduzir pela metade a incidência nos estados com expansão da transmissão, sejam aquelas classificadas com transmissão

intensa, controlável/estável ou esporádica. O Ministério da Saúde ainda afirmou que iniciará a distribuição de coleiras impregnadas com inseticidas como forma de combate ao vetor, não havendo, entretanto, qualquer menção à iniciativa no âmbito da produção de imunizantes ou medicamentos para o combate à doença em humanos ou animais (Brasil, 2021).

A LC apresentou número de casos um pouco menor, quando comparado ao observado na LV. Em 2019, foram confirmados 15.484 casos novos de LC, distribuídos em todo o território brasileiro e concentração de casos (42,8%) na região Norte (Brasil, M. D. S., 2021). Durante o período de 2017 a 2019, oito municípios aprestaram transmissão muito intensa: Rio Preto da Eva, Presidente Venceslau e Manaus (AM), Serra do Navio (AP), Teolândia, Valença, Venceslau Guimarães e Taperoá (BA), conforme pode ser observado na figura 12.

Figura 12 – Estratificação de risco da LC por município brasileiro durante o período de 2017-2019.



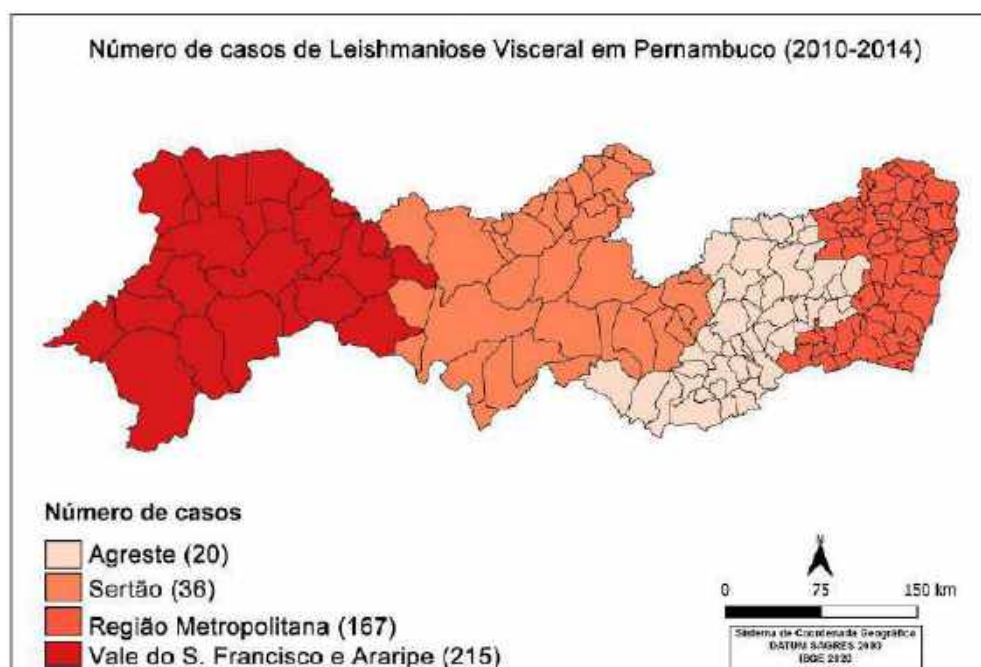
Fonte: SINAN/Ministério da Saúde (2021).

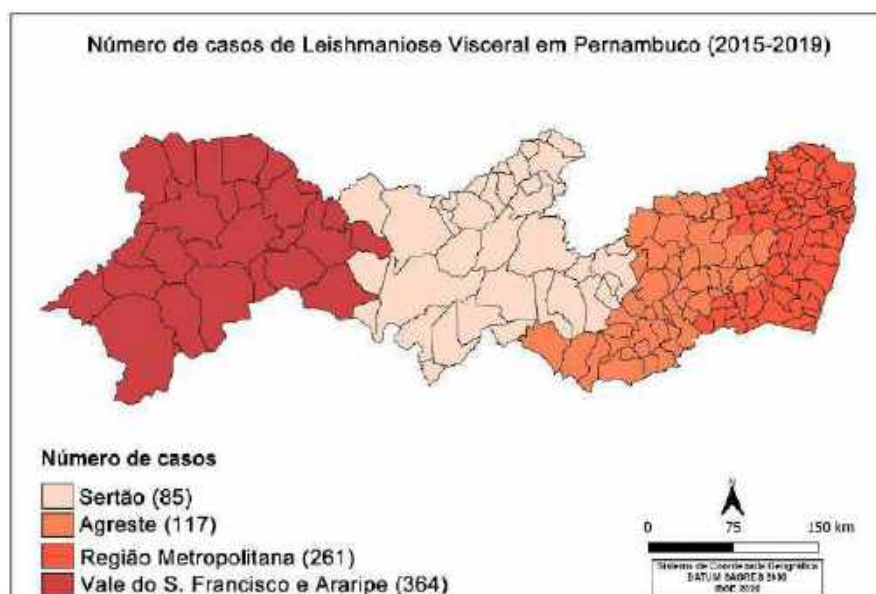
Em 30 de outubro de 2018 o Ministério da Saúde incorporou o uso da miltefosina como estratégia no combate a LC no âmbito do Sistema Único de Saúde, resultando num investimento de R\$ 3,5 milhões na compra de quase 200 mil cápsulas

do medicamento que serão destinadas a 2.300 pessoas com diagnóstico de LC confirmado no Brasil (Brasil, 2020). Dentre metas da vigilância da LC, a redução do número de crianças menores 10 anos com LC e a redução da mortalidade em 90% foram destacadas pelo Governo do Brasil (Brasil, 2021).

No período que compreende de 2010 a 2019, o Estado de Pernambuco notificou cerca de 1.265 casos de LV (Figura 13). As regiões com maior número de casos da forma visceral da doença, entre 2010 e 2014 foram Região do Vale do São Francisco e Araripe (215 casos) e Região Metropolitana (167 casos). Quando observado o período de 2015 a 2019, é possível notar um aumento de 65,84% no número total de casos de LV, dos quais as regiões do Vale do São Francisco e Araripe e Metropolitana do Recife apresentaram 364 e 261 casos, respectivamente (Brasil, 2021). Esses dados sugerem que a incidência de LV está concentrada no interior do estado e com tendência de crescimento, como observado no aumento em 31,73% na região do Vale do São Francisco e Araripe entre os períodos analisados

Figura 13 – Distribuição do número de casos de LV por macrorregiões de saúde do Estado de Pernambuco

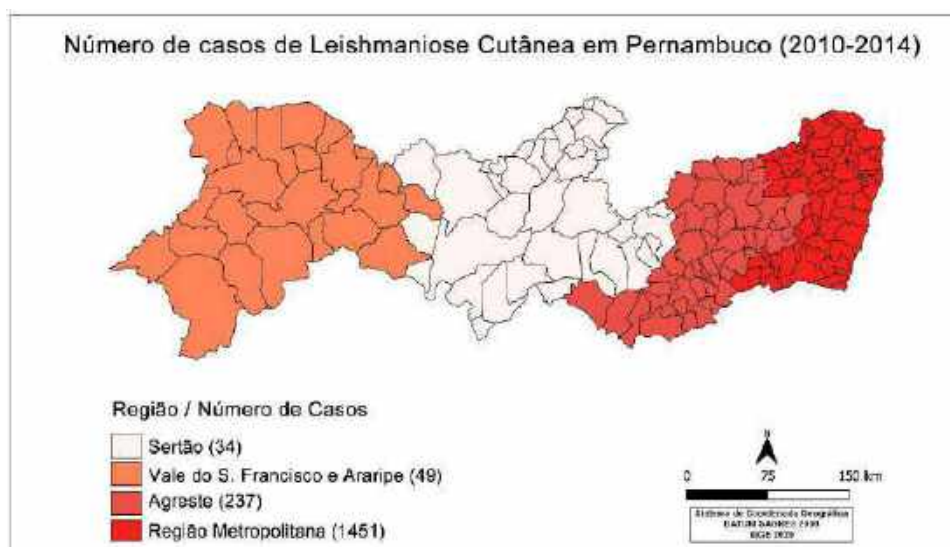


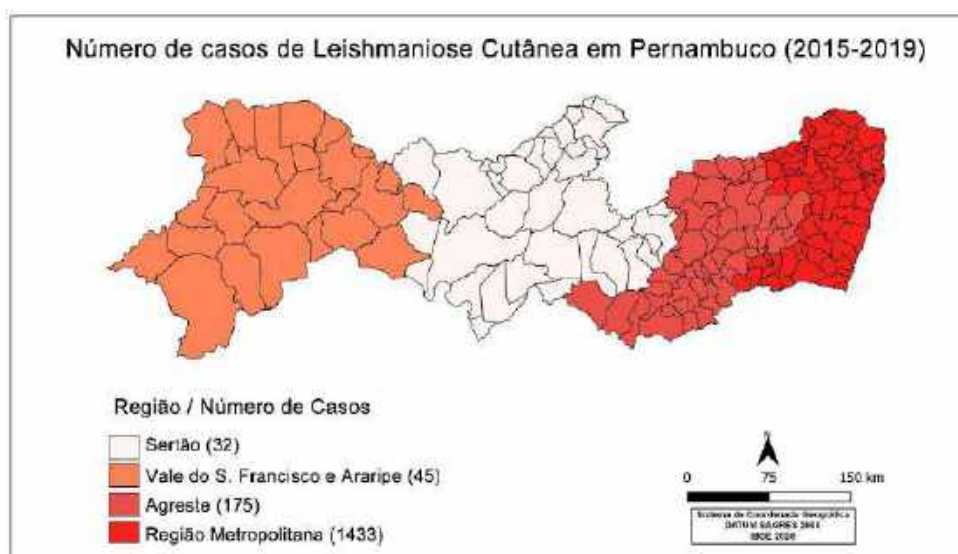


Fonte: O autor (2022).

Diferente da LV, a LC apresentou maior incidência com cerca de 3.456 casos de LC notificados para o período de 2010 a 2019 (Figura 14). Entre os primeiros cinco anos, a Região Metropolitana concentrou 81,93% dos casos de LC, seguida pela região Agreste com 13,38%. Esse perfil se manteve nos cinco anos posteriores, nos quais a RM reuniu, sozinha, 85% dos casos de LC, seguida pelo Agreste com 10,3%. A análise desses dados sugere que a forma cutânea da leishmaniose tende a ocorrer com maior frequência nas áreas próximas ao litoral pernambucano, especificamente na região da Zona da Mata e assim como a LV, apresentou tendência de crescimento.

Figura 14 – Distribuição do número de casos de LC por macrorregiões de saúde do Estado de Pernambuco





Fonte: O autor (2022).

Dentre as atividades de controle das leishmanioses realizadas pelo Estado de Pernambuco em 2018 estão o exame de 5.390 cães e o combate ao mosquito vetor com inseticida através do borrifamento de 1.243 domicílios (Pernambuco, 2018). Medidas adicionais como a realização de treinamento de entomologia para os municípios das Gerências Regionais de Saúde (GERES), seminários de atualização sobre leishmaniose com médicos, enfermeiros e coordenadores municipais de vigilância em saúde e atenção básica e assessoramento dos municípios com prioritários e não-prioritários do SANAR também foram realizadas (Pernambuco, 2018). O diagnóstico precoce e o controle do vetor têm sido considerados medidas importantes dos casos de LV e LC no Brasil, sobretudo devido às características geográficas, climáticas e ambientais que constituem o habitat natural dos mosquitos transmissores das leishmanioses (Claborn, 2010; Werneck, 2010).

3.3 MEDIDAS DE CONTROLE DAS LEISHMANIOSES: DIAGNÓSTICO, CONTROLE DO VETOR E FORMAS DE TRATAMENTO

Ao longo da descoberta das leishmanioses, várias formas de controle foram desenvolvidas com o objetivo de minimizar as possibilidades de que alguém seja infectado pelos parasitos e, conseqüentemente, desenvolvam a doença (Costa *et al.*, 2018). Apesar de ser uma etapa fundamental no rastreamento e identificação dos casos, o diagnóstico clínico e laboratorial das leishmanioses não consiste em uma tarefa fácil

devido à similaridade com outras doenças e falta de especificidade e sensibilidade de alguns testes (Cerutti *et al.*, 2017).

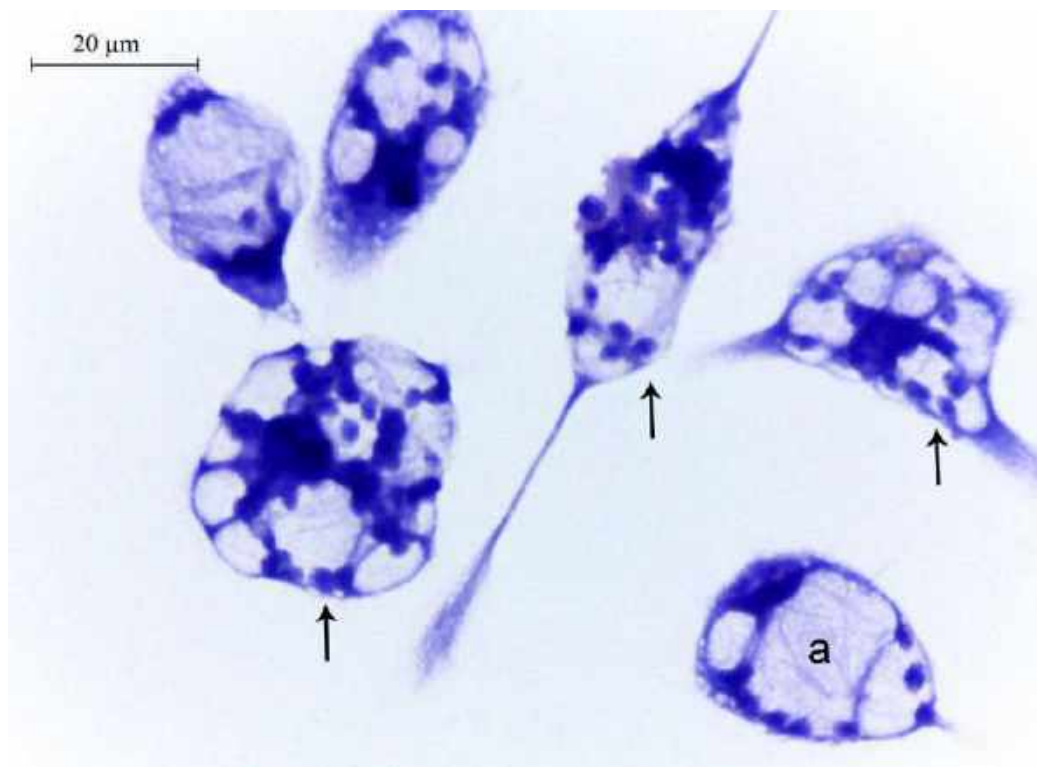
As características clínicas da LV podem ser facilmente confundidas com outras doenças febris como malária e febre entérica, enquanto que a LC pode ser confundida com esporotricose, infecção por *Mycobacterium marinum*, úlceras de estase venosa, blastomicose, sarcoidose, goma treponêmica, sarcoma de Kaposi, hanseníase, cromoblastomicose e várias outras condições infecciosas e não infecciosas que se manifestam como nódulos ulcerados ou exofíticos, por exemplo (Vega-López, 2003; Srivastava *et al.*, 2011).

3.3.1 Diagnóstico das Leishmanioses

O diagnóstico parasitológico ainda costuma ser uma das primeiras condutas no manejo do paciente com suspeita de leishmaniose por conta da alta especificidade da técnica (Desjeux, 1996). No contexto da LV, podem ser realizados esfregaços esplênicos que costumam apresentar até 98,7% de sensibilidade, entretanto esta é uma técnica invasiva e que pode resultar em sangramento grave ou fatal (Sundar e Rai, 2002).

Para a forma cutânea o material da margem da úlcera é coletado e analisado sob microscopia para a identificação de formas amastigotas *Leishmania* sp. no interior de macrófagos (Figura 15), podendo ainda ser realizada a análise histopatológica de acordo com a necessidade de avaliação laboratorial (Vries *et al.*, 2015). Uma variável presente na realização do diagnóstico parasitológico e que deve ser levada em consideração é a necessidade de um microscopista com expertise suficiente para identificar as estruturas parasitárias (Srivastava *et al.*, 2011).

Figura 15 – Formas amastigotas de *Leishmania* sp. no interior de macrófagos. Parasitos representativos estão demonstrados nas setas pretas. Vacúolo parasitóforo ocupando quase todo o espaço intracelular do macrófago (a).



Fonte: O autor (2022).

Outra técnica possível a partir da coleta de material biológico com suspeita de leishmaniose é a cultura dos parasitos *in vitro*. A cultura de *Leishmania* tem sido empregada com menos frequência pois consiste em uma metodologia de alto custo e maior tempo para a liberação dos resultados (Pourmohammadi *et al.*, 2010). Nesse contexto, uma modificação da técnica possibilitou a detecção sensível e reproduzível de parasitos a partir da utilização de *buffy coat*, uma camada rica em leucócitos e células mononucleares do sangue periférico (PBMC) isoladas de indivíduos infectados (Maurya *et al.*, 2010). Os meios de cultura comumente utilizados para o cultivo artificial de *Leishmania* são Novy-McNeal Nicolle, Tobies, Schneider, M199 ou meio de Grace (Rasti *et al.*, 2016).

Assim como a pesquisa do parasito, a investigação sorológica também é uma ferramenta útil para o diagnóstico da leishmaniose. Essa metodologia consiste na presença de uma resposta humoral específica, que pode ser avaliada a partir de análises como teste intradérmico de Montenegro (Antonio *et al.*, 2014),

imunofluorescência indireta (Sassi *et al.*, 1999), ensaio imunoenzimático (Bracamonte *et al.*, 2020), *immunoblotting* (Menu *et al.*, 2021), teste de aglutinação (Bangert *et al.*, 2018), teste com tira imunocromatográfica (Mniouil *et al.*, 2018) e a detecção do antígeno (Bennis *et al.*, 2018). Mesmo compreendendo diferentes abordagens, essas técnicas diagnósticas podem não oferecer evidências clínicas suficientes a conclusão de infecção por *Leishmania*, podendo ainda ser necessária uma investigação molecular.

O diagnóstico molecular consiste em ensaios *in vitro* baseados na reação da polimerase em cadeia (PCR) com primers direcionados a vários genes como genes de rRNA e kDNA. Essa metodologia possibilita a identificação da espécie com sensibilidade de até 95,7% e a quantificação da carga parasitária, no caso da PCR em tempo real, fornecendo dados precisos para o diagnóstico mais específico a partir de amostras clínicas (Antinori *et al.*, 2007). Devido à falta de um teste padrão-ouro para o diagnóstico das leishmanioses, a associação entre técnicas moleculares, sorológicas e microscópicas é recomendada (Thakur *et al.*, 2020).

O rastreio dos casos de forma precoce pode ser uma medida útil no controle da doença a conduzir as ações sanitárias direcionadas ao combate ao mosquito vetor (Pinto e Vargas, 2017). No Brasil, as políticas públicas visam primariamente a eliminação do mosquito que transmite a leishmaniose, ainda que o país, de forma geral, seja composto por fatores ambientais, climáticos e ecológicos que favoreçam o desenvolvimento e multiplicação desses insetos em ambientes urbanos, rurais ou silvestres (Karunaweera e Ferreira, 2018). A eutanásia de cães soropositivos para leishmaniose ainda continua a ser realizada, apesar de ser uma medida controversa quando se avalia o real impacto na redução de casos e o bem-estar animal (Machado *et al.*, 2016).

3.3.2 Entre o Amor ao Animal e à Saúde Pública: Medidas Sanitárias Controversas de Controle das Leishmanioses No Brasil

Os insetos vetores das leishmanioses são popularmente conhecidos como mosquito-palha, cangalhinha, flebótomo, arrepiado, asa-de-palha, etc. (Silva *et al.*, 2010; Pinto e Vargas, 2017). Devido ao intenso desmatamento e ocupação de áreas de floresta ou mata, esses insetos passaram a existir em ambientes urbanos e o cão doméstico e o ser humano passaram a ser hospedeiros dos parasitos transmitidos

durante o repasto sanguíneo (Rufino, 2011). Com o passar do tempo, o cão foi constituído como o principal reservatório da leishmaniose na região urbana e devido à falta de tratamento ou vacina para impedir a infecção, esses animais têm sido sentenciados à morte (Silva e Winck, 2018).

A campanha contra as leishmanioses teve início em 1953 com foco primariamente no combate à LV e posteriormente o decreto nº 51.838 de 14 março de 1963 estabelecia as medidas a serem tomadas de forma prática e pragmática (Brasil, 1963). Ao longo dos anos iniciais a iniciativa foi pautada na vigilância entomológica, aplicação de inseticidas nos diversos domicílios e identificação de cães soropositivos com consequente eliminação, além do rastreio diagnóstico em humanos (Oliveira e Araújo, 2003). A proximidade dos cães aos humanos associada a coexistência da doença foi considerada critério para sugerir que o cão possa manter e ampliar o ciclo do parasito no peridomicílio e então, confirmar o papel do cão como principal reservatório das leishmanioses no Brasil (Machado *et al.*, 2016).

É importante destacar que o vetor das leishmanioses é altamente adaptável aos centros urbanos e, nesse contexto, o cão doméstico se constitui de um animal exposto e vulnerável, sobretudo àqueles que têm acesso à rua ou não estão abrigados em lares ou instituições de apoio aos animais (Pinto e Vargas, 2017). Em virtude da redução indiscriminada da população canina, a OMS destacou que a eliminação dos cachorros selvagens e de rua poderia ser justificada por muitos motivos relacionados à saúde e que a ocorrência de leishmaniose consiste em uma razão adicional, mas que se deve reconhecer as particularidades de cada país (OMS, 1984).

De fato, a taxa de incidência para a LV no Brasil não reduziu mesmo com ações de combate e controle, como a pulverização das casas e morte de 20.000 cães por ano e isso poderia estar relacionado a graves falhas de amostragem, diagnóstico correto, seleção e falha no número de casos notificados (OMS, 2004). Nesse contexto, emergiu a falta de vontade dos donos de cães em permitir que seus animais sejam abatidos (Pinto e Vargas, 2017). Devido às evidências não apontarem que a eutanásia de cães diminui a prevalência local de leishmaniose, a insistência dessa medida através das orientações previstas no Manual de Vigilância e Controle da Leishmaniose Visceral do Ministério da Saúde viola o princípio da precaução, pautado na adoção de medidas cautelares com base científica (Machado *et al.*, 2016).

A utilização do tratamento farmacológico empregado na leishmaniose humana é proibida em cães, em virtude das restrições de uso com o objetivo de reduzir o

surgimento de cepas resistente (Brasil, 2008). Dessa forma, é possível realizar a profilaxia a partir do uso de inseticidas e repelentes como 10% de imidacloprida e 4,5% de flumetrina foi que podem ser impregnados nas coleiras dos animais (Otranto *et al.*, 2013). O tratamento tópico com inseticidas para impedir que o ciclo da *Leishmania* se complete e inclua o cão como reservatório também tem sido destacado com sucesso por grupos de pesquisa ao redor do mundo (Reguera *et al.*, 2016). As principais medidas profiláticas recomendadas (tabela 2) são produzidas com piretróides e formulações à base de deltametrina, disponíveis em coleiras de plástico, loções, dispositivos para aplicação local e sprays, para facilitar o manejo por donos de cães (Otranto e Dantas-Torres, 2013).

Tabela 2 – Medidas profiláticas atuais para combater vetores da *Leishmaniose* canina

Formulação	Duração	Referência
4% Deltamentrina	4 a 6 meses	(Killick-Kendrick <i>et al.</i> , 1997)
65% Permetrina	2 semanas	(Wylie <i>et al.</i> , 2014)
50% de permetrina + 10% de imidacloprida	3 a 4 semanas	(Otranto <i>et al.</i> , 2010)
36,08% de permetrina + 4,95% de dinotefurano + 0,44% de piriproxifeno	1 mês	(Franc <i>et al.</i> , 2012)
61% permetrina + 54,5% fipronil	1 mês	(Franc <i>et al.</i> , 2015)
10% de imidacloprida + 4,5% de flumetrina	8 meses	(Brianti <i>et al.</i> , 2014)

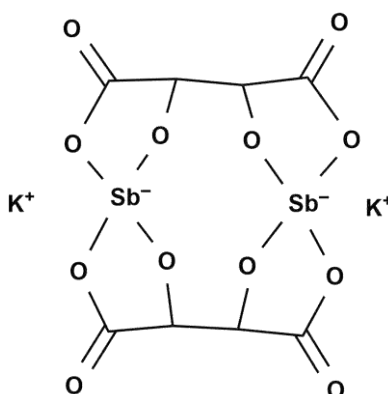
Fonte: O autor, (2022)

Apesar de acometer a humanidade há muitos anos, e de existir esforço considerável por parte da comunidade científica na busca de uma vacina, não existe um imunizante que impeça a infecção em humanos e animais (Mohammed *et al.*, 2020). Nesse contexto, as ações de educação em saúde, monitoramento das áreas endêmicas, realização do diagnóstico precoce e desenvolvimento de alternativas terapêuticas são medidas eficazes no combate à doença (Borges *et al.*, 2020). Atualmente, os medicamentos pertencentes ao grupo dos antimoniais pentavalentes são as drogas de primeira escolha, tendo a miltefosina sido introduzida recentemente no Sistema Único de Saúde como tratamento eletivo para a LC (Brasil, 2021).

3.4 TRATAMENTO FARMACOLÓGICO DAS LEISHMANOSES

Os fármacos de primeira linha utilizados no tratamento das leishmanioses são compostos conhecidos há muitos anos e o uso clínico no combate à infecção por *Leishmania* data do século passado (Frézard *et al.*, 2009). Um dos primeiros relatos sobre a eficácia dos derivados do antimônio contra a leishmaniose foi feito pelo pesquisador Gaspar Viana, ao apresentar a eficácia do tártaro emético (Sb^{3+}) – figura 16, no tratamento da LMC (Vianna, 1912). Com o passar do tempo, novos derivados antimonialis foram introduzidos como medicamentos de referência para as leishmanioses, seguidos de fármacos alternativos provenientes do reposicionamento, como anfotericina B, pentamidina e, mais recentemente a miltefosina (Tiuman *et al.*, 2011).

Figura 16 – Estrutura química do tártaro de antimônio e potássio



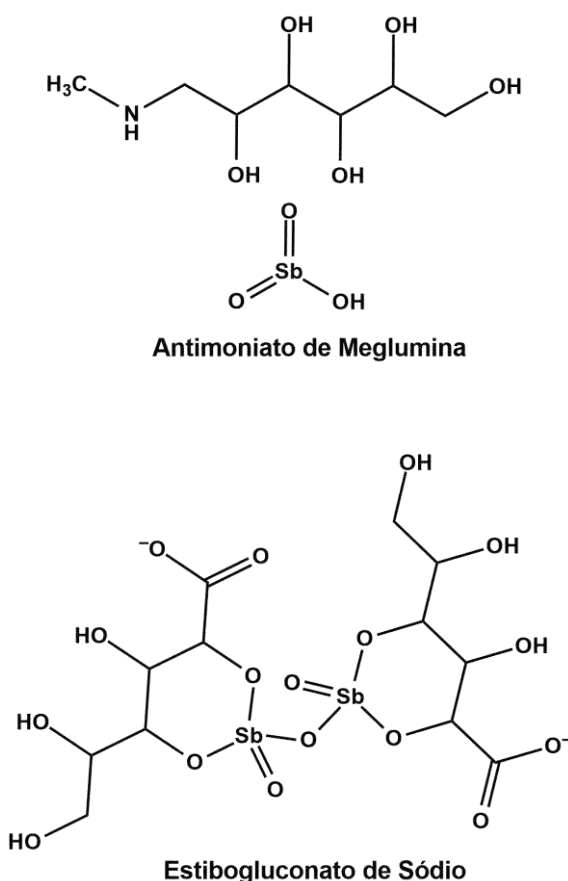
Fonte: O autor (2022).

3.4.1 Antimoniais Pentavalentes

Os antimoniais pentavalentes (Sb^{V}) são os medicamentos preconizados pelo Ministério da Saúde do Brasil para o tratamento das leishmanioses (Brasil, 2017b) e, este grupo é formado por dois fármacos (figura 16), o antimoniato de meglumina (Glucantime®) e o estibogluconato de sódio (Pentostam®), mas apenas o primeiro está disponível para uso no sistema público de saúde brasileiro (Holanda *et al.*, 2019). Essas drogas são derivadas do metal pesado antimônio e administradas por via parenteral no mínimo por 20 dias, podendo as concentrações da substância chegarem a 20/mg/kg/dia ou mais (Oyama *et al.*, 2018). Apesar do espectro clínico das

leishmanioses, a administração tópica dessas drogas ainda tem sido realizada de forma limitada (Nilforoushzadeh *et al.*, 2017).

Figura 17 – Estrutura química dos medicamentos de primeira linha contra as leishmanioses. Antimoniato de meglumina e Estibogluconato de sódio.



Fonte: O autor (2022).

O mecanismo de ação dos antimoniais contra a leishmaniose ainda é motivo de discussão entre os pesquisadores da área de farmacologia, bioquímica e química medicinal (Frézard *et al.*, 2009). Dessa forma, duas teorias gerais apontam prováveis vias de inibição dos protozoários. A primeira delas destaca a possibilidade do antimonial pentavalentes agir como um pró-fármaco e que no ambiente fisiológico seria convertido para antimonial trivalente, com maior efeito e toxicidade (Shaked-Mishan *et al.*, 2001).

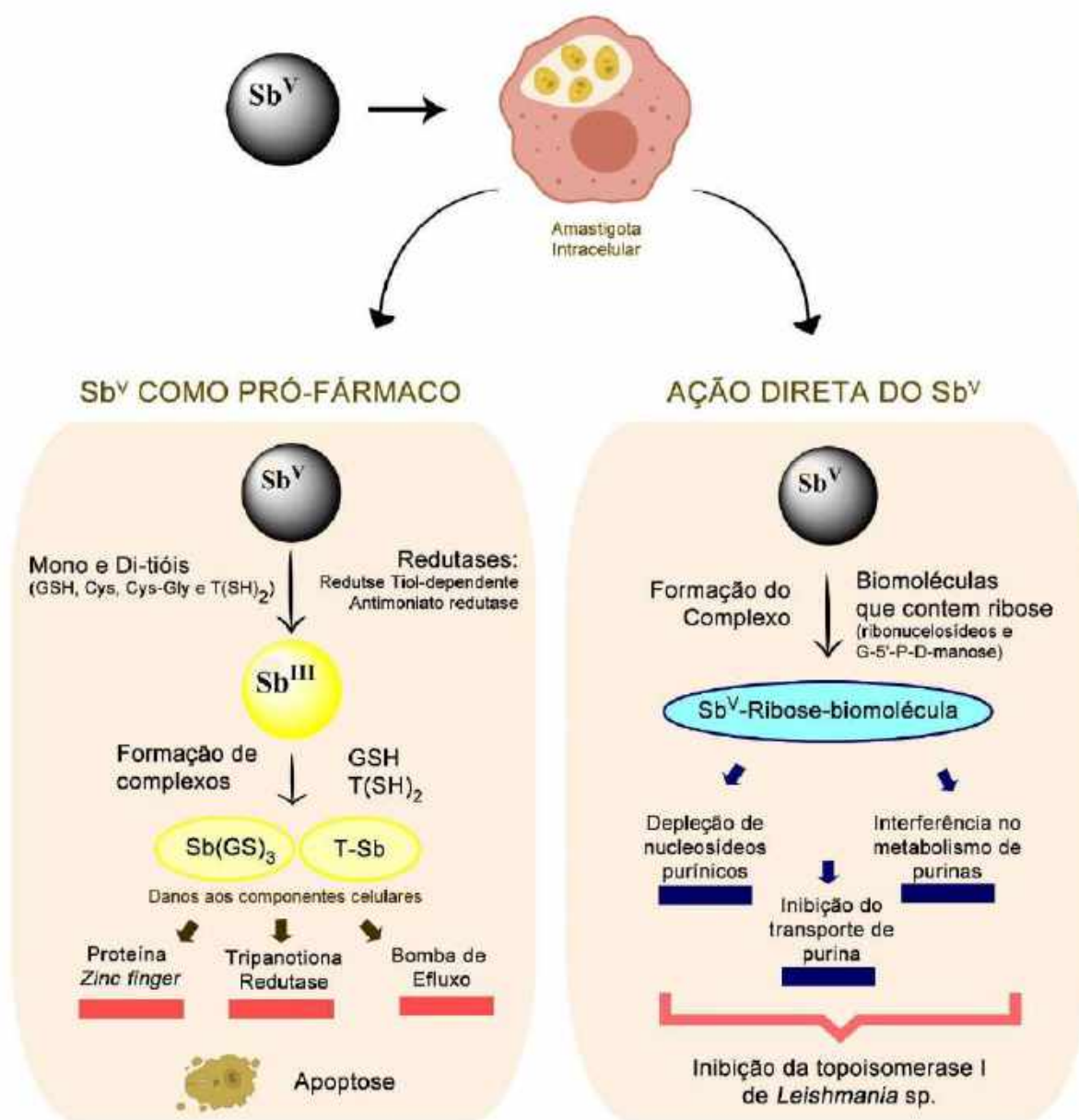
Nesse seguimento, a teoria aponta para a possibilidade de que derivados tióis presentes no ambiente intracelular como a glutatona, cisteína, cisteinil-glicina e

glutathiona-espermina reduzem o Sb^{V} para Sb^{III} a 37 °C (Ferreira *et al.*, 2003). Outros estudos também destacam as enzimas redutase tiol-dependente e antimoniatoreductase, presente nos parasitos, podem atuar como possíveis catalisadores da conversão do antimonial (Denton *et al.*, 2004; Zhou *et al.*, 2004). O antimonial trivalente consiste em um íon metálico e possui alta afinidade por grupos compostos por nitrogênio e sulfidril e a ação leishmanicida desse composto deve estar relacionada a interação com biomacromoléculas que apresentam esses grupos na composição (Ge e Sun, 2007).

A segunda teoria acerca do mecanismo de ação dos antimoniais pentavalentes sugere que o efeito anti-*Leishmania* ocorre devido a ação do próprio Sb^{V} , a partir da inibição da topoisomerase tipo I de *Leishmania donovani* (Jean-Moreno *et al.*, 2006). O estibogluconato de sódio apresentou potencial inibitório específico sobre a referida topoisomerase através do desenrolamento e clivagem do plasmídeo superenrolado pBR322, estabilizando e complexos covalentes de DNA (Chakraborty e Majumder, 1988).

O efeito do estibogluconato de sódio sobre a topoisomerase de *Leishmania* tem sido corroborado a partir de estudos de sensibilidade e resistência *in vivo* considerando o complexo como alvo de investigação (Lucumi *et al.*, 1998). Adicionalmente a esta hipótese, foi observado que o Sb^{V} forma complexos com ribonucleosídeos de adenina e possivelmente os grupos hidroxilas (OH) na ribose podem ser os pontos de ligação com o antimônio (Demicheli *et al.*, 2002). Os possíveis mecanismos de ação dos antimoniais pentavalentes estão representados na figura 18.

Figura 18 – Mecanismos de ação propostos para o efeito leishmanicida dos antimoniais pentavalentes



Fonte: O autor (2022).

Apesar de certa eficácia, ao longo dos anos várias limitações quanto ao uso das drogas de primeira linha contra as leishmanioses foram surgindo (Rodrigues *et al.*, 2020). Diversos estudos têm apontado as dificuldades encontradas durante a terapia farmacológica com os derivados do antimônio, seja pela necessidade de administração parenteral e, em muitos casos internação hospitalar, seja pelos amplos efeitos tóxicos relatados pelos usuários ou identificados por médicos durante o acompanhamento clínico (Azim *et al.*, 2021). Dentre os efeitos colaterais de maior

preocupação se destaca o acometimento a órgãos vitais como fígado, rim e coração (Roatt *et al.*, 2020). Não obstante, décadas atrás já havia dados na literatura científica demonstrando desfechos fatais atribuídos ao uso de antimoniato de meglumina durante o tratamento da leishmaniose cutânea (Oliveira *et al.*, 2005; Lima *et al.*, 2007).

Adicionalmente ao efeito comprovadamente tóxico dos antimoniais, a ocorrência de espécies de protozoários resistentes tem sido uma realidade há muitas décadas e contribuído para o insucesso do tratamento (Mukhopadhyay *et al.*, 1996; Potvin *et al.*, 2021). Devido aos severos efeitos colaterais ou apenas pelo desconforto de se submeterem à administração parenteral dessas drogas, muitos usuários abandonam o tratamento farmacológico assim que os sintomas regredem, o que os leva a falsa percepção de cura e reforça a descontinuidade da farmacoterapia (Queirão *et al.*, 2019). Na maioria dos casos, após algum tempo, ocorre recidiva da doença com maior gravidade e possibilidade de acometimento sistêmico, seja na forma visceral inicialmente apresentada como cutânea localizada (Pereira *et al.*, 2015), seja como cutânea disseminada após um quadro prévio de LV (Calvopina *et al.*, 2006).

Diversos estudos avaliaram a eficácia e risco toxicológico do uso dos antimoniais a partir da análise de variáveis como dose terapêutica, tempo de tratamento e efeitos clínicos adversos (Oliveira-Neto *et al.*, 1997; Oliveira-Neto e Mattos, 2006). Dentre as reações colaterais mais relatadas estavam dor musculoesquelética, náusea, vômito, diarreia, dor abdominal, cefaleia, anorexia, astenia, fadiga, febre, exantema, eritema e urticária (Oliveira *et al.*, 2011). A administração intramuscular foi associada a dor leve a moderada no local da aplicação (Krolewiecki *et al.*, 2007). Em alguns casos, o desconforto local era intenso e exigiu a interrupção do tratamento ou mudança da via de administração. Foram observados ainda o surgimento de pancreatite, elevação das enzimas hepáticas, alteração na função renal, eosinofilia, leucopenia e trombocitopenia que, em determinadas situações, levou a interrupção do tratamento (Seaton *et al.*, 1999; Soto *et al.*, 2004).

Em virtude da problemática ainda vivenciada desde o início do uso dos antimoniais pentavalentes, outras drogas como anfotericina, pentamidina, miltefosina e paromicina foram incluídas como segunda linha para o tratamento das leishmanioses, sobretudo nos casos de resistência ou quando não há resposta terapêutica (Serenio *et al.*, 2000). A tabela 3 sumariza 14 publicações, ao longo dos últimos anos, que destacam a ineficácia ou ocorrência de efeitos adversos severos durante o uso dos derivados do antimônio.

Tabela 3 – Distribuição dos estudos que abordam a ineficácia, toxicidade ou desfecho fatal em usuários de antimoniato de meglumina (AM) ou estibogluconato de sódio (ES) durante o tratamento das leishmanioses.

Medicamento	Dose	Desfecho	Referência
AM	13.5 mg/kg	Paciente tratado por via intravenosa desenvolveu cardiotoxicidade, pancreatite aguda e choque cardiogênico que se relacionaram aos níveis plasmáticos de AM.	(Marques <i>et al.</i> , 2019).
AM	5 a 20 mg/kg	Ocorrência de mialgia e artralgia em 50% (n = 23) pacientes e um terço apresentou alteração cardíaca, além de febre e elevação da enzima ALT.	(Neves <i>et al.</i> , 2009).
AM	20 mg/kg	Cerca de 56% dos pacientes (n = 25) em tratamento com AM apresentaram efeitos colaterais que resultaram na descontinuação do tratamento em 28% dos usuários. 12% morreram durante a terapia farmacológica.	(Delgado <i>et al.</i> , 1999).
AM e ES	20 mg/kg	Toxicidade neurológica grave, mas reversível, foi atribuída ao tratamento com antimonial pentavalentes.	(Maristany Bosch <i>et al.</i> , 2021).
AM	20 mg/kg	Elevação das enzimas hepáticas e pancreáticas e ocorrência de falha terapêutica em 15% (n = 40), seguida de recidiva.	(Andersen <i>et al.</i> , 2005).
AM	20 mg/kg	Alterações no eletrocardiograma foram observadas em um grupo de 41 usuários de AM.	(Paula <i>et al.</i> , 2003).
AM e ES	15 mg/kg	O grupo de pacientes que recebeu o tratamento com ES (n = 31) apresentou redução de 4% na eficácia quando comparado ao grupo tratado com AM (n = 32). TGO, TGP, amilase, e lipase foram mais elevadas no grupo que usou o ES (p < 0,05).	(Deps <i>et al.</i> , 2000).
AM e ES	20 mg/kg	Foi observada a elevação de enzimas hepáticas, pancreáticas e marcadores da função renal durante o tratamento de 47 pacientes com AM e 64 com ES. 43% dos usuários de AM e 54% dos que receberam ES apresentaram alterações eletrocardiográficas.	(Saldanha <i>et al.</i> , 2000)
AM	20 mg/kg	Todos os pacientes do estudo que receberam AM de forma isolada ou combinada com creme de imiquimode 7,5% apresentaram recidiva após a descontinuação do tratamento (20 dias).	(Arevalo <i>et al.</i> , 2007).
AM	15 mg/kg	Paciente de 58 anos com histórico de hipertensão e diabetes evoluiu para óbito no 18º dia de tratamento com AM.	(Lima <i>et al.</i> , 2007).
AM	20 mg/kg	Paciente de 45 anos acometido com LC recebeu tratamento com AM por 30 dias, sem haver remissão da lesão e dez dias após o primeiro esquema terapêutico, o usuário sofreu parada cardíaca evoluindo para óbito.	(Oliveira <i>et al.</i> , 2005).
AM	850 mg/Kg	Mulher de 19 anos, em período gestacional, realiza tratamento com AM contra LV por 20 dias. No 5º dia de tratamento, ocorreu o parto prematuro seguido de óbito da criança.	(Silveira <i>et al.</i> , 2003).
AM	15 mg/kg	20% dos usuários de AM (n = 74) relataram efeitos adversos, principalmente artralgia durante o tratamento.	(Neves <i>et al.</i> , 2011).

AM	10 a 20 mg/kg	Um estudo de coorte com 150 usuários de AM destacou que 59,6% dos pacientes apresentaram pelo menos um sinal ou sintoma atribuído aos efeitos colaterais como: artralgia, mialgia, cefaleia e febre.	(Rodrigues <i>et al.</i> , 2006).

Fonte: O autor (2022).

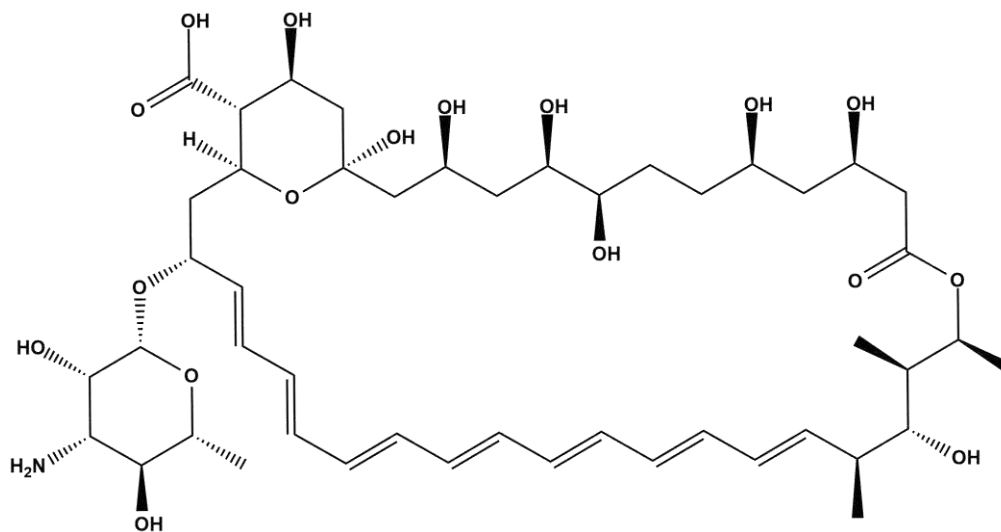
Mesmo diante do questionável custo-benefício dos antimoniais pentavalentes, o antimoniato de meglumina ainda permanece sendo a droga de referência orientada pelo Ministério da Saúde (Brasil, 2017b). As drogas de segunda linha também têm sido utilizadas apesar das limitações que também permeiam o âmbito da toxicidade aos usuários e, especificamente no âmbito da gestão pública, o alto custo na aquisição e distribuição dessas drogas pelo sistema público de saúde (Kanezaki *et al.*, 2019). No Brasil, fármacos como pentamidina e anfotericina B se destacam como alternativas terapêuticas após a falha terapêutica dos antimoniais (Félix *et al.*, 2019).

3.4.2 Anfotericina B

A anfotericina B (AmB) é um antimicrobiano da classe dos polienos, purificado inicialmente a partir da cultura de *Streptomyces nodosus* (Falci e Pasqualotto, 2015). Dentre o espectro de uso clínico da AmB está o tratamento de infecções fúngicas, concentrando a maior parte das indicações de uso, e também para o tratamento das leishmanioses quando não há resposta à terapia com antimoniais (Balasegaram *et al.*, 2012). No final da década de 1950, este medicamento já era utilizado em alguns casos clínicos e posteriormente, em 1965, se constituiu o primeiro agente antifúngico aprovado pela agência estadunidense *Food and Drug Administration* (Filippin e Souza, 2006).

O uso da AmB no tratamento das leishmanioses, através das formulações de desoxicolato de anfotericina B ou lipossomal, tem sido realizado para os casos de resistência às drogas de primeira linha (Brasil, 2017b). O mecanismo de ação desse fármaco no combate aos parasitos se dá pela formação de poros na membrana plasmática do parasito, o que resulta no aumento da permeabilidade das membranas celulares a íons e outras moléculas e leva os protozoários à morte (Chattopadhyay e Jafurulla, 2011). A estrutura química da AmB está representada na figura 19.

Figura 19 – Estrutura química da Anfotericina B



Fonte: O autor (2022).

A estrutura química da AmB apresenta característica anfipática e sua solubilidade aquosa se dá pela formulação de desoxicolato de sódio com carreadores lipídicos associados (Mesa-Arango *et al.*, 2012). A absorção no trato gastrointestinal é baixa e em alguns casos, resulta na necessidade de aplicação endovenosa. A concentração plasmática pode reduzir até 75% após 24 horas da administração, sendo posteriormente depurada pela via renal. Apesar de efetiva, a aplicação do esquema terapêutico com a AmB pode requerer internação hospitalar, o que pode limitar o uso dessa droga (Neves *et al.*, 2011; Brasil, 2017b).

Assim como os antimoniais, a anfotericina também apresenta amplo potencial toxicológico aos usuários, em especial a probabilidade de causar nefrotoxicidade. Devido a eficiência contra infecções fúngicas e parasitárias, diversos pesquisadores ao redor do mundo têm se dedicado a pesquisa de formulações a partir de desoxicolato de anfotericina B que apresente menos efeitos colaterais (Mishra *et al.*, 2013).

Dentre as formulações alternativas estão a dispersão coloidal AmB (González *et al.*, 2004), composta por estruturas semelhantes a disco; complexo lipídico-AmB (Clemons e Stevans, 1992), formado por uma concentração de estruturas semelhantes a fitas de uma camada dupla membrana e AmB lipossomal (Moen *et al.*, 2009), que consiste em vesículas unilamelares contendo AmB. Apesar dos benefícios observados como a redução da toxicidade, a aquisição de tais formulações resulta em

um custo adicional aos serviços públicos de saúde, tornando-se muitas vezes, inviável (Rex e Walsh, 1999).

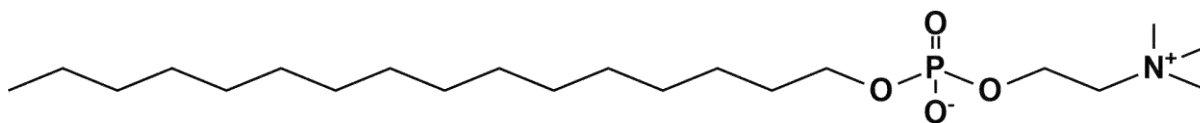
Somado às limitações relacionadas a aquisição de formulações menos tóxicas, o desoxicolato de anfotericina B tem se mostrado potencial de causar efeitos adversos como febre, vômitos, hipertensão, e mais gravemente, acometimento renal agudo (Capella *et al.*, 2013). Estes efeitos colaterais estão relacionados à modulação de mediadores inflamatórios e estudos demonstraram a ocorrência de doença cardíaca durante o uso da anfotericina, como também anemia, neutropenia, plaquetopenia e toxicidade hepática (Laniado-Laborín e Cabrales-Vargas, 2009; Falci e Pasqualotto, 2015). Diante dessas limitações, outras drogas como a miltefosina, paromicina e pentamidina também compõem o rol de drogas possíveis de serem utilizadas como “plano b”.

3.4.3 Miltefosina, Paromicina e Pentamidina

Além dos antimoniais e AmB, outros medicamentos têm mostrado potencial contra parasitas do gênero *Leishmania*, como a miltefosina, paromicina e pentamidina. A miltefosina apresenta efeito deletério na membrana de promastigotas de *Leishmanias*, a partir da interação com fosfolipídios, afetando assim o metabolismo do inositol, ativação da fosfolipase, proteína quinase C e outras vias mitogênicas, que levam as células à apoptose (Croft *et al.*, 1987; Verma e Dey, 2004).

A miltefosina (figura 20) pertence ao grupo das alquifofocolinas e apresenta efeito antineoplásico e antiparasitário (Gontijo *et al.*, 2015). Inicialmente o fármaco foi planejado para o tratamento contra o câncer e posteriormente, reposicionado para o tratamento da LC não responsiva aos antimoniais (Sundar *et al.*, 2002; Fernández *et al.*, 2014). O mecanismo de ação da miltefosina na leishmaniose tem sido investigado e publicações preliminares sugerem que o fármaco interfere no metabolismo lipídico, na síntese de proteínas e desencadeia apoptose (Dorlo *et al.*, 2012). A miltefosina é disponibilizada como medicamento veterinário na Europa e recentemente foi liberada no Brasil (Brasil, 2021).

Figura 20 – Estrutura química da miltefosina



Fonte: O autor (2022).

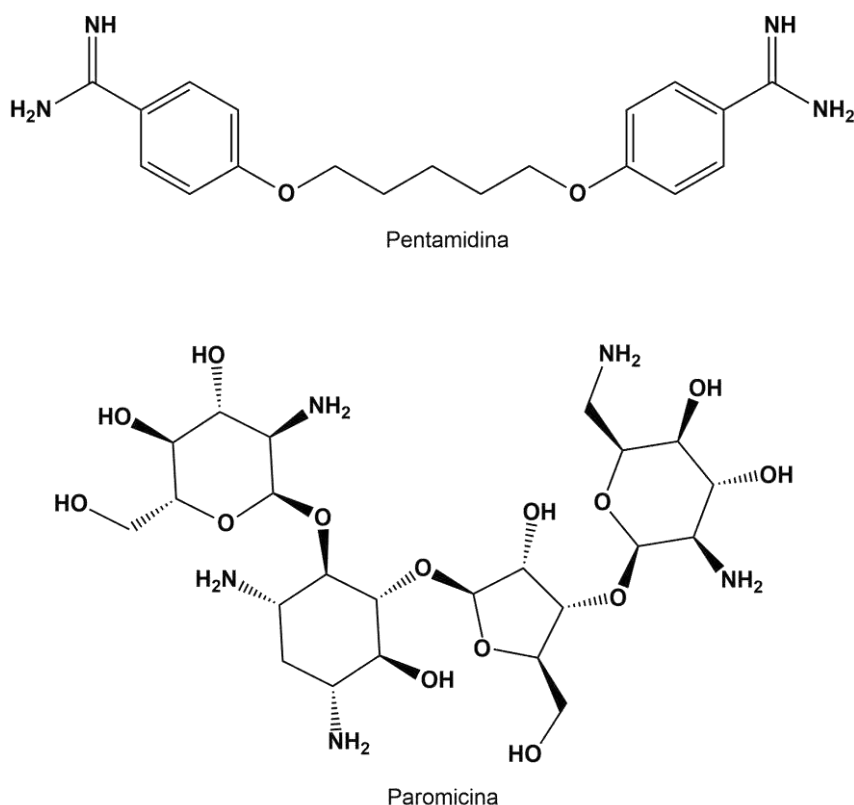
A paromicina e a pentamidina são antimicrobianos reposicionados para o tratamento da leishmaniose (Seifert *et al.*, 2011). Em 2007 a paromicina foi licenciada para o uso no tratamento da leishmaniose na Índia e assim como os demais aminoglicosídeos, esse composto causa reações adversas comumente associadas à toxicidade renal e vestibular (Davidson *et al.*, 2009).

Não obstante, a pentamidina apresenta efeitos adversos graves em pacientes com disfunção hepática, renal, hipertensão ou anemia (Au *et al.*, 2002; Andersen *et al.*, 2005). O mecanismo de ação da pentamidina tem sido relacionado ao dano no metabolismo de ácidos nucleicos, fosfolipídios e proteínas (Bray *et al.*, 2003; Alvar *et al.*, 2012). A pentamidina, uma diamidina aromática, entra nas formas promastigota e amastigota das células de *Leishmania* através de um processo mediado por carreador pelo qual as diamidinas de alta afinidade são reconhecidas (Basselin *et al.*, 2002).

A mitocôndria é um importante alvo da pentamidina e esta droga está envolvida na ligação e desintegração do DNA do cinetoplasto (Croft *et al.*, 2006). Essa característica é particularmente importante tendo em vista que a *Leishmania* possui uma única mitocôndria, o que a torna um alvo importante para o direcionamento na ação de drogas (Fidalgo e Gille, 2011).

Mesmo não sendo amplamente utilizadas como os antimoniais pentavalentes, a existência de cepas resistentes às drogas de segunda linha como a AmB (Mwenechanya *et al.*, 2017), miltefosina (Carnielli *et al.*, 2019), paromicina (Coser *et al.*, 2020) e pentamidina (Piccica *et al.*, 2021) já tem sido reportada. Devido às limitações aqui apresentadas para as drogas de primeira e segunda linha, a busca por fármacos alternativos mais seguros e com menores chances de causar efeitos adversos tem sido extensivamente realizada. As estruturas químicas da paromicina e pentamidina estão representadas na figura 21.

Figura 21 – Estruturas químicas da pentamidina e paromicina



Fonte: O autor (2022).

Devido às limitações aqui apresentadas para as drogas de primeira e segunda linha, a busca por fármacos alternativos mais seguros e, que possam ser obtidos e/ou produzidos com menores custos financeiros e com poucas chances de causar efeitos adversos tem sido extensivamente realizada (Holanda *et al.*, 2020).

3.5 PESQUISA E DESENVOLVIMENTO DE NOVAS DROGAS CONTRA AS LEISHMANIOSES

O desenvolvimento de novos medicamentos contra as diversas doenças envolve uma variedade de profissionais e diversas áreas do conhecimento como bioquímica, química medicinal, química computacional e farmacologia (Zulfiqar *et al.*, 2017). As etapas de pesquisa de novos fármacos (figura 22) se estendem desde o planejamento e análise de compostos bioativos a partir de ferramentas *in silico*, até a experimentação *in vivo* e posteriormente realização de estudos clínicos em seres humanos (Holanda, 2021).

Figura 22 – Etapas do processo de pesquisa e desenvolvimento de novos fármacos



Fonte: O autor (2022).

Especificamente no âmbito das leishmanioses, as etapas de desenvolvimento de novas drogas podem envolver análises de triagem fenotípica, que consiste numa abordagem que avalia a capacidade de determinada substância reduzir a viabilidade do parasito (Swinney e Anthony, 2011) e/ou triagem baseada em um alvo específico, que compreende a produção de drogas com foco em uma estrutura vital para o parasito (Gilbert, 2013). A utilização simultânea dessas duas abordagens pode fornecer evidências mais detalhadas sobre o potencial leishmanicidas de novos compostos bioativos (Njogu *et al.*, 2016). As moléculas com potencial leishmanicida previamente avaliado apresentam ampla diversidade estrutural e dentre os grupos químicos com destaque na prospecção de antiparasitários, os heterocíclicos imidazol,

imidazóis e triazóis têm ganhado destaque na produção de moléculas seletivas e com menor índice de toxicidade *in vitro* e *in vivo* (Dheer *et al.*, 2017; Fernandes *et al.*, 2020).

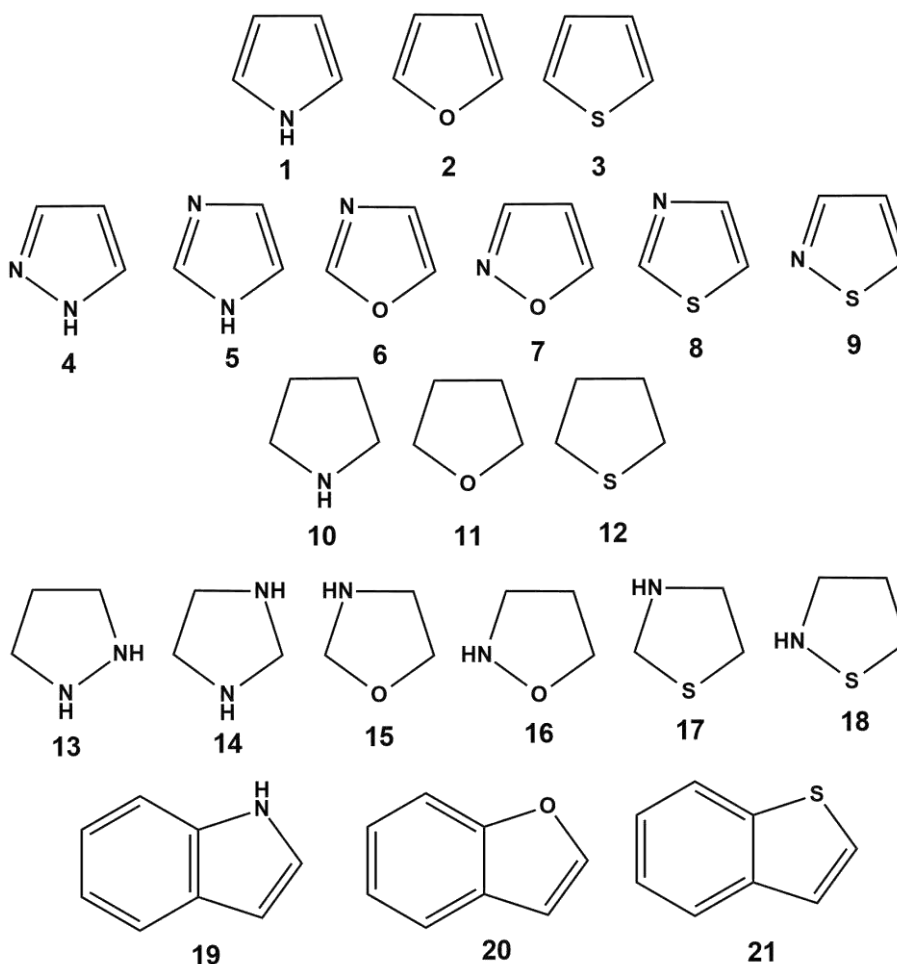
3.5.1 Compostos Heterocíclicos: Grupos Funcionais Privilegiados na Descoberta de Novos Medicamentos

Os compostos heterocíclicos constituem um grupo de moléculas cíclicas compostas de um ou mais heteroátomos, com alto grau de diversidade estrutural e economicamente úteis no desenvolvimento de agentes terapêuticos (Kumar e Narasimhan, 2018). As moléculas de cinco membros com um heteroátomo mais comuns são pirrol, furano e tiofeno, enquanto os mais comuns contendo dois heteroátomos são pirazol, imidazol, oxazol, isoxazol, tiazol e isotiazol (Verevkin *et al.*, 2011).

Devido a variedade estrutural, a classe de compostos heterocíclicos tem apresentado grande importância no contexto químico, biológico e tecnológico (Mermer *et al.*, 2021). Essas moléculas desempenham papel importante nos sistemas biológicos e podem ser encontrados nas formas de alcaloides como a morfina (Arora *et al.*, 2012), quinina a partir da *Cinchona* e campotecina, um potente inibidor da topoisomerase (Dai *et al.*, 2020). No campo da bioquímica e fisiologia, os heterocíclicos desempenham papel fundamental na regulação e funcionamento dos organismos vivos, compondo estruturas como aminoácidos, bases nitrogenadas do DNA, neurotransmissores endógenos, proteínas e vitaminas (Saraswat *et al.*, 2016).

Ao longo de décadas de pesquisa as valiosas contribuições da química orgânica sintética possibilitaram a síntese dos heterocíclicos já conhecidos e de novas substâncias com melhorias estruturais que ampliaram o potencial biológico (Gomtsyan, 2012). O potencial farmacológico dessas substâncias tem revelado importantes avanços na descoberta e desenvolvimento de drogas anticâncer, anti-inflamatória, antibacteriano, antiviral, antidiabético, antifúngico e antiparasitário (Mendgen *et al.*, 2012). Os principais heterocíclicos de cinco membros e seus análogos estão representados na figura 23.

Figura 23 – Heterocíclicos de cinco membros contendo 1 ou 2 heteroátomos e seus respectivos homólogos saturados. Compostos: 1 - pirrol; 2 - furano; 3 - tiofeno; 4 - pirazol; 5 - imidazol; 6 - oxazol; 7 - isoxazol; 8 - tiazol; 9 - isotiazol; 10 - pirrolidina; 11 - tetrahydrofurano; 12 - tetrahidrotiofeno; 13 - pirazolidina; 14 - imidazolidina; 15 - oxazolidina; 16 - isoxazolidina; 17 - tiazolidina; 18 - isotiazolidina; 19 - indol; 20 - benzofurano; 21 - benzotiofeno.

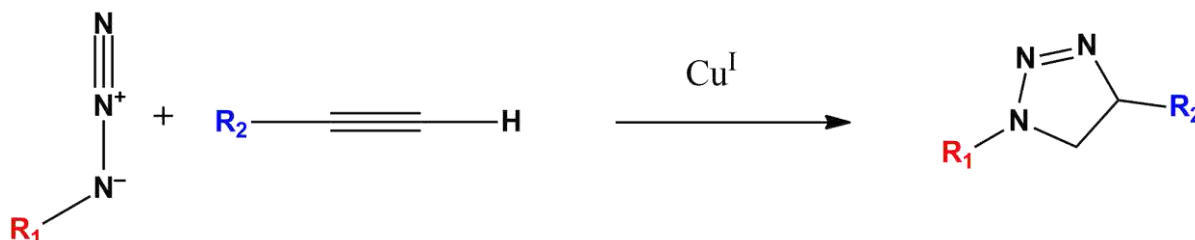


Fonte: O autor (2022).

Diversos heterocíclicos apresenta potencial antiparasitário reportado, o que resultou no uso dessas moléculas para a produção de medicamentos para o tratamento de doenças causadas por parasitos, como o caso do benzonidazol na doença de Chagas (Pinazo *et al.*, 2010). Outros derivados de heterocíclicos foram produzidos e aprimorados por métodos de síntese que fornecem diversas vantagens importantes na pesquisa de novas drogas contra doenças negligenciadas como o baixo custo, a rapidez das reações, facilidade na separação dos produtos devido à regioseletividade, altos rendimentos e possibilidade de serem reproduzidos em larga

escala. Em conjunto, essas características podem ser reunidas nas reações de *click chemistry* (Figura 24), como por exemplo a cicloadição 1,3-dipolar entre uma alcino e uma azida catalisada por cobre para a síntese do heterocíclico 1,2,3-triazol (Hou *et al.*, 2012).

Figura 24 – Reação de *Click Chemistry*

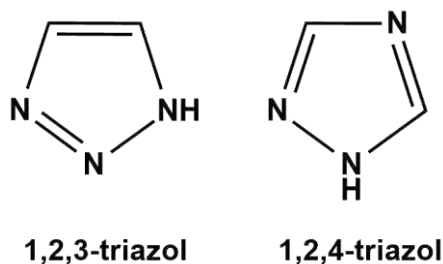


Fonte: O autor (2022).

A descoberta da cicloadição azida-alcino catalisada por metal por Meldal, Sharpless e Fokin há 20 anos revolucionou a química dos triazóis e possibilitou a síntese heterocíclicos 1,2,3-triazóis 1,4- ou 1,5-disubstituídos em altos rendimentos sob condições facilitadas (Moura e Tomé, 2021). O triazol por vez, tem se mostrado cada vez mais uma molécula versátil para utilização como grupo espaçador, unindo outros grupos farmacofóricos e com amplo espectro biológico (Bozorov *et al.*, 2019).

Os triazóis são compostos orgânicos heterocíclicos formados por um anel de cinco membros, com três átomos de nitrogênio e dois de carbono e compreendem duas formas isoméricas: 1,2,3-triazol e 1,2,4-triazol (Figura 25). Os 1,2,3-triazóis têm sido alvos de pesquisas no âmbito da química medicinal para produção de medicamentos e rápido avanço nessa área tornou o alcance a essa tecnologia inevitável (Dheer *et al.*, 2017). Esses compostos são notavelmente estáveis ao ambiente fisiológico pois resistem à hidrólise, oxidação, redução e degradação enzimática (El-Sagheer e Brown, 2012). Adicionalmente a estas características, os triazóis têm se mostrado substitutos eficazes de amidas em moléculas bioativas e efeitos bioisotéricos na ligação peptídica, anel aromático, ligações duplas e no anel imidazol (Li *et al.*, 2013).

Figura 25 – Estruturas químicas dos isômeros triazólicos: 1,2,3-triazol e 1,2,4-triazol



Fonte: O autor (2022).

Os derivados do triazol possuem uma vasta e diversificada aplicação biológica como ação antituberculosa (Srinivasarao *et al.*, 2020), antifúngica (Chen *et al.*, 2020), antitumoral (El-Azab *et al.*, 2021), antibacteriana (Xu, 2020), antiviral (Holanda *et al.*, 2021) e antiparasitárias (Pertino *et al.*, 2020), comumente são atribuídas à porção 1,2,3-triazólica pelas interações não covalentes que possibilitam a ligação a alvos biomoleculares. Especificamente no contexto de substâncias antiparasitárias, o núcleo 1,2,3-triazol se destaca pelos resultados promissores em estudos de novas drogas contra malária, toxoplasmose, doença de Chagas e leishmanioses (Bozorov *et al.*, 2019).

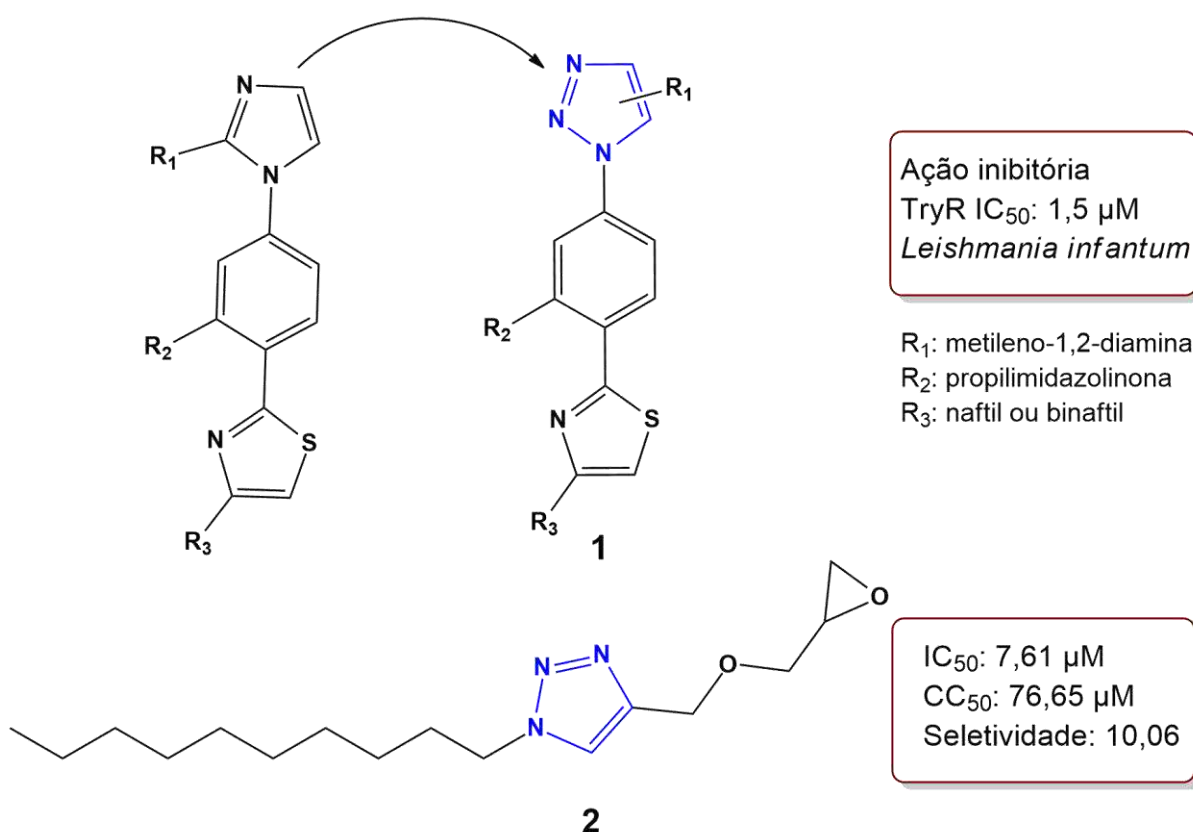
3.5.2 Potencial Leishmanicida de Compostos Bioativos Derivados do 1,2,3-Triazol: do Planejamento Estrutural à Compreensão dos Mecanismos de Ação

Dentre as atividades biológicas de moléculas orgânicas contendo o núcleo triazólico, o efeito antiparasitário *in vitro* e *in vivo* tem chamado a atenção de pesquisadores e indústrias farmacêuticas, sobretudo no seguimento das doenças negligenciadas como as leishmanioses (Meinel *et al.*, 2020). Dessa forma, diversos grupos farmacofóricos têm sido sintetizados juntamente com o 1,2,3-triazol na busca de potentes agentes leishmanicidas (Stroppa *et al.*, 2017).

O motivo pelo qual os derivados triazólicos ocupam lugar de destaque na química medicinal devido à sua contribuição para o desenvolvimento de diversos agentes terapêuticos contra diversas doenças (Dheer *et al.*, 2017). Devido à presença de três nitrogênio e outras características estruturais, esse composto tem sido explorado em grande escala para preparar compostos com significativa atividade anti-*Leishmania* (Braga, 2019). A seguir, serão apresentados compostos bioativos contendo o grupo triazol com ação anti-leishmania investigada.

A produção de derivados triazólicos também ocorre a partir do aprimoramento de moléculas existentes. Nesse sentido, com o objetivo de melhorar a atividade leishmanicida de derivados imidazólicos, o grupo 1,2,3-triazol foi inserido para a produção de compostos mais potentes (**1**) e com ação inibitória seletiva sobre a tripanotona redutase (TryR) de *Leishmania infantum* (Revuelto *et al.*, 2021). Em outro estudo no qual uma biblioteca de sais 1,2,3-triazólicos foi produzida, diferentes compostos com perfis de inibição de *Leishmania amazonensis* foram avaliados e o mais potente (**2**) apresentou a constante de inibição de 50% da cultura de parasitos (IC₅₀) de 7,61 µM, com mecanismo de ação associado ao aumento de espécies reativas de oxigênio (ERO) e despolarização mitocondrial (Figura 26).

Figura 26 – Derivados triazólicos com ação leishmanicida produzidos a partir da modificação estrutural de imidazóis (composto 1) e pela incorporação de derivados epóxido (composto 2).



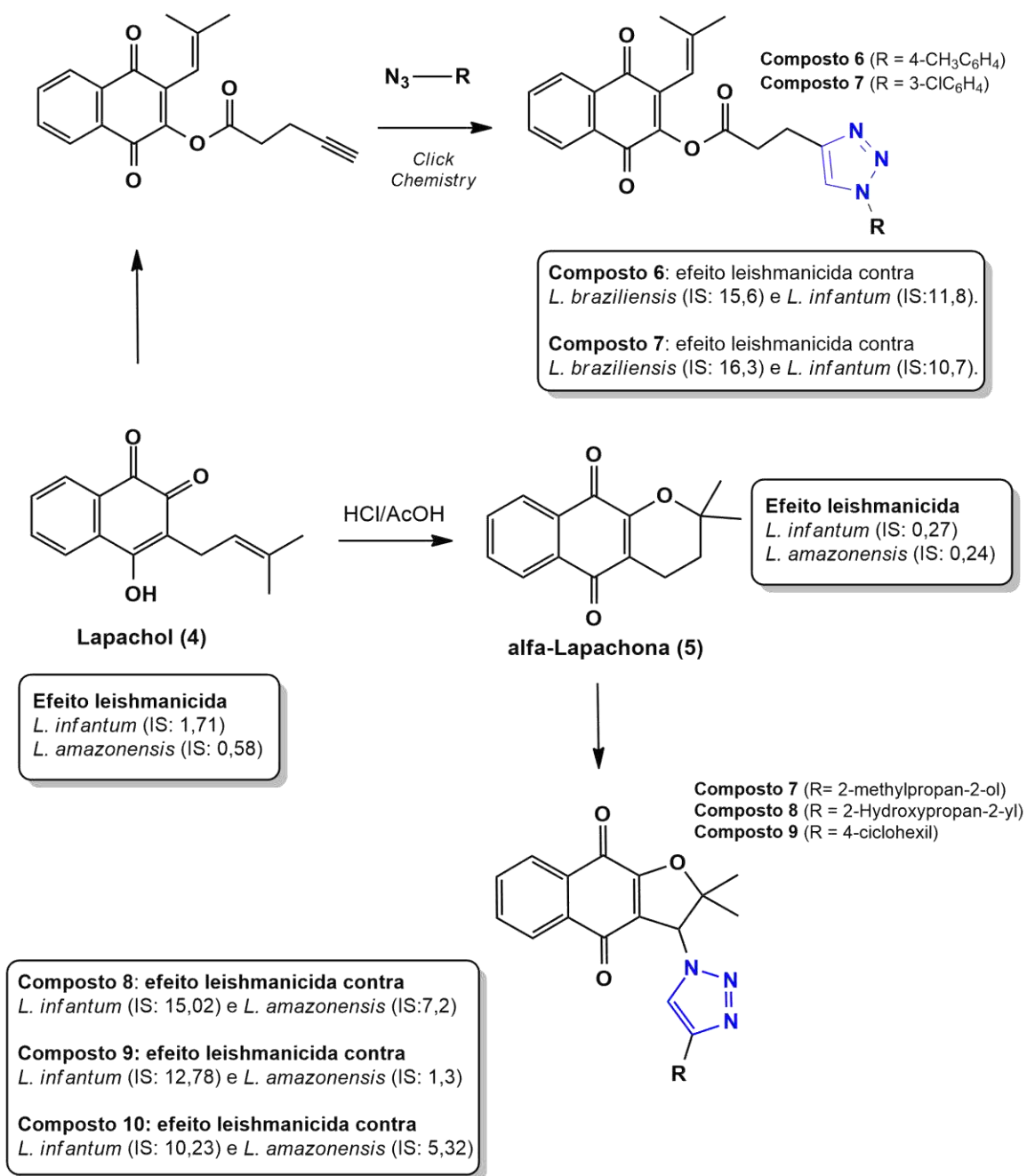
Fonte: O autor (2022).

Grupos farmacofóricos pertencentes a classe das quinonas, como o próprio lapachol (**4**) e α-Lapachona (**5**) são conhecidos pelo moderado efeito leishmanicida

(Araújo *et al.*, 2019). Nesse sentido, novas moléculas híbridas formadas pela união do lapachol com o triazol têm se mostrado promissoras na busca de agentes antiparasitários mais potentes e menos tóxicos (Erasmus *et al.*, 2021). Pertino *et al.* (2020) sintetizaram e avaliaram o potencial leishmanicida e tripanocida de uma série de derivados de triazol-lapachol e notaram que a união do triazol com os referidos grupos melhorou a atividade antiprotzoária das moléculas, obtendo moléculas (**6** e **7**) com toxicidade seletiva de até 16,3 vezes mais para *Leishmania braziliensis* do que para fibroblastos da linhagem NCTC929.

A obtenção de derivados de naftoquinona-1,2,3-triazol a partir da metodologia de *click chemistry* também foi descrita com eficiência na produção de potentes agentes antiparasitários (Oramas-Royo *et al.*, 2019). Novos derivados triazólicos produzidos a base de α - e nor- α -lapachona (**8**, **9** e **10**) por cicloadição azida-alcino catalisada por cobre apresentaram efeito anti-leishmania superior a droga antimonial e seletividade de até 15 vezes mais toxicidade aos parasitos, incluindo cepas resistentes aos fármacos de referência (Guimarães *et al.*, 2013). As moléculas **4-10** e os respectivos potenciais leishmanicida *in vitro* estão representados na figura 27.

Figura 27 – Derivados de lapachol-1,2,3-triazol produzidos por *click chemistry* com efeito leishmanicida *in vitro*.

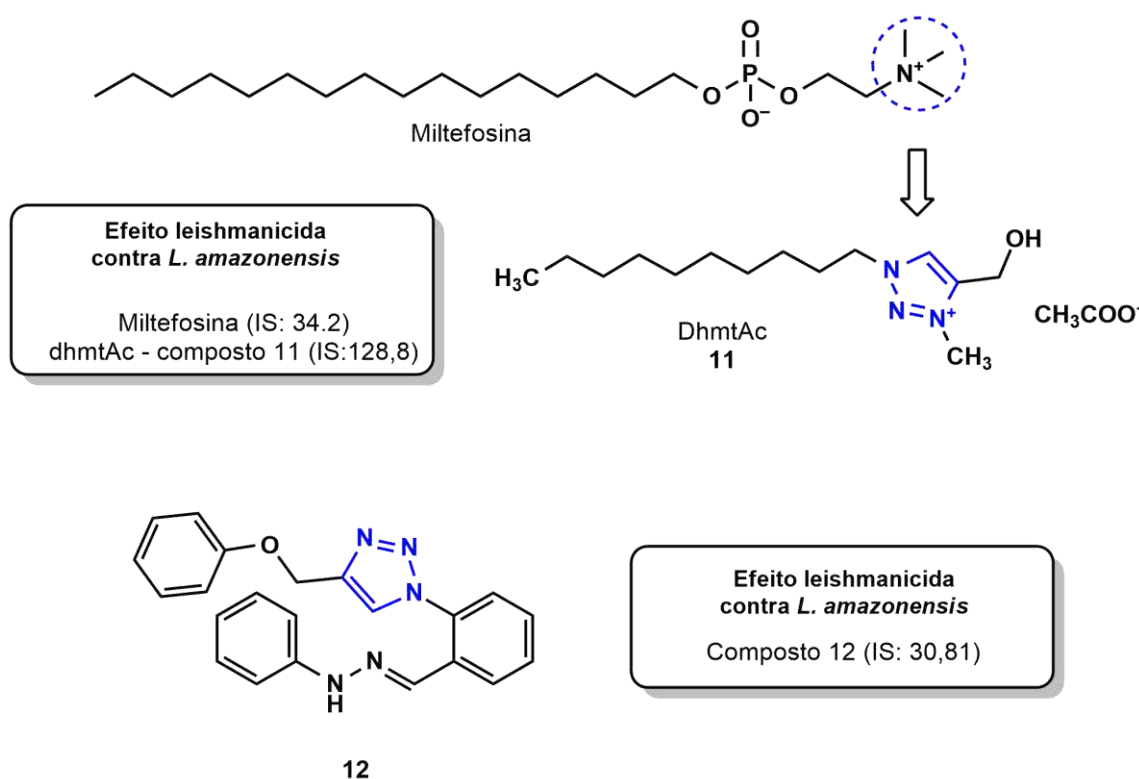


Fonte: O autor (2022).

A união do grupo triazol com outros grupos também foi realizada incluindo drogas que já estão em uso clínico, para a produção de moléculas bioisósteras não-clássicas. Stroppa *et al.* (2017) sintetizaram e avaliaram o potencial leishmanicida de 15 bioisósteros de miltefosina contendo 1,2,3-triazol e destacaram o derivado dhmtAc

(11) com atividade superior ao fármaco de referência. O potencial anti-*Leishmania* do grupo triazólico também foi explorado a partir da investigação do efeito de 1,2,3-triazóis 1,4 substituídos sobre *L. amazonensis*, demonstrando que mesmo sem estar em associação a grupos farmacofóricos específicos, o anel triazólico (12) consegue apresentar seletividade contra os parasitos de até 30 vezes (Almeida-Souza *et al.*, 2020). Ambos os derivados 11 e 12 (figura 28) apresentaram efeito superior a miltefosina.

Figura 28 – Derivados triazólicos bioisósteros da miltefosina (11) e 1,4 substituídos (12) com potente efeito leishmanicida sobre *L. amazonensis*



Fonte: O autor (2022).

Ao longo da investigação do potencial leishmanicida de derivados triazólicos, muitos grupos de pesquisa têm buscado apontar o mecanismo central pelo qual esses grupos químicos combatem seletivamente estes parasitos, seja nas formas promastigota ou amastigota (Revuelto *et al.*, 2021). Devido à presença de apenas uma mitocôndria, essa organela é comumente investigada como possível alvo do efeito de drogas. A mitocôndria em protozoários tripanossomatídeos são organelas vitais para a sobrevivência dessas células e desempenham papel fundamental na regulação de

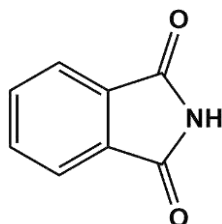
processos fisiológicos como a bioenergética, geração de ATP, controle do metabolismo redox, homeostase do cálcio, crescimento e diferenciação celular (Monzote e Gille, 2010).

Nesse contexto, os mecanismos comumente associados ao potente efeito leishmanicida de moléculas que contém o grupo 1,2,3-triazol envolvem a alteração no potencial de membrana mitocondrial ($\Delta\Psi_m$) e aumento das EROs, que em conjunto contribuem para o colapso da organização celular dos parasitos (Glanzmann *et al.*, 2021). Visando potencializar esses efeitos ou ainda ampliar o potencial biológico dos derivados do triazol, o grupo ftalimida tem sido utilizado na obtenção de compostos bioativos com maior amplitude de ação farmacológica (Silva *et al.*, 2019). A união desses grupos através da metodologia de *click chemistry* tem mostrado resultados promissores no campo da química medicinal, em especial na pesquisa de novos medicamentos para o tratamento de doenças infecciosas (Phatak *et al.*, 2019).

3.5.3 Potencial Terapêutico e Atributos Medicinais de Compostos Bioativos Derivados de Ftalimida e 1,2,3-Triazol

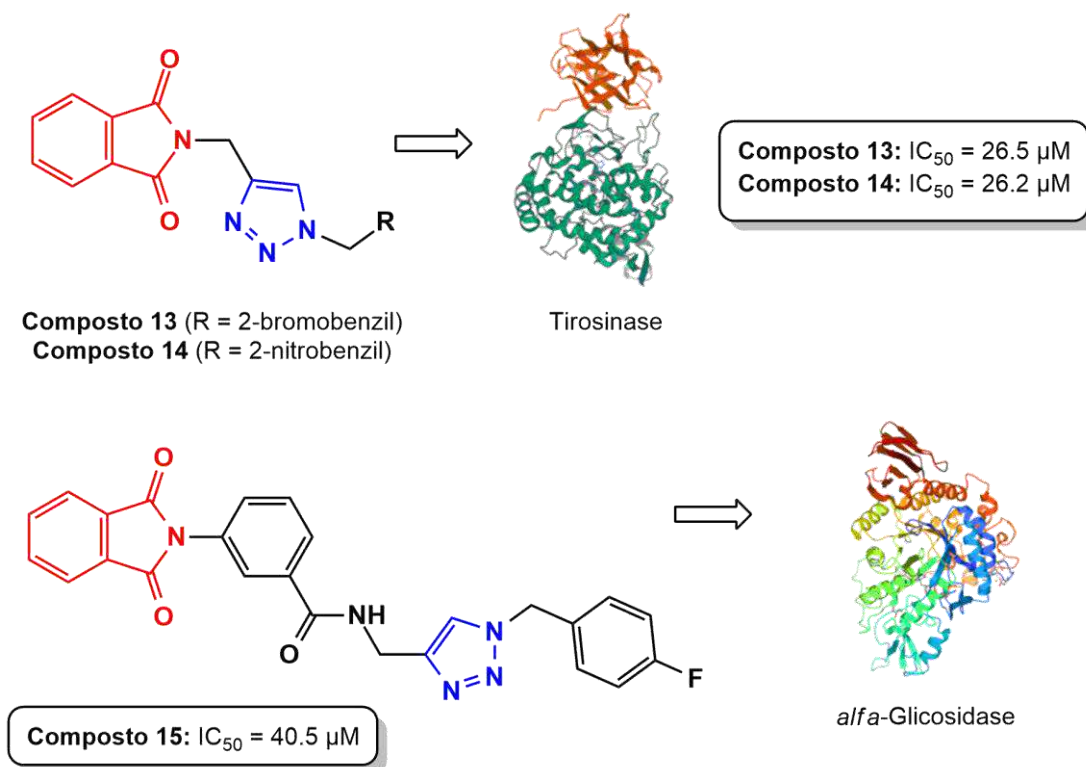
A ftalimida consiste um dos principais dos grupos farmacofóricos com aplicações biológicas e propriedades importantes na produção de compostos bioativos (Sharma *et al.*, 2010). Esse composto consiste numa imida cíclica aromática com dois grupos carbonilas ligados a fração funcional da amina e possui vasto potencial biomédico, o que a torna merecedora de destaque. Dentre as atividades biológicas investigadas, destacam-se os efeitos: analgésico (Banarouei *et al.*, 2019), hipolipidêmico (Sena *et al.*, 2003), anti-inflamatório (Assis *et al.*, 2012), antibacteriano (Arif *et al.*, 2018), antifúngico (Othman *et al.*, 2019) e antiparasitário (Dos Santos Aliança *et al.*, 2017). Em virtude desse conjunto de atividades biológicas, a ftalimida tem sido comumente associada ao anel triazólico na produção de novas substâncias bioativas híbridos com ação inibitória sobre enzimas, patógenos de importância médica e veterinária e mais recentemente, até com potencial anti-SARS-CoV-2 investigado (Holanda *et al.*, 2021). A estrutura química da ftalimida está representada na figura 29.

Figura 29 – Estrutura química da ftalimida (1,3-isoindolindiona)



Fonte: O autor (2022).

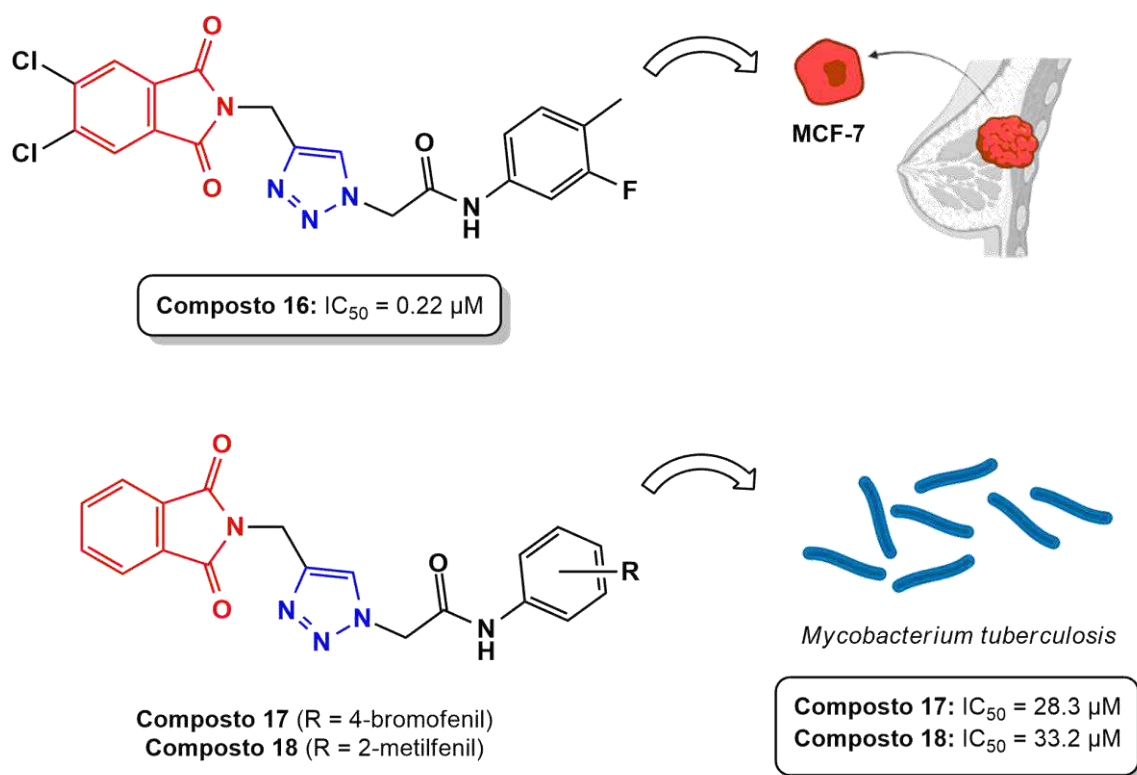
As moléculas compostas pela ftalimida e 1,2,3-triazol têm cada se tornado objeto de estudo para o desenvolvimento de fármacos (Silva *et al.*, 2012). Em um estudo no qual foi sintetizada uma série de derivados de ftalimida-1,2,3-triazol, dois compostos (**13** e **14**, figura 30) apresentaram efeito inibitório sobre a enzima tirosinase, importante para diversos seguimentos como cosmética, medicina, agricultura e indústria alimentar (Tehrani *et al.*, 2019). Outro derivado ftalimídico-1,2,3-triazólico (**15**) sintetizado por Sadat-Ebrahimi *et al.* (2020) apresentou excelente efeito sobre a enzima α -glicosidase, importante na catálise de carboidratos complexos e alvo terapêutico de fármacos anti-diabéticos.

Figura 30 – Moléculas híbridas derivadas de ftalimida e 1,2,3-triazol com efeito inibitório sobre as enzimas tirosinase (13 e 14) e α -glicosidase (15). Vermelho: ftalimida; azul: 1,2,3-triazol.

Fonte: O autor (2022).

A atividade anticâncer também foi descrita para moléculas híbridas de ftalimida e triazol. O derivado **16**, sintetizado juntamente a uma série formada por essa classe de moléculas ligadas a fragmentos lipofílicos terminais, apresentou citotoxicidade seletiva para células de câncer de mama MCF-7, figura 31 (Ihmaid *et al.*, 2021). No âmbito dos agentes microbicidas, os derivados ftalimídicos-triazólicos se destacam pela atividade contra bactérias e protozoários. Recentemente dois derivados triazólicos contendo ftalimida (**17** e **18**) foram descritos com potente atividade anti-*Mycobacterium* e o mecanismo de ação foi atribuído ao potencial inibitório da enoíl redutase, enzima importante na biossíntese de ácidos micólicos, componentes da parede celular bacteriana (Phatak *et al.*, 2019).

Figura 31 – Derivados de ftalimida e 1,2,3-triazol com atividade anticâncer (16) e anti-*Mycobacterium* (17 e 18). Vermelho: ftalimida; azul: 1,2,3-triazol

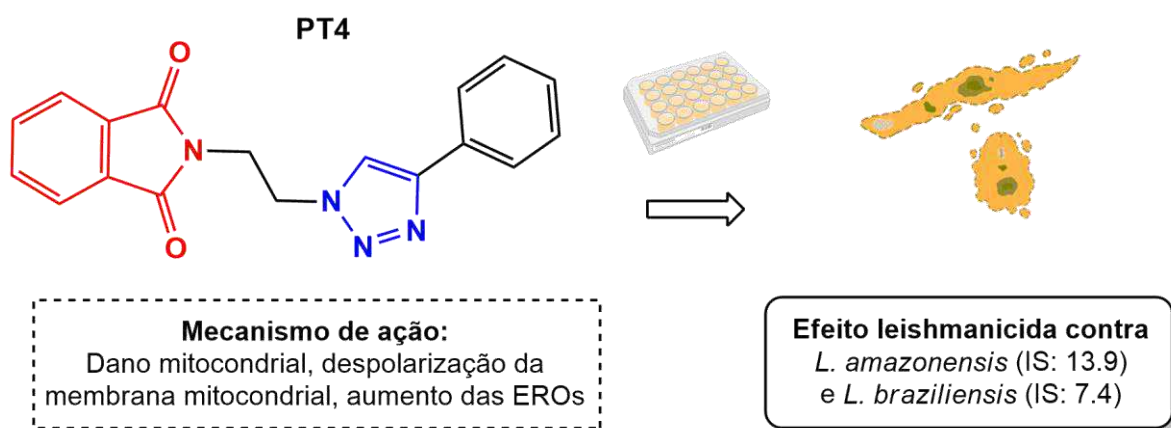


Fonte: O autor (2022).

À vista do abrangente espectro medicinal e potencial terapêutico das moléculas compostas por ftalimida e 1,2,3-triazol seja de forma isolada ou híbrida, nosso grupo

de pesquisa tem se dedicado a produção e avaliação do potencial leishmanicida de compostos bioativos contendo o triazol e a ftalimida como principais grupos farmacofóricos. Nesse contexto descrevemos, pela primeira vez, o efeito leishmanicida de uma molécula derivada de triazol-ftalimida (PT4) sobre as formas evolutivas de *Leishmania amazonensis* e *Leishmania braziliensis* (Holanda *et al.*, 2020). O derivado PT4 foi escolhido devido as propriedades físico-químicas, farmacocinéticas e toxicológicas e ao potencial anti-inflamatório, fator importante na terapia farmacológica da LC. O mecanismo do efeito leishmanicida dessa molécula (figura 32) está relacionado ao dano mitocondrial e a capacidade de inibir a enzima lanosterol 14 α -demetilase, essencial para a síntese de lipídios nas células de *Leishmania*.

Figura 32 – Estrutura química e mecanismo de ação do derivado PT4 sobre as formas evolutivas de *L. amazonensis* e *L. braziliensis*. Vermelho: ftalimida; azul: 1,2,3-triazol



Fonte: O autor (2022).

Diante do exposto, a investigação do efeito antiparasitário de novos derivados ftalimídicos-triazólicos se constitui de um campo aberto em expansão. Especificamente no que diz respeito às leishmanioses, as vantagens observadas no processo de produção dessas moléculas através da *click chemistry*, se mostra um diferencial, sobretudo no âmbito das doenças negligenciadas (Porta *et al.*, 2014). Dessa forma, a presente tese de doutorado visa a expansão da busca por derivados ftalimídicos-1,2,3-triazólicos e moléculas análogas úteis no desenvolvimento de novos fármacos leishmanicidas menos tóxicos e mais seletivos.

4 MATERIAIS E MÉTODOS

A metodologia a seguir apresentada compreende todas as etapas dos ensaios realizados para a elaboração dos trabalhos centrais da presente tese de doutorado, os quais envolvem a avaliação do potencial de derivados de ftalimida-1,2,3-triazol como novos agentes leishmanicidas. Com o objetivo de proporcionar uma maior discussão no que diz respeito aos grupos farmacofóricos, foram considerados compostos que continham apenas o grupo 1,2,3-triazol e derivados no qual o grupo ftalimida foi substituído por uma naftoquinona, estruturalmente semelhante.

Mesmo com a temática definida e delimitada à busca de novos fármacos contra a Leishmaniose Cutânea, foram realizadas importantes colaborações no campo da bioquímica, fisiologia, química de produtos naturais, química medicinal e nanotecnologia que possibilitaram a expansão das técnicas a seguir descritas para a produção de outros trabalhos que, em conjunto, também compõem o resultado desta tese e contribuem com as diversas áreas do conhecimento. Adicionalmente, as principais colaborações e publicações no âmbito desta tese de doutorado podem ser encontrados nos apêndices do A ao C.

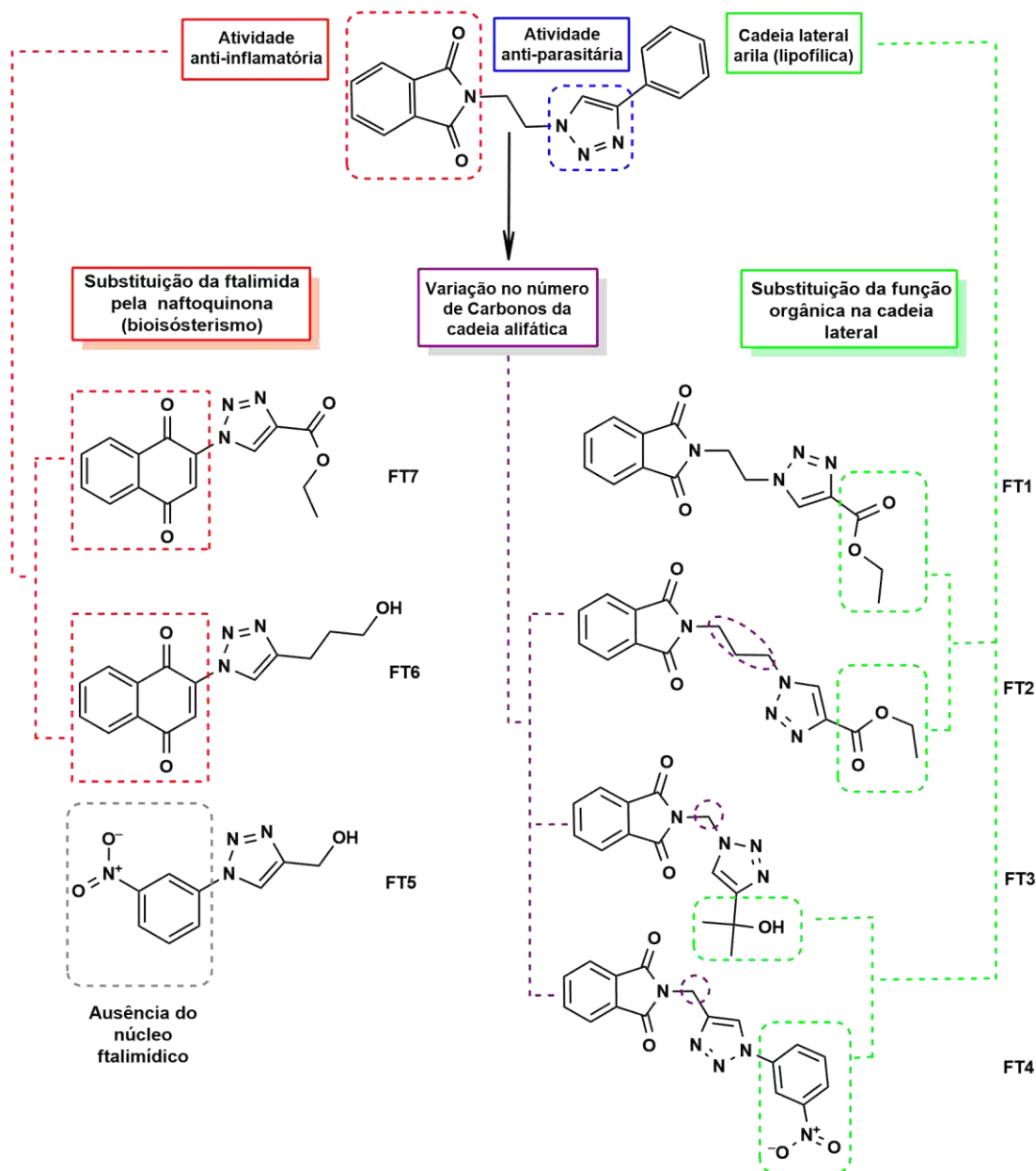
4.1 SÍNTESE DOS DERIVADOS DE 1,2,3-TRIAZOL E FTALIMIDA

Sete derivados triazólicos foram produzidos a partir da reação de cicloadição 1,3-dipolar, seguindo os protocolos previamente estabelecidos e descritos por Nascimento *et al.* (2011), Silva *et al.* (2012) e Valença *et al.* (2012). Quatro derivados apresentam um grupo ftalimida (FT1-FT4), um derivado apenas o anel triazólico como principal grupo farmacofórico (FT5) e dois apresentam o grupo naftoquinona (FT6 e FT7). As cadeias laterais dos compostos variaram entre os grupos funcionais álcool, éster e nitro.

Para a produção dos compostos FT1 a FT3, foram adicionados 1,5 mmol dos respectivos alcinos terminais; 0,15 mmol de CuI e duas gotas de Et_3N em 1,5 mL de DMF, seguido da adição da azida-ftalimida (1 mmol). Os derivados FT4 e FT5 tiveram como reagentes de partida uma nitroaril-azida (1 mmol) que reagiu com alcinos álcool e 2-propinil-ftalimida. Já no preparo dos compostos FT6 e FT7, foi utilizada a azida-naftoquinona (1 mmol) em substituição a azida-ftalimida e, no caso do derivado FT6, o alcino but-3-in-1-ol (2 mmol) foi utilizado. As modificações estruturais propostas para

as moléculas do presente estudo foram definidas com base no derivado PT4 (Holanda *et al.*, 2020), previamente descrito pelo nosso grupo como potencial agente leishmanicida. A estratégia sintética está representada na figura 32.

Figura 33 – Planejamento quimioestrutural e farmacofórico de novos agentes leishmanicidas formados pelo núcleo ftalimídico-1,2,3-triazólico

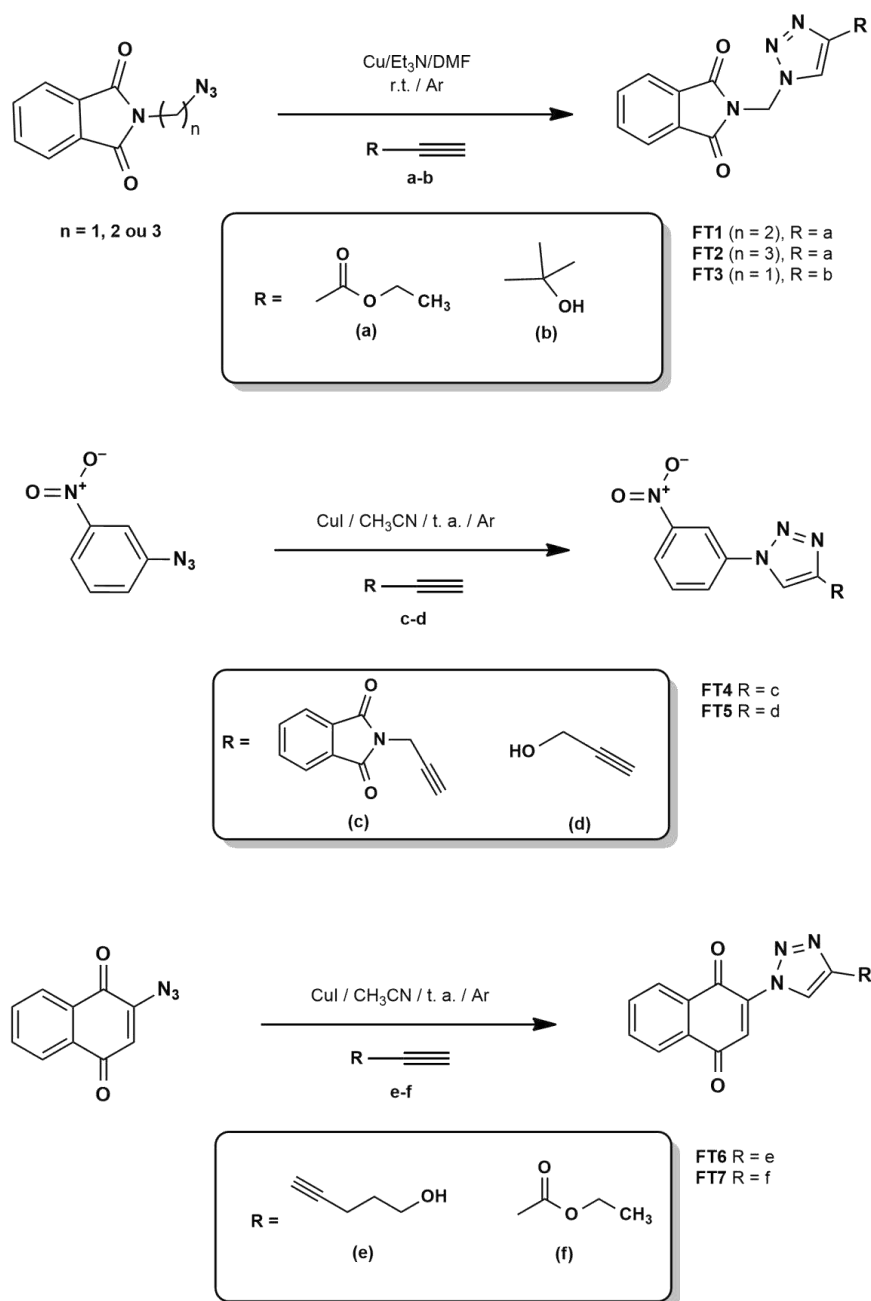


Fonte: O autor (2022).

A caracterização dos compostos ocorreu a partir do registro dos espectros infravermelho, com um espectrofotômetro IFS66 Bruker usando discos KBr e de ressonância magnética nuclear (RMN) de ^1H e ^{13}C , obtidos em espectrômetro Varian

Unity Plus 400 MHz usando DMSO- d_6 como solvente. Os derivados foram purificados por cromatografia em coluna em sílica gel 60 da Merck (malha 70–230), com uma razão de sistema hexano:EtOAc. A pureza das frações foi monitorada por análise de TLC em uma placa GF₂₅₄. As reações de cicloadição 1,3-dipolar para a formação dos triazóis e síntese dos compostos orgânicos deste trabalho estão apresentadas no esquema 1.

Esquema 1 – Reações de *click chemistry* para a síntese dos derivados FT1-FT7



Fonte: O autor (2022).

4.2 ANÁLISE *IN SILICO* DO PERFIL FÍSICO-QUÍMICO, FARMACOCINÉTICO E TOXICOLÓGICO

Após o preparo dos compostos foi realizada a análise *in silico* do perfil físico-químico, farmacocinético e toxicológico (ADMET) de todos os derivados e comparados aos fármacos de referência utilizados no tratamento das leishmanioses. Para isso, a estrutura química dos derivados FT1 - FT7 foram construídas no ACD/ChemSketch 2.1 e os *smiles* foram obtidos para cada um dos compostos. Os *smiles* dos fármacos de referência foram utilizados a partir dos dados disponíveis na plataforma PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) sob os seguintes códigos: miltefosina (CID: 3599), anfotericina B (CID: 5280965), pentamidina (CID: 4735) e antimoniato de meglumina (CID: 64953).

No perfil ADMET os compostos foram avaliados de acordo com os parâmetros da “Regra dos 5” estabelecidos por Lipinski (2004): apresentar até 5 doadores de hidrogênio, 10 aceptadores de hidrogênio, peso molecular menor ou igual a 500 g/mL e coeficiente de partição octanol/água menor ou igual a 5. As análises dos perfis toxicológico e de excreção foram realizadas através das plataformas pkCSM (Pires *et al.*, 2015) e ProTox-II (Banerjee *et al.*, 2018), nas quais foram investigadas as previsões para: teste de Ames, inibição do gene humano *ether-a-go-go* (hERG), hepatotoxicidade e dose letal 50% (LD₅₀).

4.3 ENSAIO DE CITOTOXICIDADE EM CÉLULAS DE MAMÍFEROS

O ensaio de citotoxicidade foi realizado em duas linhagens de células de mamífero: fibroblastos ATCC® PCS-201-012™ e macrófagos J774A.1. ATCC® TIB-67™. As células foram obtidas de uma cultura previamente estabelecida em meio *Dulbecco's Modified Eagle Medium* (DMEM, Sigma-Aldrich, EUA) suplementado com 10% de soro fetal bovino (SBF) e mantidas a 37 °C numa atmosfera de 5% de CO₂. Nesse contexto, fibroblastos e macrófagos foram cultivados em placas de 96 poços (5 × 10⁵ células/poço) e tratadas com diferentes concentrações dos derivados FT por 48 horas. O grupo não tratado foi considerado controle e representou 100% de viabilidade celular.

Após a incubação com as drogas, as células foram lavadas com PBS estéril e então incubadas com uma solução de 0,5 mg/mL de 3-(4,5-dimetiltiazol-2-il)-2,5-

difeniltetrazolio brometo) (Sigma-Aldrich, EUA) durante 3 horas para a formação dos cristais de formazan, conforme estabelecido por Mosmann (1983). A reação de oxidação do MTT foi acompanhada através da observação em microscópio óptico. Após esta etapa, os cristais de formazan foram solubilizados com uma solução acidificada de isopropanol (0,04 M HCl + isopropanol absoluto) e posteriormente submetidos a leitura em espectrofotômetro (Bio-Rad®, California, EUA) a 540 nm. As análises foram realizadas em quadruplicata, em dois experimentos independentes. Os dados da absorbância foram utilizados para o cálculo da concentração citotóxica de cada derivado através de regressão linear no SPSS (Versão 18.0. IBM Corp. EUA).

4.4 AVALIAÇÃO DO EFEITO DOS DERIVADOS DE FTALIMIDA E 1,2,3-TRIAZOL SOBRE FORMAS PROMASTIGOTAS DE *Leishmania amazonensis* E *Leishmania braziliensis*

O potencial leishmanicida dos derivados FT foi avaliado em formas promastigotas de *Leishmania amazonensis* (LTB0016) e *Leishmania braziliensis* (LTB2903). As promastigotas foram cultivadas em meio Schneider's (Sigma-Aldrich, EUA) suplementado com 10% de SBF, penicilina (100 IU) e estreptomicina (0,01 mg/mL). Em todos os experimentos foram utilizadas células em crescimento exponencial. Dessa forma, as promastigotas de ambas as espécies foram incubadas na ausência (controle) e presença de diferentes concentrações das moléculas deste estudo.

A análise do efeito leishmanicida ocorreu através da quantificação de ATP com o kit *Molecular ATP determination* (Thermo Fisher, EUA) seguindo a determinação do fabricante. O efeito dos compostos sobre o crescimento foi estimado através da IC₅₀ (concentração da droga capaz de inibir em 50% a proliferação dos parasitas após 48h de tratamento). Foram realizados três ensaios independentes em triplicata. Após isto, as moléculas foram ranqueadas de acordo com o índice de seletividade (IS) obtido pela fórmula: $\frac{CC_{50}}{IC_{50}}$. Os dois derivados com maior IS para ambas as células de mamífero (macrófago e fibroblastos) simultaneamente, foram escolhidos para as etapas seguintes de investigação.

4.5 INVESTIGAÇÃO DO POTENCIAL HEMOLÍTICO DOS DERIVADOS FTALIMÍDICOS-TRIAZÓLICOS DE MAIOR SELETIVIDADE CONTRA *Leishmania* sp.

A investigação do potencial hemolítico foi realizada em amostras de hemácias obtidas de humanos saudáveis dos diferentes fenótipos: A, B, O e AB. Os procedimentos seguiram a metodologia descrita por Yang *et al.* (2005). O presente estudo foi realizado seguindo os princípios éticos adotados pela legislação brasileira (Lei Nº 11.974/2008) e pelo Comitê Nacional de Ética em Pesquisa com Seres Humanos (CEP/CONEP), estando aprovado e registrado pelo número CAAE: 38726620.6.0000.5048 (anexo A).

As hemácias foram diluídas em uma solução salina (NaCl 0,9%) e incubadas por 30 minutos a 37 °C em microtubos contendo diferentes concentrações dos compostos (1:1) de forma que o volume final resultasse em 1 mL. Após essa etapa, os microtubos foram centrifugados (1.500 × g por 4 min) e o sobrenadante foi coletado para análise de espectrofotometria de UV-Vis a 412 nm. Para esse ensaio, foi considerado controle negativo (ausência de hemólise) as células que foram incubadas apenas com solução salina e controle positivo (presença de hemólise), as amostras tratadas com Triton X-100 (0,1%). A taxa de hemólise foi determinada a partir do seguinte cálculo: $\%Hemólise = \frac{As - An}{Ap - An} \times 100$, no qual As, Ap e An são as absorbâncias da amostra testada, controle positivo e controle negativo, respectivamente. Cada análise foi realizada em três experimentos independentes em triplicata.

4.6 AVALIAÇÃO DO EFEITO LEISHMANICIDA DOS DERIVADOS FT1 E FT2 SOBRE FORMAS AMASTIGOTAS DE *Leishmania amazonensis* E *Leishmania braziliensis*

A investigação do efeito leishmanicida nas formas amastigotas de *L. amazonensis* e *L. braziliensis* foi realizado nas formas amastigotas axênicas e intracelulares. No ensaio utilizando as formas axênicas, as promastigotas de ambas as espécies foram cultivadas e utilizadas no início da fase estacionária. Após o 5º de cultivo, as promastigotas foram transferidas para o meio amastigota-axênica (MAA). Para isso, uma solução de MAA foi produzida de acordo com o método proposto por

Sereno *et al.* (2001). Dessa forma, uma solução salina de HBSS previamente preparada foi suplementada com 0,5% de triptocaseína, 15 mM de D-glicose (Sigma-Aldrich, EUA), 5 mM de glutamina (Gibco®, Thermo Fisher Scientific, EUA), 4 mM de NaHCO_3 , 0,023 mM de hemina bovina (FLUKA) e 25 mM de HEPES para um pH final de 5,5 e suplementado com 20% de soro bovino fetal inativado (Gibco®, Thermo Fisher Scientific, EUA).

As promastigotas em fase estacionária (2×10^6 células/mL) foram cultivadas no meio MAA e submetidas a variações crescentes de temperatura, 24 horas em 28 °C e posteriormente, 96 horas em 37 °C. O processo de diferenciação foi acompanhado através de microscopia óptica. As células foram utilizadas para experimentação quando o percentual de formas amastigotas ultrapassou 80% das células em cultura. Dessa forma, as amastigotas axênicas (1×10^6 células/mL) foram incubados na ausência e presença de diferentes concentrações dos derivados FT1 e FT2 por 24h a 37 °C. Após o tratamento, a viabilidade das células foi verificada através da dosagem de ATP. Foram realizados três ensaios independentes em triplicata.

A etapa seguinte consistiu na investigação do efeito de FT1 e FT2 sobre o modelo de infecção *in vitro* de *L. amazonensis* e *L. braziliensis* em macrófagos de exsudato peritoneal murino. Com o objetivo de reduzir o número de animais utilizados na experimentação, apenas nesta etapa foram utilizados os macrófagos peritoneais obtidos de camundongos BALB/c machos e saudáveis. Os protocolos do presente trabalho foram submetidos a apreciação pela Comissão de Ética no Uso de Animais (CEUA) do Instituto Aggeu Magalhães/FIOCRUZ-PE, no qual obteve parecer aprovado sob os registros 146/2019 (anexo B).

Dessa forma, os macrófagos peritoneais foram coletados e cultivados em lamínulas de vidro presentes em placas de cultura de 24 poços (5×10^5 células/mL), contendo meio RPMI suplementado com 10% FBS. As células foram incubadas a 37 °C, em uma atmosfera de 5% de CO_2 , durante 3h para adesão. Em seguida, todos os poços foram lavados e as células não aderidas retiradas. Posteriormente, foi realizada a infecção dos macrófagos com formas promastigotas de *L. amazonensis* e *L. braziliensis*, utilizando uma razão 15 parasitas para 1 macrófago, por 14 horas. Após este período, a cultura foi lavada 3 vezes com meio de cultura e os parasitas não-interiorizados foram removidos.

Os macrófagos infectados, contendo amastigotas intracelulares, foram tratados com três concentrações determinadas a partir da IC_{50} , estabelecida previamente para

as respectivas formas promastigotas. Sendo assim, os grupos tratados consistiram em $\frac{1}{2}$, 1 e 2x os valores da IC₅₀, submetidos as mesmas condições de cultura por mais 24 horas. Neste experimento, o controle negativo consistiu nos macrófagos infectados não tratados e os controles positivos receberam os valores correspondentes a IC₅₀ do antimoniato de meglumina (70 μ M) e anfotericina B (1 μ M). Após o tratamento, o sobrenadante foi coletado e reservado para dosagem de óxido nítrico (NO). As células foram fixadas com metanol P. A. e coradas com a técnica de May-Grünwald-Giemsa.

A percentagem de células infectadas foi determinada pela contagem de 150 macrófagos escolhidos aleatoriamente em duplicata. O índice de sobrevivência foi determinado pela multiplicação da porcentagem de macrófagos infectados pelo número médio de parasitas por célula infectada. A concentração que inibiu o crescimento das amastigota intracelulares (IC₅₀/ama) em 50% foi determinada por análise de regressão como já descrito.

4.7 INVESTIGAÇÃO DO EFEITO DOS DERIVADOS FT1 E FT2 NA PRODUÇÃO DE ÓXIDO NÍTRICO EM MACRÓFAGOS DE EXSUDATO PERITONEAL

O sobrenadante da cultura de macrófagos infectados com as formas amastigotas de *L. amazonensis* e *L. braziliensis* foi utilizado para dosagem de NO. Para isso, foi preparada uma solução de reagente de Griess conforme descrito na literatura (Ugucione et al., 2002), através da solubilização de 1 g de sulfanilamida e 100 mg de dicloridrato de N-(1-naftil)-etilenodiamina em 100 mL H₃PO₄ a 2,5%. A reação foi deixada à temperatura ambiente por 10 min protegida da luz. Em seguida, a absorbância foi medida em 540 nm no leitor de placas múltiplas GloMax (Promega, Califórnia, EUA). A concentração de NO foi determinada de forma indireta, através da curva padrão de nitrito de sódio.

4.8 ANÁLISE DO EFEITO DOS DERIVADOS FT1 E FT2 SOBRE A ULTRAESTRUTURA DE PROMASTIGOTAS DE *Leishmania amazonensis* E *Leishmania braziliensis*

Para a investigação das alterações ultraestruturais, as formas promastigotas de *L. amazonensis* e *L. braziliensis* foram tratadas com as concentrações correspondentes a ½, 1 e 2x o valor da IC₅₀ para cada composto, por 48 horas. Após este período, as células foram lavadas com tampão PBS e então fixadas com Karnovski (2,5% de glutaraldeído + 4% de paraformaldeído, em tampão cacodilato de sódio 0,1 M, pH 7,2). Em seguida, foi realizada a pós-fixação por 1 hora com 1% de tetróxido de ósmio, 0,8% de ferricianeto de potássio, 5 mM de CaCl₂ e tampão cacodilato 0,1 M. Posteriormente, as células foram desidratadas em baterias crescentes de etanol e secadas em ponto crítico. Por fim, as células foram metalizadas com ouro e observadas no microscópio SEM 510 Jeol.

4.9 AVALIAÇÃO DO POTENCIAL DE MEMBRANA MITOCONDRIAL, PRODUÇÃO DE ESPÉCIES REATIVAS DE OXIGÊNIO E COMPARTIMENTOS ÁCIDOS EM PROMASTIGOTAS TRATADAS COM OS DERIVADOS FT1 E FT2

A avaliação do efeito do tratamento dos derivados FT1 e FT2 sobre a fisiologia mitocondrial e compartimentos ácidos das promastigotas de *L. amazonensis* e *L. braziliensis* ocorreu através da marcação fluorescente e análise em citômetro de fluxo. Dessa forma, as células foram previamente tratadas conforme já descrito anteriormente. Após, as promastigotas foram lavadas em tampão PBS e marcadas com Rodamina 123 (10 µg/mL), por 15 minutos e MitoSOX (5 µM), durante 10 minutos. A solução de MeOH 70% foi utilizada como controle positivo para dano celular. As alterações no potencial de membrana mitocondrial foram determinadas através do cálculo do índice de variação: $\frac{MT-MC}{MC}$, no qual MC corresponde a mediana da fluorescência do controle negativo (sem tratamento) e MT a mediana da fluorescência das células tratadas.

A avaliação dos compartimentos ácidos ocorreu através da marcação com Laranja de Acridina (LA). Dessa forma, as células tratadas e controles foram lavados e incubados com uma solução de 10 µg/mL de LA por 15 minutos. Todas as

marcações ocorreram na ausência de luz e à temperatura de 26 °C. Após as marcações, as células foram lavadas 3 vezes em PBS. Os dados foram analisados no citômetro de fluxo FACS Calibur, equipado com o *software* Quest Cell. Um total de 20.000 eventos foram adquiridos na região previamente estabelecida como a correspondente às formas viáveis das promastigotas. Os valores obtidos serão analisados com o *Windows Multiple Document Interface* (versão 2.8) *software* para a determinação da porcentagem da média de marcação para cada situação.

4.10 ANCORAGEM MOLECULAR E ESTUDO DAS INTERAÇÕES QUÍMICAS ENTRE OS DERIVADOS FT1 E FT2 SOBRE OS PRINCIPAIS ALVOS FARMACOLÓGICOS EM *Leishmania* sp.

Para investigar o potencial inibitório e o perfil das interações químicas com os principais alvos farmacológicos em tripanossomatídeos, os ligantes FT1 e FT2 foram confrontados com as proteínas de *Leishmania*. Dessa forma, as estruturas químicas dos compostos foram construídas no Discovery Studio v.20.1.0.19295 e otimizadas utilizando o método semiempírico PM3 (Stewart, 1989), implementado no Mercury 4.3.1 (Macrae *et al.*, 2008). Os estados de protonação dos ligantes foram calculados pelo *software* MarvinSketch 20.19.0 assumindo um pH fisiológico (Butcher *et al.*, 2015).

O docking molecular foi realizado com a utilização do AutoDock 4.2. A proteína esterol 14-alfa-demetilase foi escolhida de acordo com os parâmetros estereoquímicos fornecidos pela análise de Ramachandran, obtidas após análise no servidor PROCHECK (Laskowski *et al.*, 2006). A estrutura do cristal da proteína foi obtida no *Protein Data Bank* (PDB) sob o código 3L4D (Hargrove *et al.*, 2011). A validação das estruturas do complexo proteína-ligante foi realizada usando o ligante co-cristalizado.

Nesse contexto, foi realizado o redocking entre a esterol 14-alfa-demetilase e o seu ligante nativo, o fluconazol. Para cada complexo ligante-proteína, 10 poses de encaixe foram geradas usando o algoritmo genético lamarckiano (López-Camacho *et al.*, 2015). A pose com a energia de ligação mais baixa foi selecionada como o resultado final do encaixe. As interações do complexo alvo-ligante foram analisadas e renderizadas usando o Discovery Studio v.20.1.0.19295.

5 RESULTADOS

Em conjunto, os resultados preliminares desta tese de doutorado foram reunidos em sete publicações das quais cinco encontram-se publicadas (artigos), um pedido de patente depositado e um manuscrito a ser submetido. Os dados sobre o potencial leishmanicida e mecanismo de ação abordados através de técnicas *in silico* e *in vitro* irão compor o seguinte manuscrito que será submetido ao periódico Journal of Medicinal Chemistry (fator de impacto 2020: 7.44):

5.1 ARTIGO 1

Phthalimide and naphthoquinone derivatives containing 1,2,3-triazoles as potential leishmanicidal agents: an *in silico*, *in vitro* and mechanism of action studies

Vanderlan Nogueira Holanda^{a,b}, Welson Vicente da Silva^b, Deyzi Caroline da Silva Barbosa^b, Elton Marlon de Araújo Lima^{b,c}, Kethully Albuquerque de Lima^d, Bruna Martins Guimarães^d, Maria Carolina Accioly Brelaz de Castro^e, César Augusto da Silva^f, Regina Celia Bressan Queiroz de Figueiredo^b, Ronaldo Nascimento de Oliveira^d, Vera Lúcia de Menezes Lima^{a*}

^aLaboratório de Lipídios e Aplicação de Biomoléculas em Doenças Prevalentes e Negligenciadas. Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, 1235, 50670-901 Recife, PE, Brazil.

^bLaboratório de Biologia Celular de Patógenos, Instituto Aggeu Magalhães, Departamento de Microbiologia, Avenida Professor Moraes Rego, 1235, 50670-901 Recife, PE, Brazil.

^cLaboratório de Polímeros Não-convencionais, Centro de Ciências Exatas e da Natureza, Universidade Federal de Pernambuco, 50670-901 Recife, Pernambuco, Brazil.

^dLaboratório de Síntese de Compostos Bioativos, Departamento de Química, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros, s/n - Dois Irmãos, 52171-900 Recife, PE, Brazil.

^eLaboratório de Parasitologia, Centro Acadêmico de Vitória, Universidade Federal de Pernambuco, 50670-901 Vitória de Santo Antão, Pernambuco, Brazil.

^fColegiado de Medicina, Universidade Federal do Vale do São Francisco, Avenida José de Sá Maniçoba, s/n - Campus Universitário, 56304-205 Petrolina, PE, Brazil.

**Corresponding author:*

Laboratório de Lipídios e Aplicação de Biomoléculas em Doenças Prevalentes e Negligenciadas. Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, 1235, 50670-901 Recife, PE, Brazil. E-mail adress: lima.vera.ufpe@gmail.com (V. L. M. Lima).

ABSTRACT

Leishmaniasis is a prevalent and neglected disease that affects many populations around the world. Due to the high toxicity and the existence of protozoa resistant to the current treatment, the search for new leishmanicidal agents is still urgent. In this study, we evaluated the leishmanicidal potential of seven triazole derivatives linked to phthalimide or 1,4-naphthoquinone obtained by click chemistry, on the evolutionary forms of *Leishmania amazonensis* and *Leishmania braziliensis*. The ADMET profile revealed that the compounds (FT1-FT7), had drug-likeness properties and a high prediction of gastrointestinal absorption. The cytotoxicity assay on macrophages and fibroblasts demonstrated that the compounds containing naphthoquinone (FT6 and FT7) were more toxic, with CC₅₀ values ranging from 20.8 to 314.2 µM, while derivatives with phthalimide were the least toxic (CC₅₀ > 2,000 µM). All compounds showed leishmanicidal activity against the promastigote forms of *Leishmania* sp., the highest selectivity indexes were found for the FT1 and FT2 derivatives (SI > 20), making them the leading compounds in this study. FT1 and FT2 derivatives did not

show hemolytic activity and were effective in reducing the survival index of axenic and intracellular amastigotes, with selectivity indices reaching 29.12. The FT1 and FT2 treatment resulted in drastic changes in the promastigotes such as the wrinkling of the plasma membrane, shrinkage, and swelling of the cell body. We also observed depolarization of the mitochondrial membrane potential and an increase in reactive oxygen species, in addition to an increase in acid compartments. These results made us consider the enzymes sterol 14- α -demethylase and trypanothione reductase as possible targets of the FT1 and FT2 molecules, and for this, we performed the molecular docking calculations. Both compounds showed high-affinity interaction with enzymes, from the binding energy values for the FT1-14 α -DM (-8.12 kcal/mol), FT2-14 α -DM (-7.36) complexes, FT1-TryR (-8.16 kcal/mol), and FT2-TryR (-6.81 kcal/mol). Cellular alterations caused by FT1 and FT2 derivatives, added to the inhibitory potential of the enzymatic targets of *Leishmania*, may be related to the leishmanicidal effect described in this study. Taken together, our results point to FT1 and FT2 molecules as potent leishmanicidal agents that are promising for use, alone or in combination, in the treatment of leishmaniasis.

Keywords: 1,2,3-triazole; Phthalimide; Naphthoquinone; Leishmaniasis; Click chemistry; Chemotherapy.

INTRODUCTION

Leishmaniasis is a prevalent and neglected disease that has affected millions of people around the world for more than a century and for which the few drugs available have great toxicity and side effects (Roatt *et al.*, 2020). Among the most common clinical manifestations of leishmaniasis are the visceral forms (LV), which are more severe and potentially fatal when left untreated (Silva *et al.*, 2020), and the tegumentary form (LT), in which the skin and mucous membranes can be severely affected through the development of ulcers with inflammatory damage (Saldanha *et al.*, 2020). Due to the limitations widely reported and attributed to reference drugs for the treatment of these diseases, the search for bioactive compounds with leishmanicidal potential is still urgent (Santos *et al.*, 2020).

The search for new leishmanicidal agents with low toxicity has been expanding and, in this context, molecules derived from 1,2,3-triazole have stood out in the

pharmacological and therapeutic field (Bozorov *et al.*, 2019). Associated with the potent anti-leishmanial effect, 1,2,3-triazole derivatives can be easily obtained by optimized organic synthesis methods, low cost, and with the formation of products with excellent yields, important characteristics in the context of neglected diseases (Sharma *et al.*, 2010). These characteristics can be gathered in the click chemistry reaction, widely used for the union of two or more pharmacophoric groups from the regioselective formation of 1,2,3-triazole (Rani *et al.*, 2020). Given this scenario, the joining of different chemical groups with proven biological activity seems to be a viable alternative in the search for new anti-leishmanial agents (Pertino *et al.*, 2020).

Among the pharmacophoric groups widely investigated in the context of anti-*Leishmania* chemotherapy, phthalimide and naphthoquinone have stood out for their selective cytotoxicity to protozoa (Dantas-Pereira *et al.*, 2021). Additionally, both groups have a diversity of biomedical applications already described *in silico*, *in vitro*, and *in vivo* studies (Silva *et al.*, 2019; Peixoto *et al.*, 2021). Phthalimide is an excellent scaffold for the production of anti-inflammatory compounds, while naphthoquinone stands out for its potent antiparasitic effect, although occasionally toxic to mammalian cells (Assis *et al.*, 2012; Guimarães *et al.*, 2013). We recently described, for the first time, the leishmanicidal effect of a hybrid molecule composed of phthalimide and 1,2,3-triazole on the evolutionary forms of *L. amazonensis* and *L. braziliensis*, etiological agents of LT in Brazil (Holanda *et al.*, 2020).

The combination of these and other chemical groups enables the production of bioactive compounds useful for the prospect of drugs against leishmaniasis. In this context, this study aimed to evaluate the leishmanicidal potential of 1,2,3-triazole analogs linked to phthalimides or naphthoquinones, structurally designed as drug-likeness, as well as the mechanism of action from an *in silico* and *in vitro* approaches.

MATERIALS AND METHODS

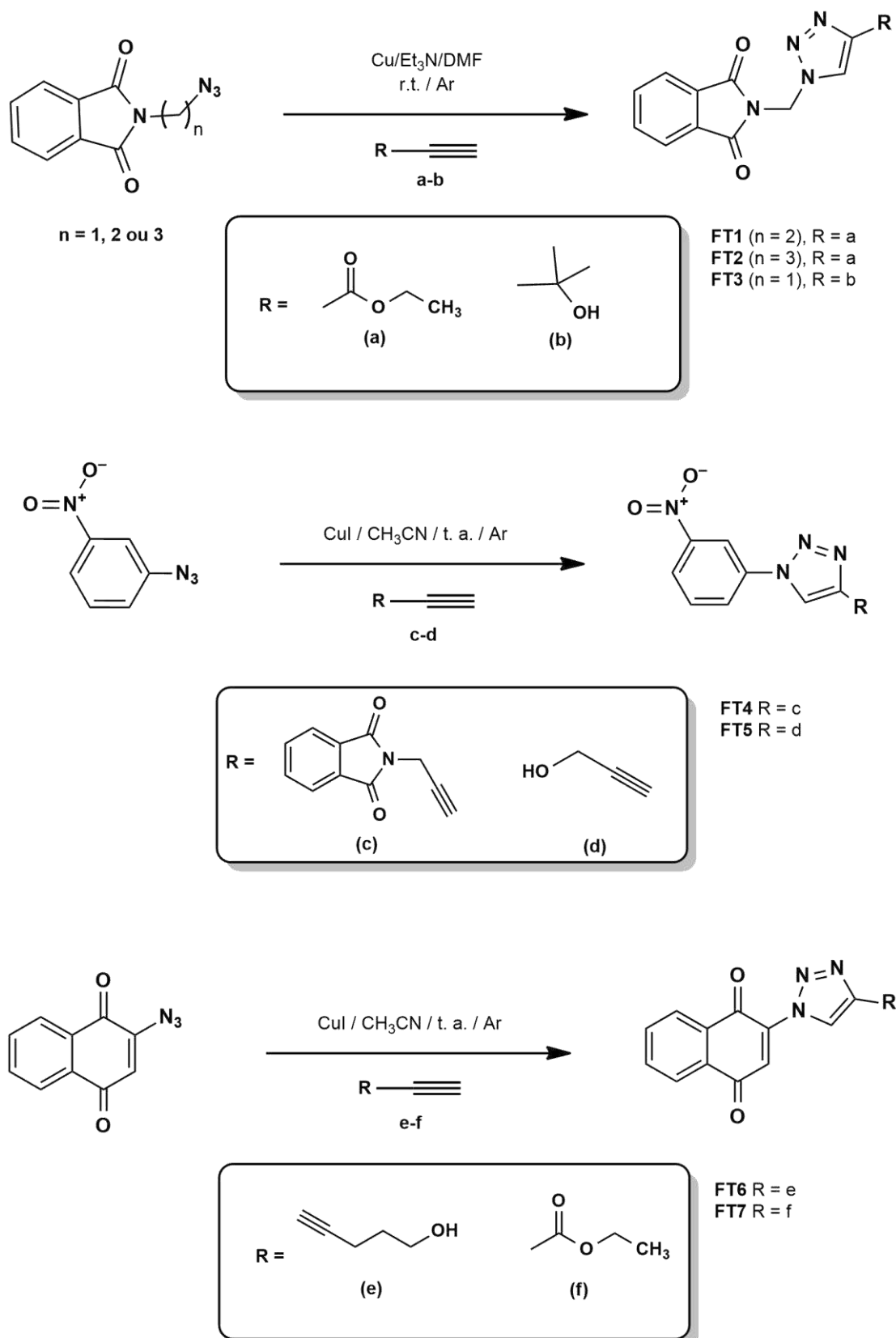
Synthesis of 1,2,3-triazoles and derivatives linked to phthalimide or naphthoquinone

Seven triazole derivatives were produced from the 1,3-dipolar cycloaddition reaction, following the protocols previously established and described by Nascimento *et al.* (2011), Silva *et al.* (2012), and Valença *et al.* (2012). Four derivatives have a phthalimide group (FT1 -FT4), one derivative only the triazole ring as the main

pharmacophoric group (FT5) and two have the naphthoquinone group (FT6 and FT7). The side chains of the compounds varied between the alcohol, ester, and nitro as functional groups. The selection of groups and structural planning of each compound followed the drug-likeness properties for drug candidate bioactive compounds (Protti *et al.*, 2021).

The infrared spectra were recorded with an IFS66 Bruker spectrophotometer using KBr disks and ^1H and ^{13}C NMR were obtained in a Varian Unity Plus 400 MHz spectrometer using $\text{DMSO-}d_6$ as solvent. Derivatives were purified by column chromatography on Merck silica gel 60 (70-230 mesh) with hexane:EtOAc system. The purity of the fractions was monitored by TLC analysis on a GF₂₅₄ plate. The 1,3-dipolar cycloaddition reactions for the formation of triazoles and synthesis of organic compounds in this work are presented in scheme 1.

Scheme 1 – 1,3-dipolar cycloaddition (Click reaction) for the synthesis of FT1-FT7 derivatives



In silico analysis of the physicochemical, pharmacokinetic, and toxicological (ADMET) profile of FT1 - FT7 derivatives

The analysis of pharmacokinetic and druglike properties of FT1-FT7 derivatives was carried out using the webserver SwissADME (Swiss Institute of Bioinformatics). The compounds were classified according to the Lipinski Rule of Five (RO5): having up to 5 hydrogen bond donors (HBD), 10 hydrogen bond acceptors (HBA), molecular weight greater than 500 g/mol, and cLogP up to five (Lipinski, 2004). The prediction of the toxicological potential of the compounds was performed using the pKCSM platforms (Pires *et al.*, 2015) and ProTOX-II (Banerjee *et al.*, 2018). The parameters of AMES toxicity, hERG inhibition potential, and lethal dose (DL₅₀) were predicted to trace the toxicological profile of the compounds.

Cytotoxicity assay in mammalian cells

The cytotoxicity assay was performed on two mammalian cell lines: macrophages J774A.1 and fibroblasts ATCC[®] PCS-201-012[™] by MTT method. Cells were obtained from a previously established culture in DMEM medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a 5% CO₂ atmosphere. Fibroblasts and macrophages were cultured in 96-well plates (5 × 10⁵ cells/well) and treated with different concentrations of FTs derivatives for 48 hours. The concentration range varied according to each molecule until the cytotoxic concentration for 50% (CC₅₀) of the culture was determined. The untreated group was considered control and represented 100% cell viability.

After incubation with the drugs, the cells were washed with sterile PBS and then incubated with a 0.5 mg/mL solution of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, USA) for 3 hours as established by Mosmann (1983). The MTT oxidation reaction was followed by observation under an optical microscope. After this step, the formazan crystals were solubilized with an acidified isopropanol solution (0.04 M HCl + absolute isopropanol) and subsequently read in a spectrophotometer (Bio-Rad[®], California, USA) at 540 nm. Analyzes were performed in quadruplicate, in two independent experiments. Absorbance data were used to calculate the cytotoxic concentration of each derivative through linear regression in SPSS (Version 18.0. IBM Corp. USA).

Evaluation of the in vitro leishmanicidal effect of FTs derivatives on promastigote forms of Leishmania amazonensis and Leishmania braziliensis

Promastigotes were cultured in Schneider's medium (Sigma-Aldrich, USA) supplemented with 10% FBS, penicillin (100 IU), and streptomycin (0.01 mg/mL). In all experiments, exponentially growing cells were used. Thus, the promastigotes of both species were incubated in the absence (control) and presence of different concentrations of the molecules in this study. The leishmanicidal effect was determined through the quantification of Adenosine triphosphate (ATP) with the Molecular ATP determination kit, Cell-Titer Glo® (Thermo Fisher, USA) following the manufacturer's determination. The effect of the compounds on growth was estimated using the IC₅₀ (drug concentration capable of inhibiting the proliferation of parasites by 50%) after 48 hours of treatment. Three independent assays were performed in triplicate. After that, the molecules were ranked according to the selectivity index (SI) obtained by the calculation: CC₅₀/IC₅₀. The two derivatives with the highest SI for both mammalian cells (macrophage and fibroblasts) simultaneously were chosen for the next stages of the investigation.

Investigation of the hemolytic potential of FTs derivatives with greater selectivity against Leishmania sp.

The investigation of the hemolytic potential was carried out in samples of red blood cells (RBC) obtained from healthy humans of different phenotypes: A, B, O, and AB. The procedures followed the methodology described by Yang et al. (2005). This study was carried out following the ethical principles adopted by Brazilian legislation (Law No. 11,974/2008) and by the National Committee for Ethics in Research with Human Beings (CEP/CONEP), being approved and registered under the CAAE number: 38726620.6.0000.5048.

RBC was diluted in a saline solution (NaCl 0.9%) and incubated for 30 minutes at 37 °C in microtubes containing different concentrations of compounds (1:1) so that the final volume resulted in 1 mL. After this step, the microtubes were centrifuged (1,500 × g for 4 min) and the supernatant was collected for analysis by UV-Vis spectrophotometry at 412 nm. For this assay, the negative control (absence of hemolysis) was considered to be the cells that were only incubated with saline solution

and the positive control (presence of hemolysis) was the samples treated with Triton X-100 (0.1%). The hemolysis rate was determined from the following calculation: %Hemolysis = $(A_s - A_n)/(A_p - A_n) \times 100$, where A_s , A_p , and A_n are the absorbances of the tested sample, positive control, and negative control, respectively. Each analysis was performed in three independent experiments in triplicate.

Evaluation of the leishmanicidal effect of FT1 and FT2 derivatives on amastigotes forms of Leishmania amazonensis AND Leishmania braziliensis

The investigation of the leishmanicidal effect of the lead compounds took place in two stages: (1) analysis of the biological activity in axenic amastigotes, that is, amastigotes outside the host cell and (2) intracellular amastigotes present inside macrophages.

Axenic amastigotes

Promastigotes of both species were cultivated and used at the stationary phase. After the 5th of cultivation, the promastigotes were transferred to the amastigote-axenic medium (MAA). For this, an MAA solution was produced according to the method proposed by Sereno *et al.* (2001). Thus, a previously prepared HBSS saline solution was supplemented with 0.5% tryptocasein, 15 mM D-glucose (Sigma-Aldrich, USA), 5 mM glutamine (Gibco®, Thermo Fisher Scientific, USA), 4 mM NaHCO₃, 0.023 mM bovine hemin (FLUKA), and 25 mM HEPES to a final pH of 5.5 and supplemented with 20% inactivated fetal bovine serum (Gibco®, Thermo Fisher Scientific, USA).

Leishmania cells (2×10^6 cells/mL) were cultivated in MAA medium and subjected to increasing temperature variations, 24 hours at 28 °C and later, 96 hours at 37 °C. The differentiation process was followed by optical microscopy. The cells were used for experimentation when the percentage of amastigote forms exceeded 80% of the cells in the culture. Thus, axenic amastigotes (1×10^6 cells/mL) were incubated in the absence and presence of different concentrations of FT1 and FT2 derivatives for 24 hours at 37 °C. After treatment, cell viability was verified by measuring ATP. Three independent trials were performed in triplicate.

Intracellular amastigotes

The next step consisted of investigating the effect of FT1 and FT2 on the *in vitro* model of infection of *L. amazonensis* and *L. braziliensis* in murine peritoneal exudate macrophages. To reduce the number of animals used in the experiment, peritoneal macrophages obtained from healthy male BALB/c mice were used only at this stage. The protocols of this study were submitted for consideration by the Committee on Ethics in the Use of Animals (CEUA) of the Aggeu Magalhães Institute/FIOCRUZ-PE, which obtained approval and authorization registered under 146/2019.

Peritoneal exudate macrophages (mPEC) were collected and cultured on glass coverslips present in 24-well culture plates (5×10^5 cells/mL), containing RPMI medium supplemented with 10% FBS. Cells were incubated at 37 °C, in a 5% CO₂ atmosphere, for 3 hours for adhesion. Then, all wells were washed and unadhered cells removed. Subsequently, the infection of macrophages with promastigote forms of *L. amazonensis* and *L. braziliensis* was carried out, using a ratio of 15 parasites to 1 macrophage, for 14 hours. After this period, the culture was washed with a culture medium and non-internalized parasites were removed.

Infected macrophages, containing intracellular amastigotes, were treated with three concentrations determined from the IC₅₀ previously established for the respective promastigote forms. Thus, the treated groups consisted of $\frac{1}{2}$, 1, and 2x IC₅₀ values, subjected to the same culture conditions for another 24 hours. In this experiment, the negative control consisted of untreated infected macrophages and the positive controls received the values corresponding to IC₅₀ of meglumine antimoniate (70 µM) and amphotericin B (10 µM). After treatment, the supernatant was collected and reserved for nitric oxide (NO) measurement.

The percentage of infected cells was determined by counting 150 macrophages randomly chosen in duplicate. The survival rate was determined by multiplying the percentage of infected macrophages by the average number of parasites per infected cell. The concentration which inhibited the growth of intracellular amastigotes (IC₅₀/ama) by 50% was determined by regression analysis as already described.

Investigation of the effect of FT1 and FT2 derivatives on the production of nitric oxide (NO) in peritoneal exudate macrophages

The supernatant from the culture of macrophages infected with the amastigote forms of *L. amazonensis* and *L. braziliensis* was used to measure NO. For this, a Griess reagent solution was prepared as described in the literature (Ugucione *et al.*, 2002), by solubilizing 1 g of sulfanilamide and 100 mg of N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 mL 2.5% H₃PO₄. The reaction was left at room temperature for 10 min protected from light. Then, absorbance was measured at 540 nm in a GloMax microplate reader (Promega, California, USA). The NO concentration was determined indirectly, through the standard curve of sodium nitrite.

Analysis of the effect of FT1 and FT2 derivatives on the ultrastructure of promastigotes of Leishmania amazonensis and Leishmania braziliensis

To investigate ultrastructural alterations, the promastigote forms of *L. amazonensis* and *L. braziliensis* were treated with concentrations corresponding to ½, 1, and 2x the IC₅₀ value for each compound, for 48 hours. After this period, cells were washed with PBS buffer and then fixed with Karnovski (2.5% glutaraldehyde + 4% paraformaldehyde, in 0.1 M sodium cacodylate buffer, pH 7.2). Afterward, post-fixation was performed for 1 hour with 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM CaCl₂, and 0.1 M cacodylate buffer. Afterward, the cells were dehydrated in crescent batteries of acetone and critically dried. Finally, the cells were metalized with gold and observed under the SEM 510 Jeol microscope.

Evaluation of the potential of mitochondrial membrane, production of oxygen reactive species, and acid compartments in promastigotes treated with FT1 and FT2 derivatives

The evaluation of the treatment effect of FT1 and FT2 derivatives on the mitochondrial physiology and acid compartments of *L. amazonensis* and *L. braziliensis* promastigotes was carried out by fluorescent labeling and analysis in a flow cytometer. Thus, the cells were previously treated as described above. Afterward, the promastigotes were washed in PBS buffer and labeled with Rhodamine 123 (10 µg/mL) for 15 minutes and MitoSox (5 µM) for 10 minutes. A 70% MeOH solution was used as a positive control for cell damage. Changes in mitochondrial membrane potential were determined by calculating the variation index: (MT-MC)/MC, in which MC corresponds to the median of the fluorescence of the negative control (no treatment) and MT the

median of the fluorescence of the treated cells. The evaluation of acidic compartments occurred through acridine orange (AO) labeling. Thus, treated and control cells were washed and incubated with a solution of 10 µg/mL of AO for 15 minutes. All markings occurred in the absence of light and at a temperature of 26 °C. After labeling, cells were washed 3 times in PBS. Data were analyzed using a FACS Calibur flow cytometer equipped with FlowJo software (BD, Biosciences, USA). A total of 20,000 events were acquired in the region previously established as corresponding to viable forms of promastigotes. The values obtained will be analyzed with the Windows Multiple Document Interface (version 2.8) software to determine the percentage of the mark average for each situation.

Molecular docking and study of chemical interactions between FT1 and FT2 derivatives on main pharmacological targets in Leishmania sp.

To investigate the inhibitory potential and the profile of chemical interactions with the main pharmacological targets in trypanosomatids, the FT1 and FT2 ligands were confronted with *Leishmania* proteins. Thus, the chemical structures of the compounds were built in Discovery Studio v.20.1.0.19 and optimized using the semi-empirical PM3 method (Stewart, 1989), implemented in Mercury 4.3.1 (Macrae *et al.*, 2008). The ligands' protonation states were calculated by the MarvinSketch 20.19.0 software assuming a physiological pH (Butcher *et al.*, 2015).

Molecular docking was performed using AutoDock 4.2. The sterol 14- α -demethylase (C14DM), trypanothione reductase (TryR), and pteridine reductase 1 (PTR1) proteins were chosen according to the stereochemical parameters provided by the Ramachandran analysis, obtained after analysis on the PROCHECK server (Laskowski *et al.*, 2006). The crystal structure of the protein was obtained from the Protein Data Bank (PDB) under the code 3L4D (Hargrove *et al.*, 2011). The validation of the protein-linker complex structures was performed using the co-crystallized linker. In this context, the redocking between the C14DM and its native ligand, fluconazole, was performed. For each ligand-protein complex, 10 fitting poses were generated using the Lamarckian genetic algorithm (López-Camacho *et al.*, 2015). The pose with the lowest binding energy was selected as the final snapping result. The target-ligand complex interactions were analyzed and rendered using Discovery Studio v.20.1.0.19295.

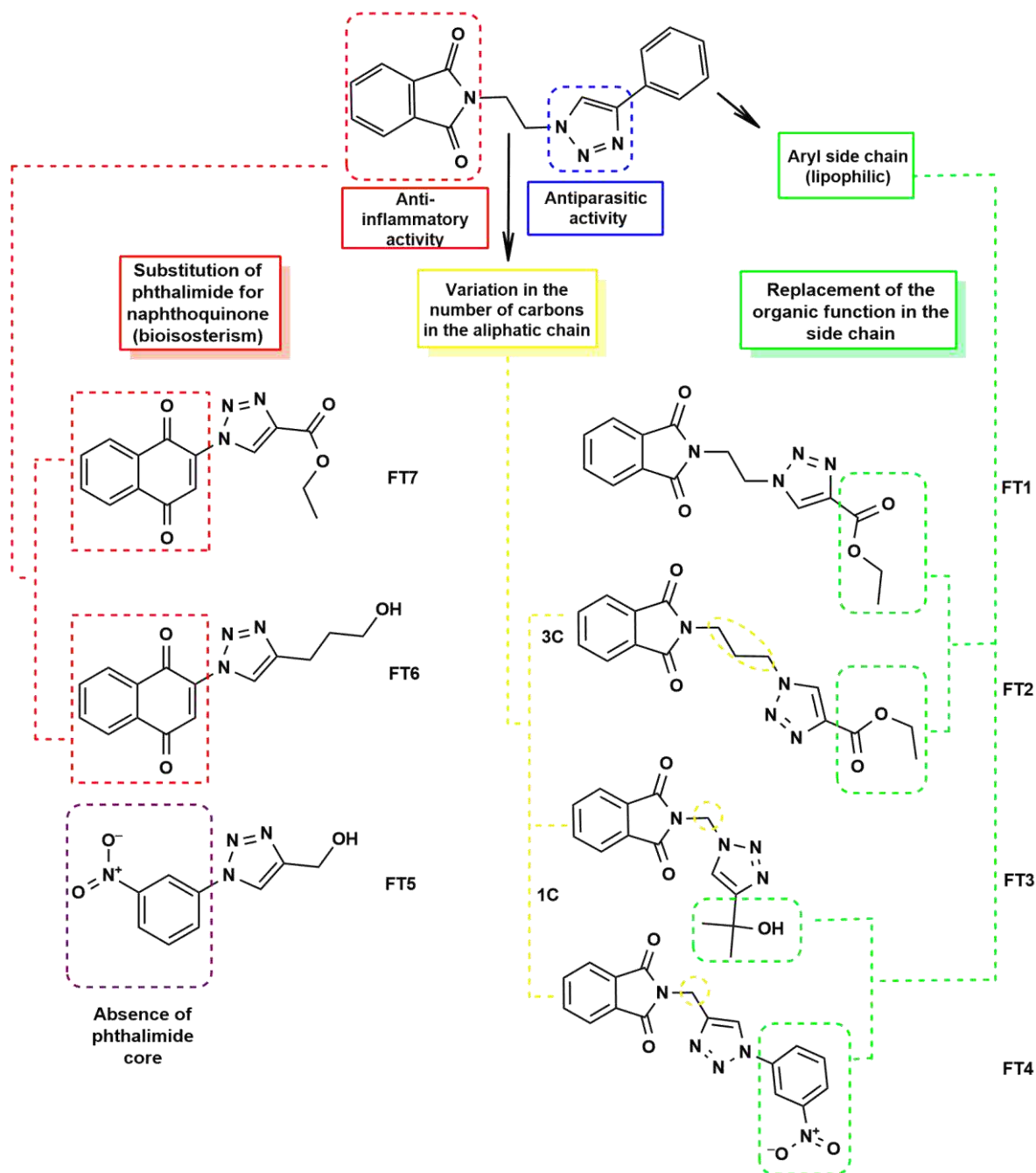
RESULTS AND DISCUSSION

Leishmaniasis is a disease caused by protozoa of the *Leishmania* genus, widely distributed around the world and affecting thousands of people, most of which are in poverty (Singh *et al.*, 2020). For almost a hundred years, pharmacological therapy for the treatment of these diseases has been used based on the use of pentavalent antimonials, drugs that cause a variety of adverse effects that can result in hepatic, renal, and cardiac involvement (Roatt *et al.*, 2020). The research and development of new drugs have pointed to a class of promising molecules in the biomedical field, the 1,2,3-triazole derivatives, easily obtained by the click chemistry reaction through fast methods and with good yields (Bonandi *et al.*, 2017). In this context, we highlight the triazole derivatives linked to phthalimide or naphthoquinone (FT1 – FT7) as candidates for anti-Leishmania pharmacological therapy.

Derivatives FT1 to FT4 have as main pharmacophoric groups phthalimide and the triazole ring, were obtained with 78, 67, 98 and 93% yield and presented the following characteristics, FT1: white solid, mp 186-188 °C, Rf 0.5 (EtOAc-hexane, 8:2); FT2: white solid, mp 124-125°C; Rf 0.5 (EtOAc-hexane, 8:2); FT3: white solid, mp 187-190°C, Rf 0.4 (EtOAc-hexane, 8:2); FT4: white solid, mp 186-188°C, Rf 0.5 (EtOAc-hexane, 8:2). Unlike the above molecules, the FT5 derivative consists only of 1,2,3-triazole as the main pharmacophoric group and has the following characteristics: white solid, mp 186-188 °C, Rf 0.5 (EtOAc-hexane, 8:2).

The compounds FT6 and FT7 (80 and 77% yield, respectively) have two main pharmacophoric groups, one being the triazole and the other the naphthoquinone ring replacing phthalimide. Derivative FT6 features yellow solid, mp 119-121 °C, Rf 0.6 (CH₂-Cl₂-EtOAc, 9:1), while derivative FT7 is a yellow solid, mp 144-150 °C, Rf 0.6 (CH₂-Cl₂-EtOAc, 9:1). ¹H and ¹³C NMR analyzes are in agreement with data previously reported by Silva *et al.* (2012), Nascimento *et al.* (2011), and Valença *et al.* (2012). The selection of compounds followed the drug-likeness criteria and was rationally delineated from the phthalimide-1,2,3-triazole derivative with previously described leishmanicidal activity, as shown in Figure 1.

Figure 1 – Chemostructural and pharmacophoric planning of potential new leishmanicidal agents formed by the phthalimide-1,2,3-triazole nucleus



After planning and production of FT1-FT7 derivatives, all compounds were submitted to *in silico* analysis of the physicochemical, pharmacokinetic, and toxicological profile (ADMET, table 1), and drug-likeness properties, important for the characterization of new drug candidates, were observed. (Jia *et al.*, 2020). None of the seven derivatives violated Lipinski's rules, which predict molar mass ≤ 500 g/mol, LogP ≤ 5 , number of hydrogen acceptors ≤ 10 , and number of hydrogen donors ≤ 5 (Lipinski, 2004). Although it was not a primary parameter in the analysis of the ADMET profile, we did not fail to notice that all compounds in this study are also within the parameters established by Veber, who highlights the need for a bioactive compound to have up to

10 rotating bonds (n-ROTB) and a value of TPSA of up to 10 Å² (Veber *et al.*, 2002). From this preliminary analysis, it is possible to consider that the FT1 to FT7 molecules have similar pharmacokinetic properties to drugs (Ursu *et al.*, 2011).

Table 1 - Analysis of the physicochemical, pharmacokinetic, and toxicological profile of FT1-FT7 derivatives.

Parameter/Drug	FT1	FT2	FT3	FT4	FT5	FT6	FT7
Lipinski Rules Violation	No	No	No	No	No	No	No
Physicochemical properties							
HBA (≤10)	6	6	5	6	5	5	6
HBD (≤5)	0	0	1	0	1	1	0
LogP (≤5)	1.13	1.49	0.74	1.41	0.32	1.20	1.39
MW (≤500) g/mol	314.30	328.32	286.83	349.30	220.18	283.23	297.27
n-ROTB (≤10)	6	7	3	4	3	4	4
TPSA (Å ²)	94.39	94.39	88.32	113.91	96.76	85.08	91.15
Absorption							
BBB	No	No	No	No	No	No	No
GIA	High	High	High	High	High	High	High
P-GP substrate	No	No	No	No	No	No	No
Metabolism							
CYP450 2C9 inhibitor	No	No	No	No	No	No	No

CYP450 2D6 inhibitor	No	No	No	No	No	No	No
CYP450 2C19 inhibitor	No	No	No	Yes	No	No	No
CYP450 3A4 inhibitor	No	No	No	No	No	No	No
CYP450 1A2 inhibitor	No	Yes	No	No	Yes	No	No
Excretion							
Renal OCT2 substrate	No	No	No	No	No	No	No
Toxicity							
AMES	No	No	No	Yes	Yes	Yes	Yes
hERG inhibitor	No	No	No	Yes	No	No	No
DL ₅₀ (mg/Kg)	2500	4000	4000	500	500	1260	1260

The *in silico* analysis of the absorption profile of the compounds revealed that none of the FTs derivatives had the potential to cross the blood-brain barrier (BBB) or act as a substrate of the P-glycoprotein (P-Gp). As expected, all molecules were predictive of high gastrointestinal absorption (GIA), an indispensable feature for drugs planned for oral administration. The negative prediction for P-Gp is particularly important since this glycoprotein acts as a limiting drug absorption from the intestine to the systemic circulation and facilitates the efflux of hepatobiliary and renal drugs, thus limiting the systemic exposure of drugs that act as its substrates (Elmeliegy *et al.*, 2020).

The drugs used for the treatment of leishmaniasis do not have the oral route as the main route of administration in the treatment and are commonly applied parenterally, which can result in pain and discomfort for users, in addition to the need

for hospitalization (Mozafari *et al.*, 2016). In this case, alternative measures such as the functionalization of nanoparticles with drugs such as Glucantime or encapsulation in liposomes, in the case of amphotericin B, can increase the cost of therapy and make access difficult for poor countries and communities, places where the disease is widespread (Borba *et al.*, 2018; Téllez *et al.*, 2020). Thus, we highlight the need to plan bioactive compounds that not only have potential against different *Leishmania* species but that have good absorption potential, especially possible to be used orally and metabolized with little or no drug interaction.

The prediction of the metabolism of FTs derivatives showed that only three of the seven molecules were shown to be potential inhibitors of the enzymes of the CYP450 family. The FT2 and FT5 compounds can inhibit the CYP450 1A2 isoenzyme and the FT4 derivative the CYP450 2C19 isoenzyme (Table 1). CYP450 1A2 corresponds to 15% of the CYP450 group, it acts on the metabolism of substances such as phenacetin, caffeine, theophylline, warfarin, verapamil, tizanidine, and its inhibition may cause an increase in the plasma concentrations of these substances (Yu *et al.*, 2017). The 2C19 isoform is related to the metabolism of drugs such as omeprazole and diazepam (Luk *et al.*, 2014; Jana *et al.*, 2018). Thus, these data suggest caution when considering the use of CYP450 inhibitors with other drugs that are widespread in clinical practice (Manikandan and Nagini, 2018). To better elucidate this potential, enzymatic inhibition and molecular docking studies may be necessary. After metabolism, we also evaluated the possibility of the derivatives acting as substrates of Organic Cation Transporter (OCT2).

OCT2 is a protein that acts on the secretion of organic cations by renal proximal tubule cells and is commonly the target of drug interactions (Song *et al.*, 2012). Approximately 40% of prescribed drugs are positively charged molecules at physiological pH, which can significantly impact the renal excretion process (Sandoval *et al.*, 2018). The *in silico* ADMET analysis showed that FT1-FT7 derivatives have a low possibility of acting as a substrate for OCT2. To confirm this data, we calculated the protonation of all compounds, considering physiological pH 7.4 (data not shown), and verified the absence of positively charged molecules. The last step of the ADMET analysis consisted of evaluating the toxicological potential.

Toxicity prediction has become a widely used tool to predict the adverse effects of small molecules (Deeb and Goodarzi, 2012). In this study, three main variables were considered: mutagenic potential (AMES), causing cardiac arrhythmia, from the

inhibition of the potassium channel linked to the ether-à-go-go-related gene (hERG), and the calculation of the lethal dose to 50 % of individuals from the same population (LD₅₀). The data showed that FT4, FT5, FT6, and FT7 derivatives were predictive for the AMES test, while only FT4 obtained a prediction of hERG toxicity.

The mutagenic potential observed for FT4-FT6 molecules may be related to the direct capacity of nitro compounds to form *N*-nitro or *C*-nitro-type carcinogens (Molognoni *et al.*, 2020) and of derivatives consisting of naphthoquinone to bind to nucleophilic compounds as RNA and DNA, intersperse the double helix DNA or by the alkylation of nucleotides (Campos *et al.*, 2012). In addition to mutagenicity, the FT4 derivative was also shown to be cardiotoxic by inhibiting hERG and resulting in an abnormal increase in the ventricular action potential (Finlayson *et al.*, 2004). The derivatives with the lowest potential for toxicity were FT1, FT2, and FT3. After analyzing the *in silico* toxicity, we carried out the investigation of the *in vitro* cytotoxic effect of FTs derivatives on J774 macrophages and fibroblast.

The macrophage is the main host cell involved in infection by *Leishmania* sp. and for this reason, it is considered an essential model for the research of new leishmanicidal drugs (Dias *et al.*, 2020). For the steps in which a lead compound had not yet been determined, we used the mouse macrophage J774A.1 instead of mPEC, to reduce the number of animals destined for experimentation. In addition to the macrophage, fibroblasts were used as a model for *in vitro* cytotoxicity analysis since, in the context of LT, these and other skin cells are extensively injured during the ulceration process and play a fundamental process in wound healing (Bogdan, 2020).

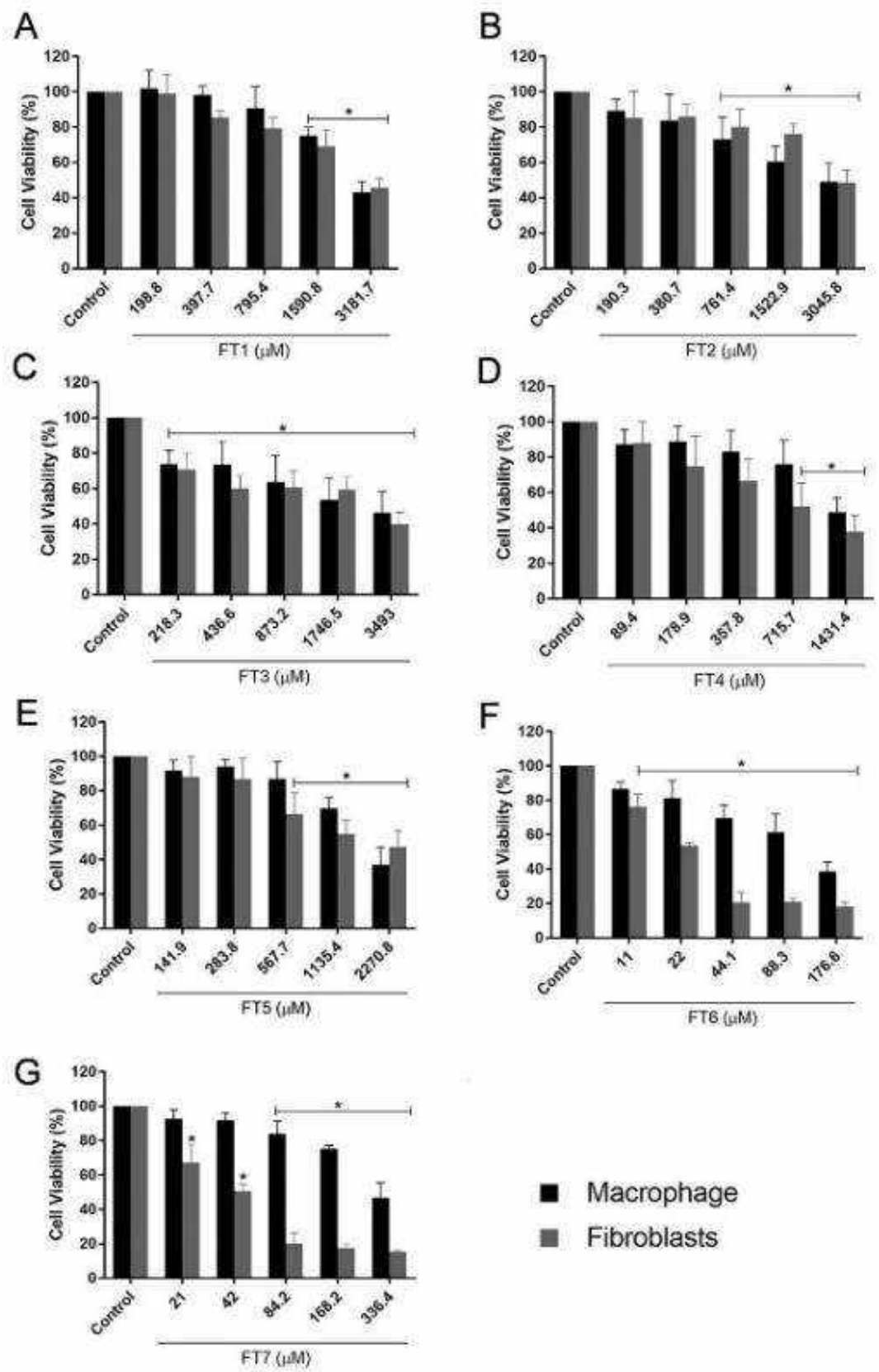
The results of the cytotoxicity assay revealed that the compounds with the greatest inhibitory effect on mammalian cells were the derivatives FT6 and FT7 (triazole-naphthoquinone), followed by the compounds FT4 and FT5 (nitro compounds). FT1, FT2 and FT3 molecules showed the lowest cytotoxic effect *in vitro* culture of J774A.1 macrophages and fibroblasts. In general, fibroblasts were more sensitive to treatment with FT4 - FT7 molecules, in which the lowest CC₅₀ values were found for FT6 (26.4 µM) and FT7 (20.86 µM) substances. On the other hand, the same pattern was not verified in the treatment with the compounds FT1 - FT3, which presented approximate and variable cytotoxic concentrations among mammalian cells.

The highest CC₅₀ values were observed for the macrophage (FT1: 2887.3 µM, FT2: 2578.4 µM and FT3: 2145.4 µM) and fibroblasts (FT1: 3030.3 µM, FT2: 2374.9 µM and FT3: 2300.1). Derivatives containing the nitro group in the side chain caused

moderate cytotoxicity when compared to the other compounds in this study. For the macrophage, the FT4 and FT5 molecules had a CC_{50} of 1396.7 and 1800.9 μM , and for the fibroblast, the values were 898.08 and 1778.2 μM , respectively.

The low cytotoxicity observed in compounds formed by triazole and phthalimide on fibroblasts and macrophages, the host cell of *Leishmania* sp. it constitutes an important property for the leishmanicidal activity (Assolini *et al.*, 2020). In both cells, the FT1 derivative was less toxic when compared to the others. The increase in the number of carbons in the aliphatic chain of the FT1 derivative (from 2 to 3 carbons) results in the FT2 derivative and seems to be a determinant for the cytotoxic effect. From 761.4 μM , the derivative with the highest aliphatic chain managed to reduce 26.9% of macrophage viability and 20% of fibroblasts, while the molecule that contains the lowest alkyl components between phthalimide and triazole needs more than twice this concentration (1590.8 μM) to cause toxic effect on said cells. The effect of TFs derivatives on the viability of mammalian cells is shown in Figure 2.

Figure 2 - Effect of treatment of FTs derivatives on J774A.1 macrophages and fibroblasts

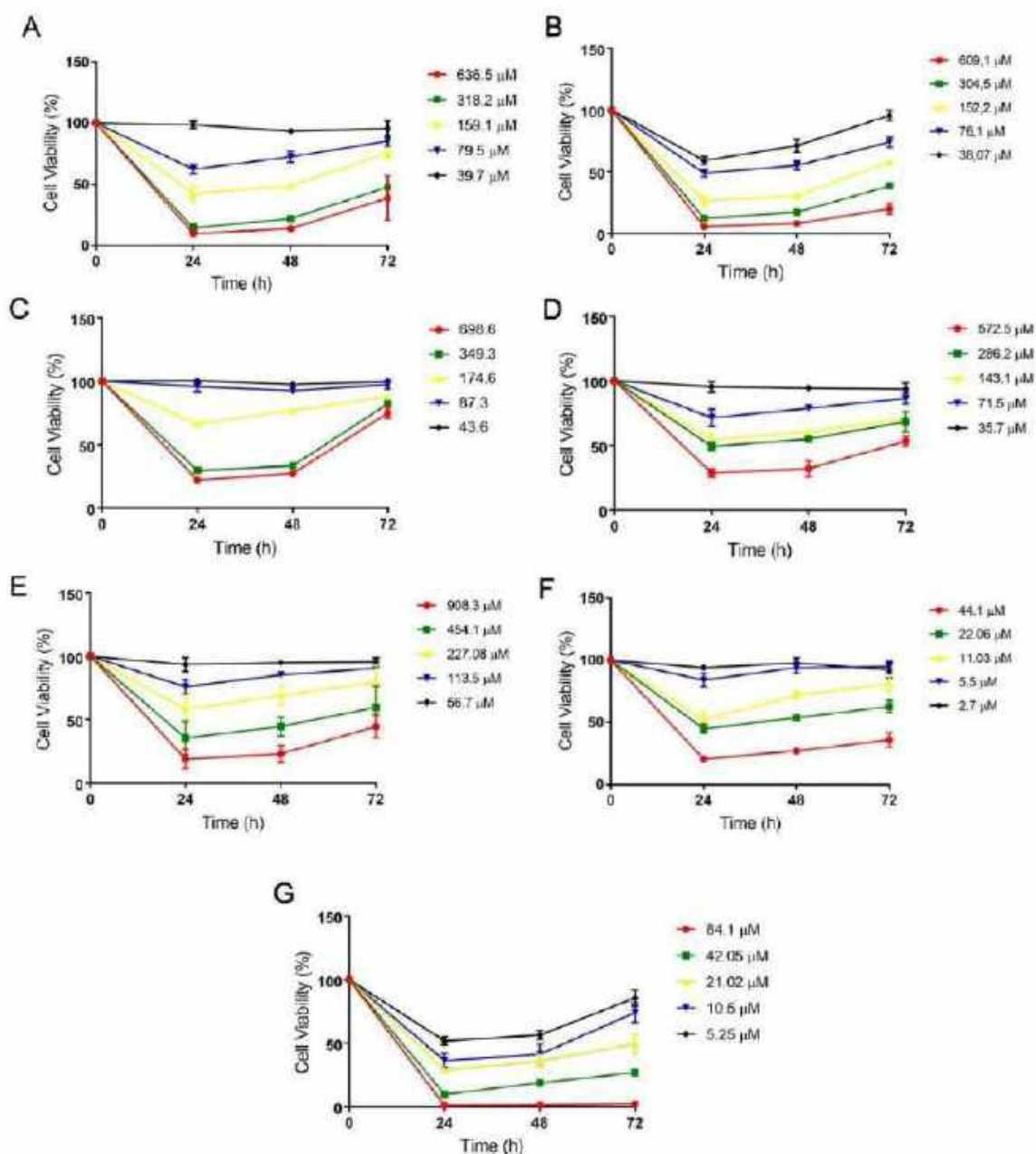


The FT3 derivative reduced the viability of both mammalian cells at all concentrations tested, reaching a maximum inhibition of 54 and 60.3% in macrophages and fibroblasts, respectively. Structurally, the FT3 compound differs from the FT1 and FT2 molecules in that it has only one carbon in the alkyl chain and the substitution of the ester side group for alcohol. By replacing the alcohol group of the FT3 derivative with a nitrophenyl, we obtain the FT4 derivative, which caused a more pronounced reduction in cell viability in both cells. The FT5 derivative also showed greater cytotoxicity when compared to compounds derived from phthalimide-1,2,3-triazole that do not have the nitro radical in the side chain. The nitro group consists of a fraction structurally associated with increased toxicity in biologically active organic molecules, which could justify the greater cytotoxic effect of the FT4 derivative when compared to FT3 (Wos *et al.*, 2017).

The triazole and naphthoquinone derivatives, FT6 and FT7, had the greatest cytotoxic effect on mammalian cells in this study. Quinones are substances widely known for their antimicrobial and also cytotoxic potential (Riaz *et al.*, 2021). The cytotoxic effect of quinone-derived molecules has been attributed to inhibition of cell division, promotion of caspase-mediated apoptosis, and reactive oxygen species (Gomes *et al.*, 2021). Results similar to those found for the compounds FT6 and FT7 were described by Guimarães *et al.* (2013) when evaluating the cytotoxic effect of naphthoquinones linked to 1,2,3-triazole.

The ester group present in side chain of FT7 derivative seemed to be determinant for a lower cytotoxic effect on macrophages when compared to the FT6 compound, which showed 2.4 times greater toxicity than its analog. In the case of fibroblasts, the structural variation between naphthoquinone-triazole derivatives did not result in decisive differences for the cytotoxic effect. It is noteworthy that the highest levels of toxicity were observed in the highest concentrations of compounds FT6 (176.6 μM) and FT7 (336.4 μM), with 81.8% and 84.6% inhibition of fibroblast culture, respectively. After tracing the cytotoxic profile of the seven triazole derivatives in this study, the next step consisted of investigating the biological effect on *Leishmania* promastigotes (Figure 3).

Figure 3 – Effect of *in vitro* treatment of FTs derivatives on *Leishmania amazonensis* promastigotes



The analysis of the biological activity of the FTs derivatives on both *Leishmania* species in this study was carried out during 24, 48, and 72 hours of *in vitro* treatment. The IC_{50} was determined during the log phase of cell development (48 hours) for *L. amazonensis* and *L. braziliensis*. All compounds were able to reduce the viability of *L. amazonensis* promastigotes for at least 48 hours of treatment. As observed for mammalian cells, molecules with naphthoquinone had the greatest cytotoxic effect for promastigotes.

The most potent effect was observed in the treatment with the compound FT7, in which *L. amazonensis* cells showed a drastic reduction in viability at concentrations from 21.02 to 81.1 μM . Only at the lowest concentrations (5.25 and 10.5 μM), promastigotes were able to re-establish the culture, up to 85.6 and 74.1%, respectively. The treatment with the highest concentration of FT6 managed to keep the viability of the *L. amazonensis* culture below 50% even after 48 hours of incubation. The FT7 compound had an IC_{50} of 8.4 μM , 3.8 times more potent than its FT6 analog, with an IC_{50} of 32.4 μM . For both molecules containing naphthoquinone, a reduction in cell viability close to 100% was observed at concentrations from 100 μM (data not shown).

Other hybrid molecules formed by 1,4-naphthoquinones bound to triazoles have been described with potent leishmanicidal effects against *Leishmania donovani* and *Leishmania major*, with IC_{50} values ranging from 0.8 to 17.1 μM (Erasmus *et al.*, 2021). The available literature suggests that the biological action of 1,4-naphthoquinones is mainly related to the naphthoquinone redox cycle, while the triazole ring contributes to the antiprotozoal effect through its wide capacity to interact with receptors and enzymes essential to these parasites (Dheer *et al.*, 2017).

The FT1, FT2, and FT3 derivatives showed anti-promastigote activity at the highest concentrations tested. Of these three molecules, the compound FT2 exhibited the greatest leishmanicidal effect on *L. amazonensis*, reaching a maximum inhibition of 80.1% at the highest concentration (609.1 μM) after 72 hours of treatment. FT1 (636.5 μM) was able to inhibit 89.9% of cells after 24 hours and this percentage reduced to 86.2% and then to 61.2% after 48 and 72 hours, respectively. The lowest inhibitory percentage was observed in cells treated with FT3, which inhibited only 77.8% of cells 24 hours and 72.7% in 48 hours (698.6 μM). The promastigotes treated with FT3 were able to re-establish the culture after 72 hours, even at the highest concentrations.

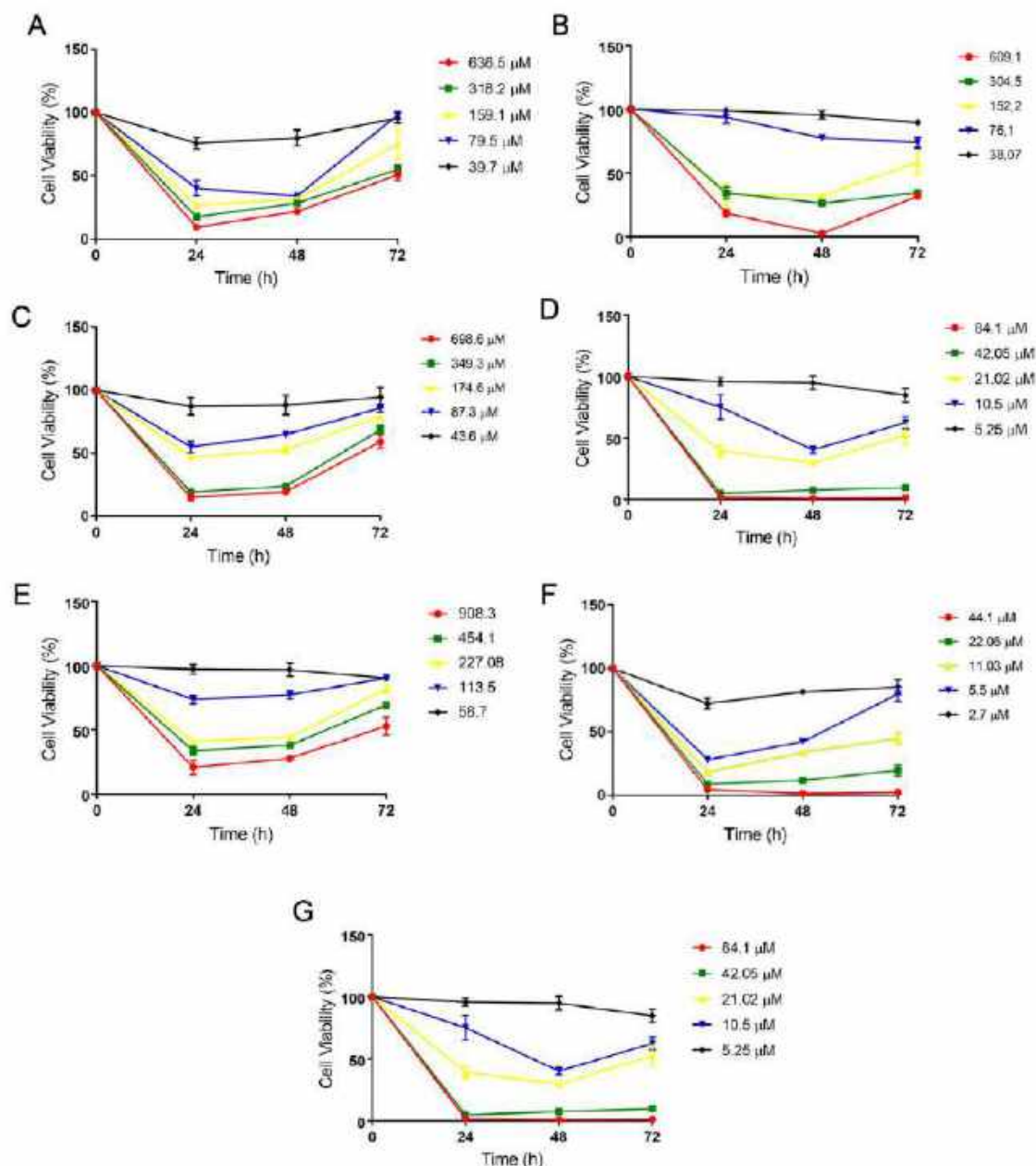
The phthalimide nucleus has been used as a pharmacophoric group in the production of antiparasitic drugs, including leishmanicidal agents (Aliança *et al.*, 2017). The IC_{50} values obtained for compounds FT1-3 on *L. amazonensis* (FT1: 205.4 μM , FT2: 85.2 μM and FT3: 360.4 μM) were remarkably higher when compared to those observed for compounds FT6-7, formed by naphthoquinones- triazole, indicating that the replacement of naphthoquinone by phthalimide significantly increases the anti-*Leishmania* effect.

The FT4 derivative (IC₅₀: 388.6 μ M) had a leishmanicidal effect similar to FT3, and this suggests that the exchange of the side chain alcohol group for nitrophenyl does not seem to determine the biological activity in *L. amazonensis*. However, when evaluating the effect of the nitrophenyl-triazole derivative without the phthalimide nucleus (FT5), we observed a higher inhibitory concentration value (IC₅₀: 547.7 μ M) and this makes us believe that phthalimide plays an important role in the biological action against promastigotes.

Considering that the cutaneous form of leishmaniasis is associated with infection by more than one species of *Leishmania* and that there is usually no specification of the species during clinical and laboratory diagnosis, we decided to evaluate the anti-promastigote potential of FTs derivatives also in *L. braziliensis* during 24, 48 and 72 hours of *in vitro* treatment (figure 4). Data observed during the log phase of cells (48 hours) were used to determine the IC₅₀.

As observed for *L. amazonensis* promastigotes, FT6 and FT7 derivatives also showed the greatest leishmanicidal effect on *L. braziliensis*, when compared to the other compounds in this study. Almost all concentrations of these substances were able to reduce *Leishmania* viability below 50% in 48 hours. Unlike what was observed for *L. amazonensis*, the compound FT6 had the lowest inhibitory concentration (IC₅₀: 5.01 μ M), followed by its analog FT7 (IC₅₀: 10.1 μ M). Thus, we note that *L. braziliensis* was more sensitive to naphthoquinone derivatives than *L. amazonensis* and this may be related to the biochemical characteristics of each species (Silva *et al.*, 2015). In both treatments, the highest concentration caused damage that led to almost 100% inhibition of promastigotes, even after 72 hours of treatment. The results obtained for compounds FT6 and FT7 are similar to the effect described by Pertino *et al.* (2020), in the hybrid molecules of naphthoquinone and 1,2,3-triazole presented IC₅₀ values between 9 and 13 μ M on epimastigote forms of *Trypanosoma cruzi*.

Figure 4 – Effect of *in vitro* treatment of FTs derivatives on *Leishmania braziliensis* promastigotes



The effect of phthalimide-1,2,3-triazole derivatives without nitrophenyl (FT1-FT3), we noticed that the effect was similar to that observed in *L. amazonensis*. Additionally, at 48 hours, FT1, FT2, and FT3 derivatives were more potent against *L. braziliensis* promastigotes, with IC_{50} of 50.4, 151.9, and 281.1 μ M, respectively. Despite this, only the cells treated with the highest concentration of the FT1 derivative (636.5 and 38.2 μ M) were able to re-establish 50% of the culture after 72 hours of

treatment, while at the highest concentrations of the FT2 derivative (609.1 and 304.5 μM), and FT3 (698.6 and 349.3 μM) the maximum percentage of viable cells after 72 hours did not exceed 42.9%.

The triazole nitro derivatives, FT4 and FT5 had the lowest inhibitory effect on *L. braziliensis* (IC_{50} 543.1 and 433.8 μM , respectively), when compared to the other compounds in this study. This effect was similar to that found for the promastigote forms of *L. amazonensis*. The FT5 compound promoted a 79.3% reduction in parasite viability in 48 hours, while its analog, FT4, inhibited 64.1% in the same treatment time. For both derivatives, *L. braziliensis* promastigotes managed to surpass 50% of the culture viability after 72 hours. Given these results, we consider that the nitro group is not a key pharmacophoric element for the leishmanicidal activity of molecules produced from phthalimide-1,2,3-triazole scaffolds. This characteristic has also been attributed to organic molecules designed for leishmanicidal action from the inhibition of trypanothione reductase (Turcano *et al.*, 2018).

The inhibitory concentration in promastigote forms of *Leishmania* sp. has been used as a parameter for the selection of promising compounds in the scope of leishmanicidal activity, however, it cannot be considered as the only parameter for the choice of antiparasitic agents (Borsari *et al.*, 2017). Thus, we determined the selectivity index (SI) of all compounds against *L. amazonensis* and *L. braziliensis* and both mammalian cells (Table 2). From the selectivity index, the most promising FTs derivatives were the FT1 and FT2 molecules, both with SI greater than 10 when considering the two mammalian cells, macrophage, and fibroblast.

Table 2 – Cytotoxicity and leishmanicidal effect on promastigote forms of *L. amazonensis* and *L. braziliensis* and cytotoxicity of FT1-FT7 derivatives.

	CC_{50} (μM)		IC_{50} (μM)		SI MØ		SI Fibroblast	
	MØ	Fibroblast	<i>La</i>	<i>Lb</i>	<i>La</i>	<i>Lb</i>	<i>La</i>	<i>Lb</i>
FT1	2887.3	3030.3	205.4	50.4	14.1	57.3	14.8	60.1
FT2	2578.4	2374.9	85.2	151.9	30.3	17.0	27.9	15.6
FT3	2145.3	2300.1	360.4	281.1	6.0	7.6	6.4	8.2
FT4	1396.7	898.08	388.6	543.1	3.6	2.6	2.3	1.7
FT5	1800.9	1778.2	547.7	433.8	3.3	4.2	3.2	4.1

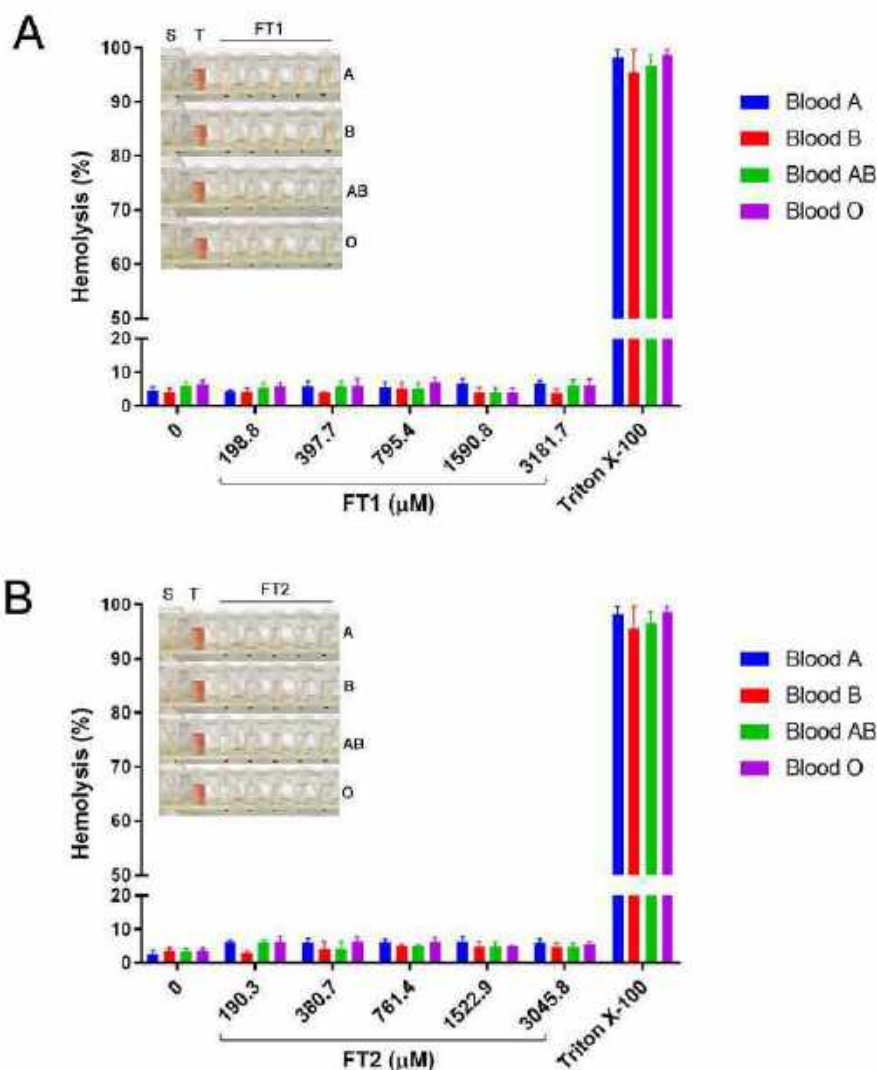
FT6	130.2	26.4	32.4	5.01	4.0	26.0	0.8	5.3
FT7	314.5	20.8	8.4	10.1	37.4	31.1	2.5	2.1

CC₅₀: cytotoxic concentration for mammalian cells; IC₅₀: inhibitory concentration for *Leishmania* sp promastigotes; SI: selectivity index; MØ: macrophage; La: *Leishmania amazonensis*; Lb: *Leishmania braziliensis*.

Even with promising results and some selectivity considering only the macrophage, the FT7 derivative was not classified as one of the hit compounds due to its low selectivity for fibroblasts (Table 1). This decision, however, does not exclude the possibility that in future studies we may produce FT7 analogs that are structurally designed to have less toxicity on these cells. Thus, the steps that followed in this study focused on the biological investigation of FT1 and FT2 compounds in the pharmacological context of leishmaniasis.

Due to the issue of cytotoxicity and side effects observed in the treatment against leishmaniasis and not only being satisfied with the *in silico* toxicity and cytotoxicity data, we decided to analyze the hemolytic potential of the leading compounds in red blood cells of different human phenotypes. Thus, red blood cells (RBC) obtained from the peripheral blood of healthy humans were incubated with different concentrations of FT1 and FT2 derivatives (Figure 4). For both molecules, no hemolytic activity was observed at any of the concentrations, when compared to the negative control (saline). Drug-induced hemolysis is a frequent problem associated with chemotherapy and this process results from the drug's interaction with the erythrocyte membrane, leading to cell lysis (Jeswani *et al.*, 2015).

Figure 5 - Evaluation of the hemolytic potential of FT1 and FT2 derivatives in human RBCs of different phenotypes.

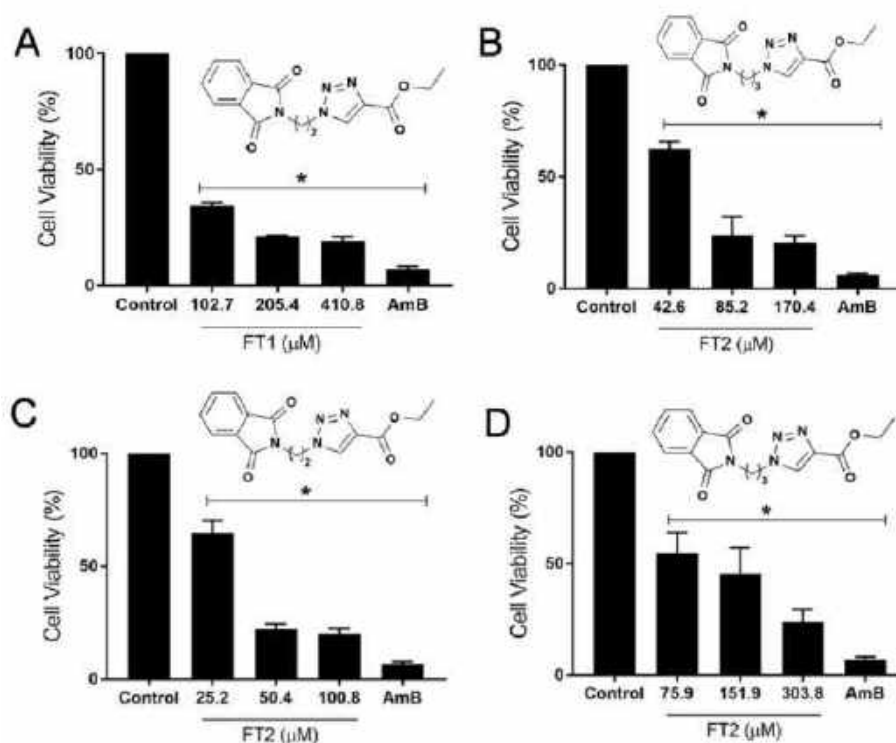


Drug-induced hemolysis can result in anemia and become a major limitation in the use of chemotherapeutic agents. Amphotericin, a second-line drug, widely used for the treatment of LT unresponsive to pentavalent antimonials, has a hemolytic activity that can cause anemia in users (Knopik-Skrocka and Bielawski, 2002). This unwanted characteristic has led pharmaceutical industries and several researchers to develop pharmacological carriers such as nanoparticles, micelles, microspheres, liposomes, etc. that reduce the deleterious effect of amphotericin on blood cells, however, this can increase the final cost of the drug and reduce acquisition by poor and developing countries (Sundar *et al.*, 2004).

After determining the selectivity of FT1 and FT2 derivatives and verifying the absence of hemolytic activity in human cells, we continued to investigate the leishmanicidal effect on amastigote forms of *Leishmania* sp. The amastigote forms are responsible for all clinical manifestations in the vertebrate host; thus vaccines and chemotherapeutic agents must be developed against this stage of the parasite's life cycle (Gupta *et al.*, 2001). The use of the axenic amastigote model has contributed to elucidate several aspects of the cell cycle of *Leishmania* sp. and this methodology has been identified as an advantageous technique for drug screening when properly applied (Zilberstein and Koren, 2019). As for the promastigote form, the viability of axenic amastigotes submitted to *in vitro* treatment with FT1 and FT2 derivatives was evaluated by quantifying the production of ATP, a method recommended due to the greater sensitivity for this assay (Dias-Lopes *et al.*, 2021).

The effect of compounds FT1 and FT2 on axenic-amastigotes is shown in figure 6. Both molecules were able to promote a significant reduction in all concentrations tested and the highest percentages of inhibition were observed in cells treated with twice the IC₅₀ value of compound FT1 for *L. amazonensis* (410.8 µM) and *L. braziliensis* (100.8 µM). The FT2 derivative promoted a similar reduction to its FT1 analog in *L. amazonensis* axenic amastigotes treated with twice the IC₅₀ value (170.4 µM). The axenic amastigotes of *L. amazonensis* were sensitive to treatment with all concentrations of the FT1 derivative, showing a reduction of 65.6% in viability, even in the treatment with half the IC₅₀ obtained in the promastigotes. As expected, the group treated with 10 µM AmB suffered a drastic decrease in the number of viable cells, with the mean percentage of inhibition obtained for *L. amazonensis* being 93.2% and for *L. braziliensis* 93.1%.

Figure 6 - Effect of FT1 and FT2 derivatives on axenic amastigote forms of *L. amazonensis* (A and B) and *L. braziliensis* (C and D).



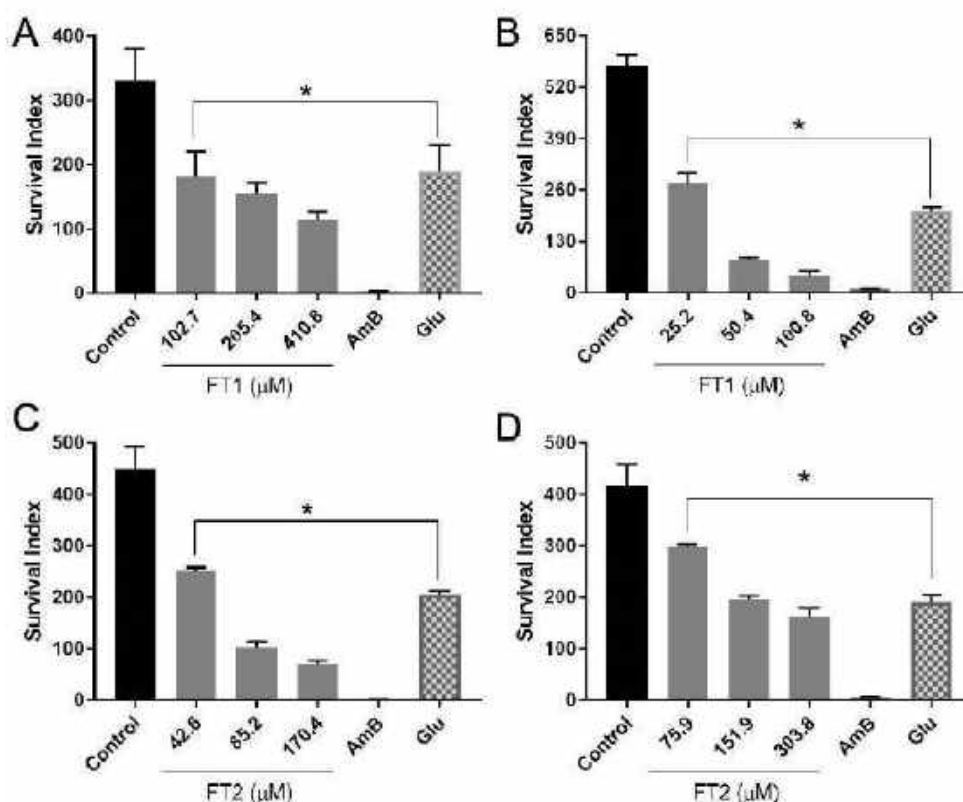
The susceptibility of *Leishmanias* of different species to drug treatment has been investigated over the years (Coelho *et al.*, 2014). In the present study, we noticed that axenic amastigotes were more sensitive to FT1 and FT2 derivatives when compared to promastigotes. This can be explained by the ability of *Leishmanias* from different species to respond differently to drug treatment and, in particular, amastigotes have shown specific susceptibility to many tested drugs (Serenio *et al.*, 2007). The FT1 derivative showed greater activity on *L. braziliensis*, while FT2 on *L. amazonensis* and, in this context, given the known biochemical and molecular differences between the species, it was not surprising that there was a variation in the sensitivity to these compounds, as also already highlighted for other drug candidates (Croft *et al.*, 2006). The difference in response to treatment to first-line drugs has also been reported in clinical studies involving patients with leishmaniasis, however, in this context, other variables such as pharmacokinetics and host immune response should also be taken into account (Coser *et al.*, 2021).

Aiming to analyze the leishmanicidal potential of FT1 and FT2 compounds in a model that approached, even more, the infection in mammals, we investigated the

biological activity of phthalimide-triazole derivatives in intracellular amastigote forms of *Leishmania* sp. For this, we chose to use macrophages obtained from the peritoneal exudate of Balb/c mice. Thus, macrophages infected with promastigotes were subjected to treatment for 24 hours, and the concentrations used were based on the IC₅₀ values previously found for each species. The reference drugs, Glucantime and amphotericin B were used as a positive control.

The results showed that both lead compounds in this study were effective in reducing the survival index (SI) of the parasites inside macrophages (Figure 7). The FT1 derivative showed greater leishmanicidal activity on *L. braziliensis* amastigotes, in which the highest concentrations (50.4 μ M and 100.8 μ M) promoted a drastic reduction in the SI of these parasites, an effect greater than that observed by Glu (Figure 7B). *L. amazonensis* amastigotes were more resistant to FT1 treatment, however, the inhibitory potential of our compound was equivalent to that observed in Glu (Figure 7A). In both tests, cells treated with AmB showed the highest rate of inhibition of survival inside macrophages, as expected.

Figure 7 – Effect of FT1 and FT2 derivatives on the intracellular amastigote forms of *Leishmania amazonensis* (A) and *Leishmania braziliensis* (B).



The FT2 derivative also showed leishmanicidal activity against the intracellular amastigote forms of *L. amazonensis* and *L. braziliensis* (Figure 7C-D). Unlike what was observed for FT1, *L. amazonensis* was more sensitive to treatment with FT2 and the higher concentrations (85.2 and 170.4 μM) resulted in a decrease in the expressive survival rate, an effect greater than that found by the reference drug, Glu (Figure 7C). All FT2 concentrations reduced the survival of *L. braziliensis* amastigotes, however, this effect was milder when compared to *L. amazonensis*. The highest concentrations of FT2 in cells infected with *L. braziliensis* (151.9 and 303.8 μM) showed a similar performance to Glu. Only amphotericin B was able to reduce almost 100% of parasite survival, regardless of species (Figure 7).

The ability to eliminate intracellular amastigotes is an important property for drug candidates for leishmanicidal drugs. For this, the compound often needs to overcome different biological barriers such as the macrophage membrane, the parasitophorous vacuole, and the amastigote itself to perform the biological activity (Aulner *et al.*, 2013).

It is possible to notice that the capacity to eliminate the parasites was similar to the assay with the axenic forms, which suggests that the leishmanicidal activity of the compounds FT1 and FT2 does not affect determinant variations by the host cell. Data obtained in ADMET analysis such as LogP (FT1: 1.13, FT2: 1.49) and TPSA (FT1 and FT2: 94.39 Å) make us believe both molecules can cross membranes and act directly on amastigotes. Additionally, the 1,2,3-triazole ring has been identified as an important pharmacophoric group, with good stability in the intracellular environment and resistance to enzymatic degradation (Dheer *et al.*, 2017).

From the assay on intracellular amastigotes, it was possible to establish the selective index for these evolutionary forms in both species, *L. amazonensis* and *L. braziliensis* (Table 3). Thus, the FT1 compound proved to be more selective against *L. braziliensis*, with selectivity indexes above 27 even when considering the two mammalian cells (macrophages and fibroblasts). The FT2 derivative also proved to be a little more selective against *L. braziliensis*, for which, regardless of the mammalian cell, the selectivity was greater than 15. Additionally, a smaller variation in the response to *in vitro* treatment with the FT1 and FT2 derivatives was found for the FT2 compound.

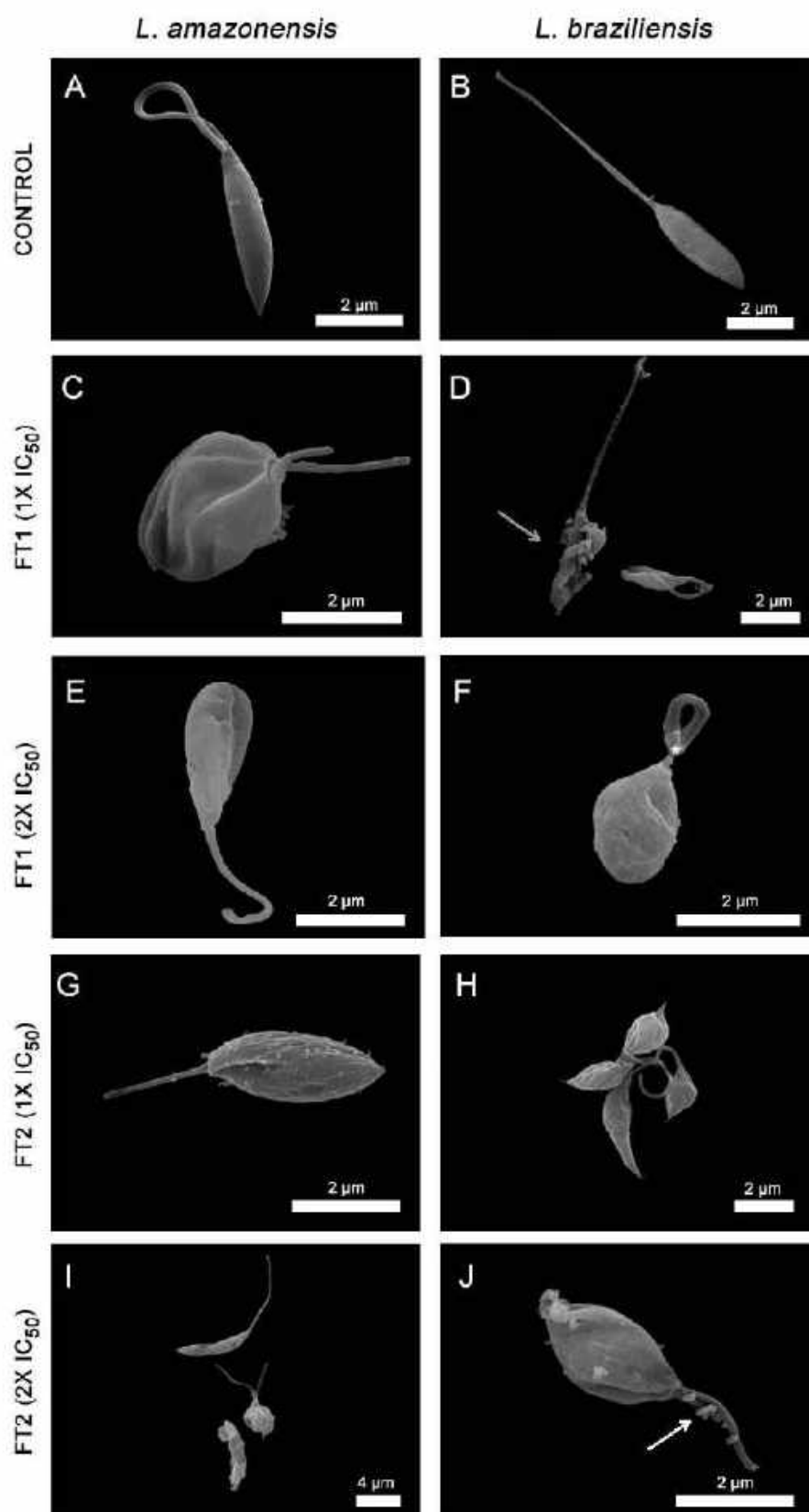
Table 3 - Leishmanicidal effect of FT1 and FT2 derivatives on intracellular amastigote forms of *L. amazonensis* and *L. braziliensis*

	<i>L. amazonensis</i>			<i>L. braziliensis</i>		
	IC ₅₀ (μM)	SI _{MØ}	SI _{Fibroblast}	IC ₅₀ (μM)	SI _{MØ}	SI _{Fibroblast}
FT1	168.65	17.12	17.96	104.06	27.74	29.12
FT2	205.40	12.55	11.56	151.98	16.96	15.62

After verifying the leishmanicidal activity of FT1 and FT2 molecules in the evolutionary forms of *L. amazonensis* and *L. braziliensis*, we decided to analyze the ultrastructure of promastigotes of both species after treatment with IC₅₀ and double this value after 48 hours of treatment. Thus, scanning electron microscopy (SEM) was performed (figure 8). The cells in the control group had the usual morphology, with the characteristic spindle shape, smooth plasma membrane, and elongated flagellum (Figures 8 A and B).

Treatment with both molecules caused drastic changes in the morphology of the promastigotes, such as reduced volume and swelling of the cell body, rupture and wrinkling of the plasma membrane, and rupture or reduction in the size of the flagellum (white asterisk, figure 8F). A promastigote with elongated flagellum but disfigured cell body can be seen after treatment with 1x IC₅₀ of compound FT1 (grey arrow, Figure 8D). In both treatments, it was possible to notice the loss of fusiform morphology characteristic of promastigotes (Figures 8C, 8E, 8F, and 8J) and this effect did not appear to be dose-dependent, as it occurred both in cells treated with the IC₅₀ value and with the double (2x IC₅₀). As with the FT1 treatment, the promastigotes treated with the FT2 compound also had the cell body disfigured, as can be seen in Figure 8I. At the highest concentration of FT2, we notice the presence of extracellular vesicles in the flagellum region of some cells (Figure 8).

Figure 8 - Ultrastructural alterations induced by FT1 and FT2 derivatives in *L. amazonensis* (left column) and *L. braziliensis* (right column) promastigotes.

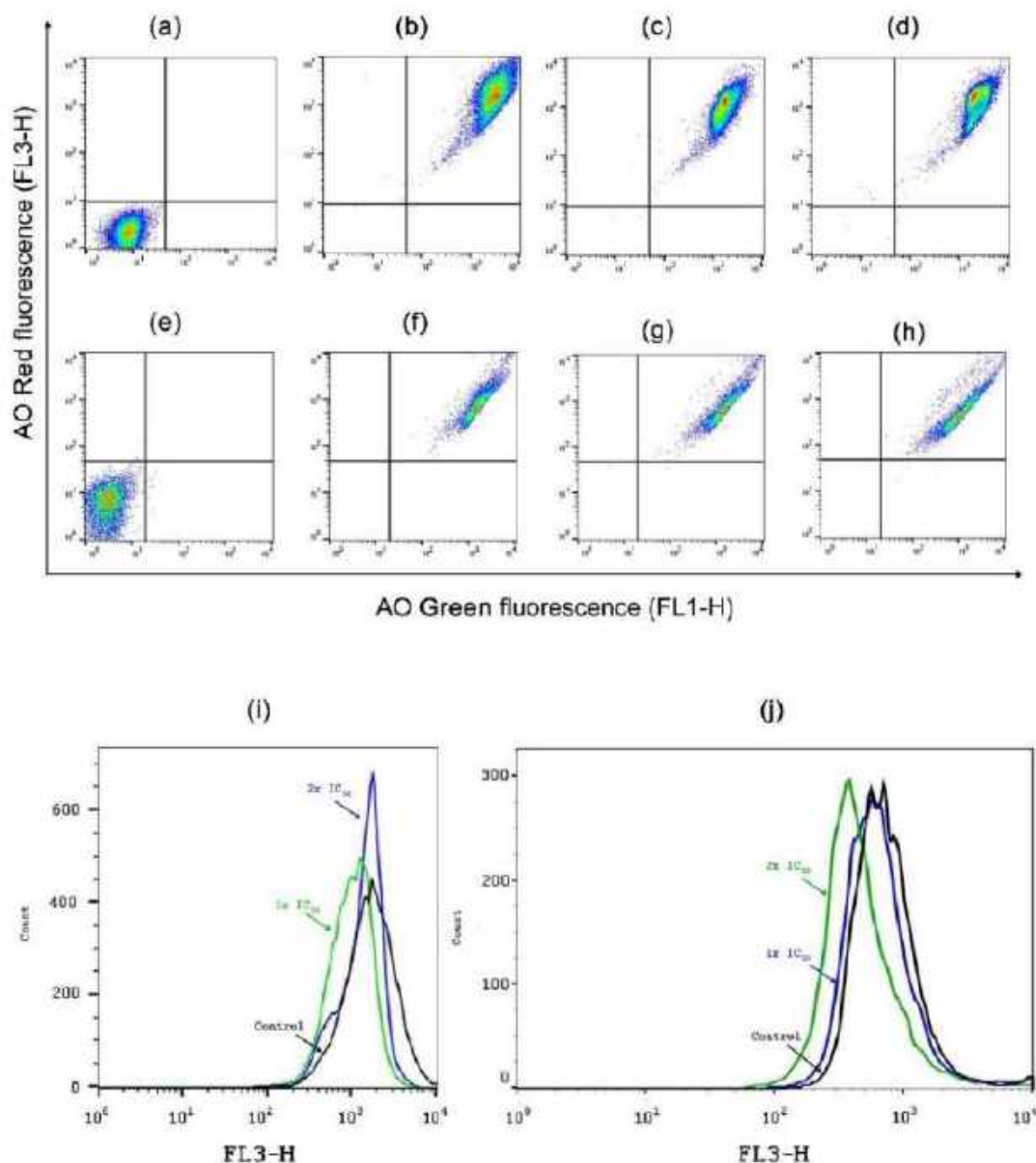


The ultrastructural changes caused by the *in vitro* treatment of FT1 and FT2 compounds may represent the occurrence of irreversible damage to *Leishmania*. Other studies have demonstrated the anti-leishmanial potential of triazole derivatives and highlighted morphological changes similar to those described in this work, in particular the presence of round cells, wrinkling of the plasma membrane, and severe disruption of the cell body (Silva *et al.*, 2018). Flagellum reduction, a characteristic observed in cells treated with the two phthalimide-1,2,3-triazole derivatives, has also been described as an important structural change caused by miltefosine, a reference drug used in the treatment of canine leishmaniasis (Scariot *et al.*, 2017).

Changes in the morphology of promastigotes suggest a high rate of intracellular damage and involvement of vital organelles for the parasite. In this context, we continued the investigation through the analysis of cell biochemistry by flow cytometry. We started the evaluation of cellular physiology from the analysis of acid compartments from acridine orange (AO) labeling. Cells were evaluated from green fluorescence, mainly by staining nuclei and cytoplasm, and in red after staining acid vesicles, which includes lysosomes and autophagolysosomes or mitophagosomes (Petit *et al.*, 2020).

L. amazonensis promastigotes treated with FT1 showed double green/red marking, with no important variations for the groups treated with the IC₅₀ value (Figure 9C) and 2x IC₅₀ (Figure 9D) when compared to the control (Figure 9A). It is important to note that the treatment with the highest concentration resulted in a greater number of labeled cells, represented by the histogram (figure 9I), and this may be related to the biochemical response to the FT1 treatment. The results observed in *L. braziliensis* were similar to those described for *L. amazonensis*, however, the higher concentration (2x IC₅₀) of FT1 resulted in a decrease in red fluorescence intensity (figure 9J). The decrease, albeit subtle, in fluorescence in AO labeling may indicate decreased green fluorescence and acidification of the cytoplasm (Sandes *et al.*, 2014).

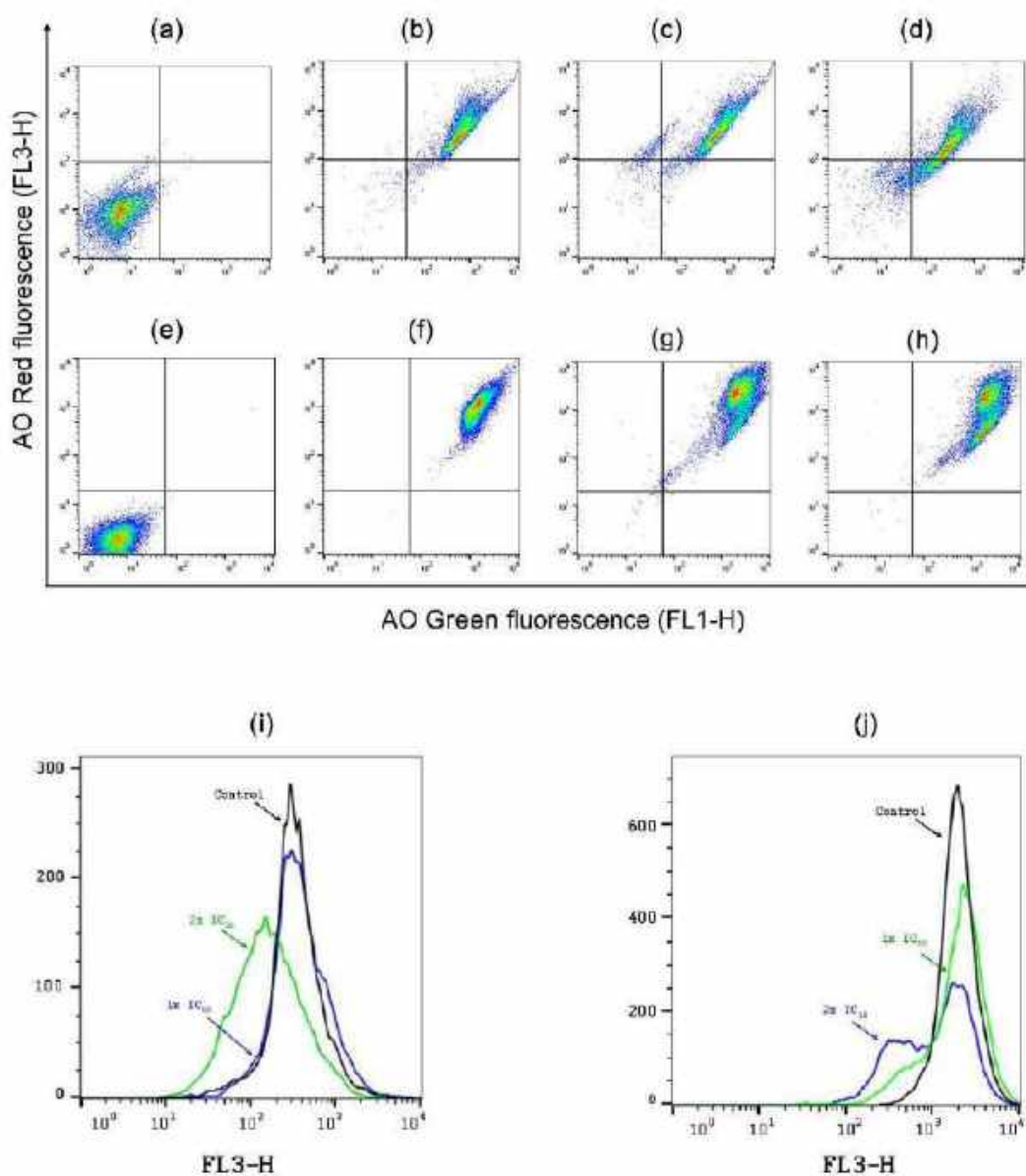
Figure 9 - Effect of the FT1 derivative on the acid compartments of *Leishmania amazonensis* (A-D, I) and *Leishmania braziliensis* (E-F, J) promastigotes. Red fluorescence histograms show highlighting for marking acidic compartments. Unlabeled cells: A and E; untreated cells: B and F; IC₅₀ treated cells: C and G; cells treated with 2x IC₅₀: D and H.



The treatment with the FT2 compound resulted in accentuated alterations in the acid compartments for both species, with prominence in *L. amazonensis*. The untreated cells showed double green/red marking, as expected (figure 10B). Treatment with IC₅₀ and 2x IC₅₀ (Fig. 10D) resulted in a decrease in double labeling and,

especially at the highest concentration, only 67.8% of cells showed usual labeling for OA. In this group, 20.9% had no acidic labeling, while 11% were negative for both fluorescent labels.

Figure 10 - Effect of the FT2 derivative on the acid compartments of *Leishmania amazonensis* (A-D, I) and *Leishmania braziliensis* (E-F, J) promastigotes. Red fluorescence histograms show highlighting for marking acidic compartments. Unlabeled cells: A and E; untreated cells: B and F; IC₅₀ treated cells: C and G; cells treated with 2x IC₅₀: D and H.

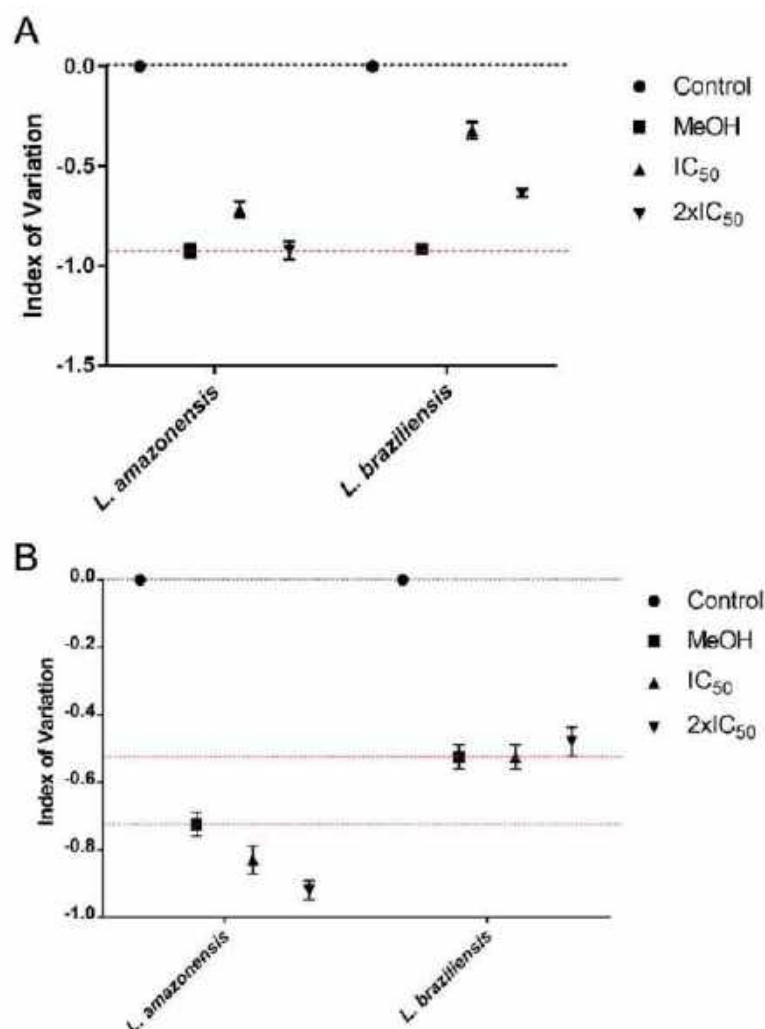


The absence of AO labeling indicates that the acidic compartments may be damaged (the acidic vesicles have become permeable or have been destroyed) and, as a consequence, it is not possible to accumulate the acidotropic dye. In this way, there are fewer AO molecules stacked up and, subsequently, a lower red fluorescence. The presence of a population of cells in the lower-left corner demonstrates that both green/red fluorescence is low, meaning that the DNA is more compacted and can no longer fix the AO molecules (Petit *et al.*, 2020). The decrease in red marking in the treated groups can be observed for *L. amazonensis* (Figure 10I) and *L. braziliensis* (Figure 10J).

The evaluation of biochemical function and mitochondrial physiology in *Leishmania* species in this study was also performed. Mitochondria are vital organelles in protozoa of the *Leishmania* genus and are commonly associated with the action of leishmanicidal drugs (Fidalgo and Gille, 2011). The analysis of the mitochondrial membrane potential ($\Delta\Psi_m$) revealed the molecules FT1 and FT2 caused depolarization of the mitochondrial membrane, observed through the negative values in the variation index (Figure 11 A-B). Both compounds showed a greater effect on *L. amazonensis*, with IV values close to or higher than those obtained for cells pre-incubated with a solution (70%) of MeOH (IV: -0.94).

The most negative IV values were obtained for *L. amazonensis* promastigotes treated with 2x IC₅₀ of FT1 (IV: -0.95) and FT2 (IV: -0.94). The change in $\Delta\Psi_m$ observed in *L. braziliensis* promastigotes was greater in cells treated with the FT2 derivative, in which we obtained IV values close to the MeOH group (IV: -0.55) for both tested concentrations, IC₅₀ (IV: -0.50) and 2x IC₅₀ (IV: -0.51). Mitochondrial damage has been related to the mechanism of potent anti-*Leishmania* agents and in this context, the evaluation of the $\Delta\Psi_m$ is particularly important because the electrochemical potential of mitochondria is the highest among intracellular organelles, and this organelle is essential for the survival of these parasites (Staderini *et al.*, 2019).

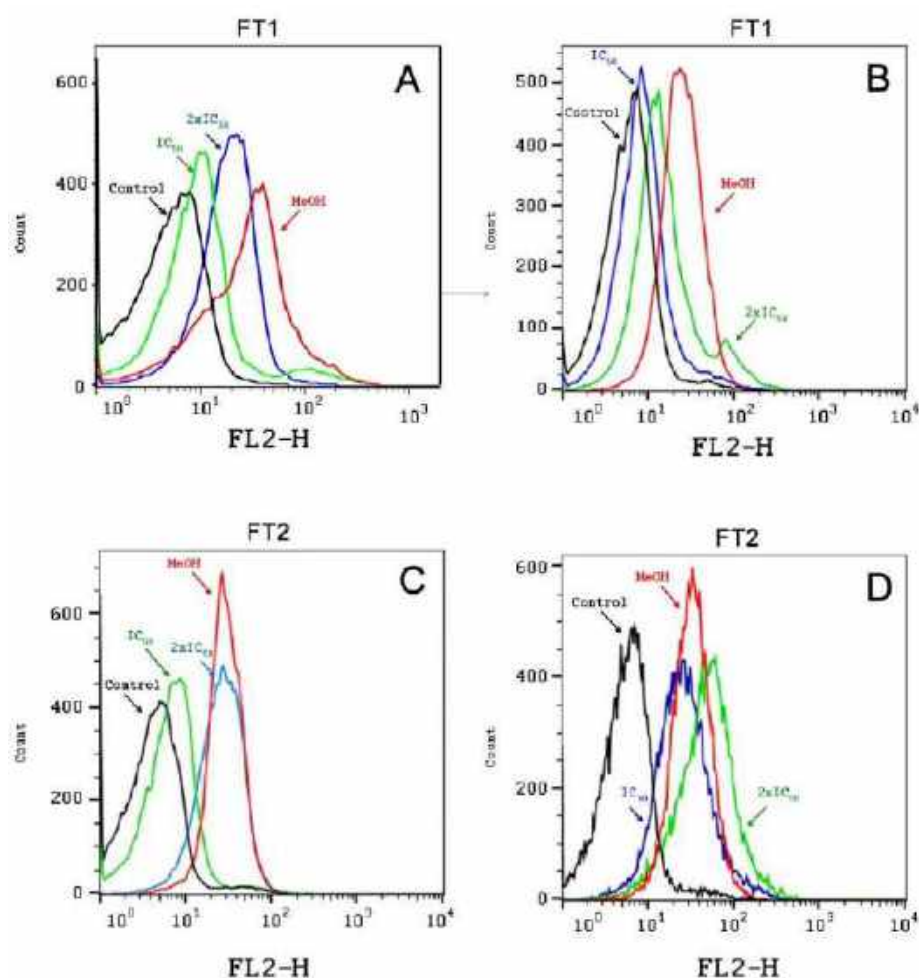
Figure 11 - Effect of FT1 and FT2 derivatives on the mitochondrial membrane potential of *Leishmania amazonensis* and *Leishmania braziliensis*. Index of Variation (IV) = $\frac{MT}{MC}$, where MT is the median of fluorescence intensity in treated cells; MC is the mean of fluorescence intensity in untreated cells.



The change at the end of the *Leishmania* species in this study, associated with a reduction in ATP levels, observed in the cell viability assay, suggests that mitochondrial damage may be determinant in the leishmanicidal activity of FT1 and FT2 compounds. Energy metabolism in these trypanosomatids is mainly based on oxidative phosphorylation reactions, which contribute most to the production of ATP (Saunders *et al.*, 2014). Thus, damage to the single mitochondria in *Leishmania* can compromise the production of energy molecules essential to cell metabolism and consequently result in death (Souza *et al.*, 2009). Continuing the investigation of the effect of phthalimide-triazole derivatives on mitochondrial biochemistry, we decided to evaluate the oxidative stress through the detection of superoxide in the mitochondria, from labeling with the MitoSOX probe.

The treatment with the FT1 and FT2 compounds resulted in an increase in mitochondrial superoxide and this effect was directly proportional to the dose used, the value of $2\times IC_{50}$ being equivalent to the oxidative stress caused by cells pre-incubated with a solution of MeOH (70%). The increase in $O_2^{\cdot-}$ was evident in both species (Figure 12). *L. amazonensis* promastigotes were more sensitive to FT1 treatment when compared to *L. braziliensis*. This same effect was not observed for cells treated with FT2, which showed similar sensitivity, regardless of species. In this case, the highest concentration ($2\times IC_{50}$) caused an increase in mitochondrial superoxide equivalent to that observed in the MeOH group. Together with the data obtained on the effect on membrane potential, we believe that the compounds FT1 and FT2 play a decisive role in the physiology of mitochondria in *L. amazonensis* and *L. braziliensis*.

Figure 12 - Effect of FT1 and FT2 derivatives on mitochondrial superoxide production in *L. amazonensis* (A-C) and *L. braziliensis* (B-D).



The increase in reactive oxygen species such as the superoxide ion ($O_2^{\bullet-}$) can be fatal in trypanosomatid protozoa due to the absence of specialized enzymes for scavenging these radicals, such as superoxide dismutase and catalase (Alzate *et al.*, 2007). During the infection in mammals, macrophages use several ways to fight *Leishmania*, of which the generation of ROS stands out as a microbicidal strategy for these defense cells (Babior *et al.*, 1973). In parasites such as *Leishmania*, the trypanothione enzyme acts as one of the main peroxidases responsible for reducing oxidative damage, despite having a catalytic activity inferior to that of superoxide dismutase. To compensate for this poor performance, trypanothione occurs in high concentrations in the intracellular environment (Krauth-Siegel *et al.*, 2003).

The depolarization of the mitochondrial membrane concomitant with the increase in reactive oxygen species has been identified as the main mechanism of action in molecules derived from triazole, in combating *Leishmania* (Meinel *et al.*, 2020). The observed results may suggest a direct or indirect action of the FT1 and FT2 derivatives on the vital structures of *Leishmania* sp., from the acid compartments to the biochemical-functional dysregulation of the mitochondria. The potential of these triazole derivatives to act as inhibitors of enzymatic targets, related to mitochondrial function and oxidative stress, has not passed our attention. Thus, we decided to compare FT1 and FT2 derivatives with three enzymes commonly related to drug action, sterol 14- α -demethylase (C14DM), trypanothione reductase (TryR), and pteridine reductase 1 (PTR1).

The C14DM enzyme consists of a protein essential for lipid metabolism in trypanosomatids protozoa, has a well-conserved structure among species, and good sensitivity to azole compounds (Lepesheva and Waterman, 2011). This target is considered particularly important because of its relationship to *Leishmania* resistance to amphotericin B (Mwenechanya *et al.*, 2017). We recently described the inhibitory potential of a phthalimide-1,2,3-triazole derivative on C14DM (Holanda *et al.*, 2020). In the present work, in addition to C14DM, we extended the analysis to TryR and PTR1 proteins.

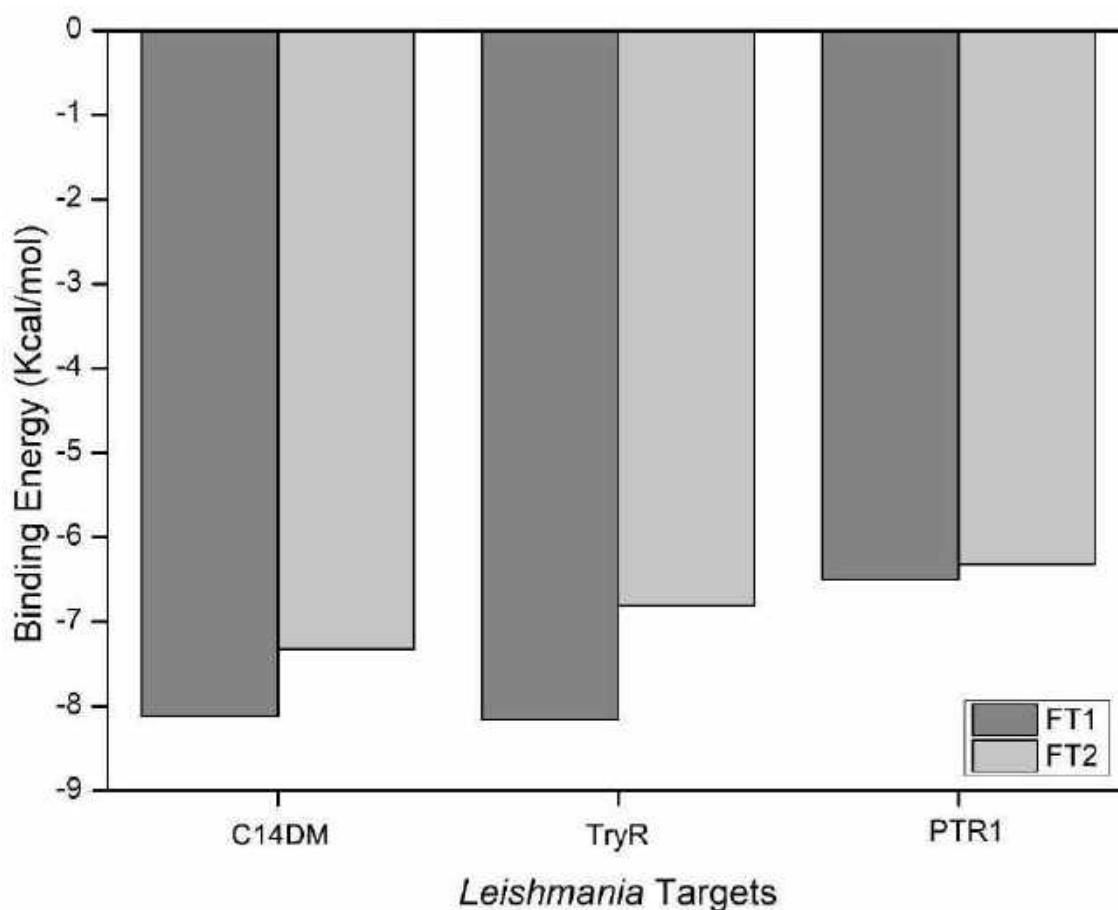
TryR is an NADPH-dependent oxidoreductase and acts in the free radical scavenging process, preventing *Leishmania* from suffering from excess free radicals in the intracellular environment (Bernardes *et al.*, 2013). The increase in the amount of ROS in cells treated with FT1 and FT2 compounds, evaluated through MitoSOX labeling, may be directly or indirectly related to TryR inhibition. Another oxidoreductase

enzyme described as a target of the action of leishmanicidal drugs is PTR1, responsible for the reduction of pteridine to biopterin and folate (Nare *et al.*, 1997). Triazole derivatives were described as potential inhibitors of this enzyme and, aiming at a better characterization of the chemical interactions with this target, we performed the molecular docking analysis.

The *Leishmania* target proteins selected for this study had the percentage of the sum of amino acid residues in the most favored and additional allowed regions is $\geq 95\%$. This result confirms that the respective structures are suitable for *in silico* analysis of molecular docking (Laskowski *et al.*, 2013). The validation of the molecular docking analyzes in this work was performed by redocking between the C14DM protein and its cocrystallized native ligand, fluconazole (PDB-ID: 34LD). The root mean square shift (RMSD) was calculated to predict the stability of the protein and the native protein-ligand complex. For this validation, we obtained an RMSD value of 1.6 Å, which indicates a good ability of the AutoDock to predict enzymatic experimental results (Huang and Zou, 2006).

The compounds FT1 and FT2 showed favorable values of binding energy (BE) for the three proteins (figure 13). The best BE score values were found for the FT1-C14DM (8.12 kcal/mol), FT2-C14DM (-7.36 kcal/mol), and FT1-TryR (-8.16 kcal/mol) complexes. Both compounds did not show good performance on the PTR1 enzyme when compared to the data obtained for C14DM and TryR. It is interesting to note that for TryR, a relatively more favorable BE value was obtained for the complex formed with the FT1 molecule than with its FT2 analog. The BE values calculated for the protein-ligand complexes in this study point to the possibility of a favorable fit, an important characteristic for enzyme inhibition (Morris and Lim-Wilby, 2008).

Figure 13 - Graphic representation of the binding energy of FT1 and FT2 compounds on molecular targets of *Leishmania* sp



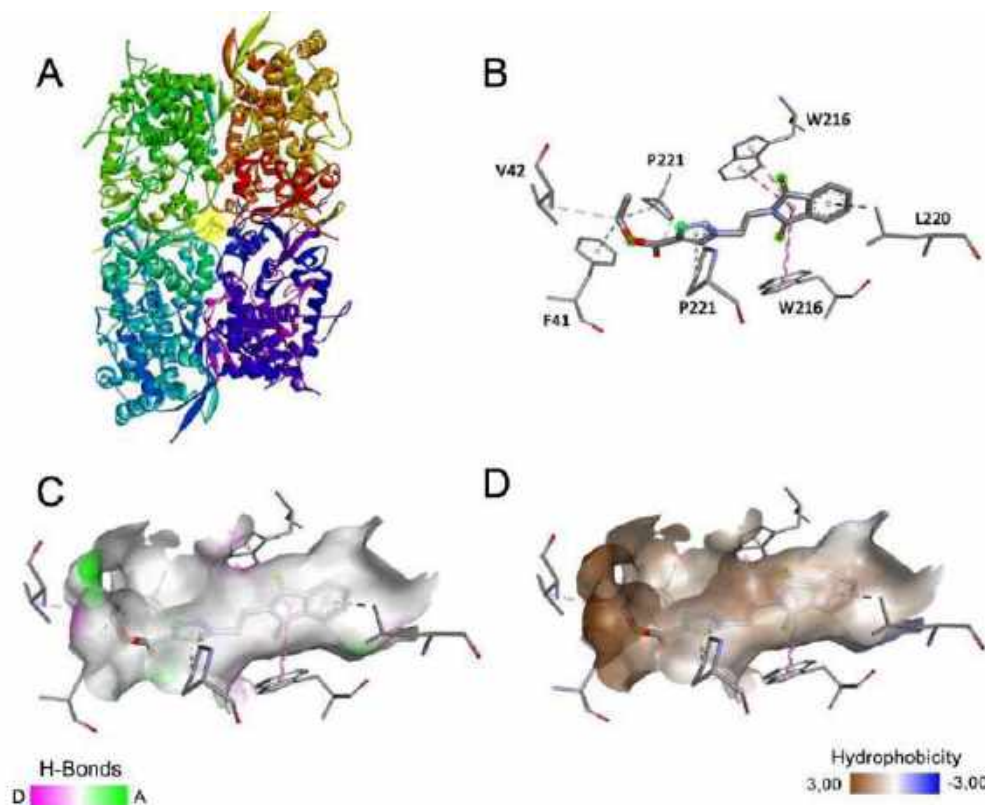
In addition to the favorable binding energy values observed for the complexes formed between FT1 and FT2 with *Leishmania* targets, we also calculated the predictive value of the enzyme inhibition constant (K_i), which represents the potential of both molecules to inhibit the activity of the respective enzymes by up to 50% (Barbosa et al., 2020). The FT1 compound had the lowest K_i values for the three proteins, C14DM (K_i : 1.13 μ M), TryR (K_i : 1.05 μ M) and PTR1 (K_i : 17.26 μ M), while the FT2 derivative had slightly higher values, C14DM (K_i : 4.02 μ M), TryR (K_i : 10.26 μ M) and PTR1 (K_i : 23.46 μ M). These data are in agreement with the affinity profile observed through BE. The binding profile between FT1 and FT2 complexes with their respective proteins is described in table 4.

Table 4 - Binding profile between FT1 and FT2 derivatives and *Leishmania* sp. Energy values are expressed in kcal/mol.

	FT1			FT2		
	C14DM	TryR	PTR1	C14DM	TryR	PTR1
Ligand efficiency	-0.35	-0.35	-0.28	-0.31	-0.28	-0.26
Inhibition constant (μM)	1.13	1.25	17.26	4.02	10.26	23.46
Intermolecular energy	-9.9	-9.95	-8.29	-9.45	-8.89	-8.4
VDW-Hb-dissolve energy	-9.9	-10.03	-8.17	-9.35	-8.87	-8.16
Electrostatic energy	0.0	-0.09	-0.11	-0.1	-0.02	-0.24
Total Internal	-0.6	-0.57	-1.81	-0.76	-1.23	-2.07
Torsional energy	1.79	1.79	1.79	2.09	2.09	2.09
Unbound energy	-0.6	-0.57	-1.81	-0.76	-1.23	-2.07

The data in table 4 demonstrate that the FT1-C14DM, FT1-TryR, FT2-C14DM, and FT2-TryR complexes show a compatible binding profile for a possible inhibitory potential of both ligands on their respective enzymatic targets. In this sense, we decided to analyze the intermolecular interactions for each case. The FT1 derivative bound at the center of C14DM, in a region of intersection between the four subunits of the enzyme and interacted with amino acids V42, F41, P221 (present in chains B and D), W126, and L220 (figure 14A-B). The phthalimide ring formed π - π stacked bonds (4.5 Å) with the indole portion present in the side chain of the tryptophan amino acids of the A and C chains. Additionally, the phthalimide group of the compound FT1 interacted with the terminal methyl of residue L220, through a π - σ bond (3.94 Å). The triazole ring formed alkyl-type bonds with the pyrrolidine ring from the P221 residue of the B chain, while the carbonyl of the ester group, in the side chain, formed a carbon-hydrogen bond with the same residue in the D chain. Other residues such as F41 and V42 also showed alkyl interactions with the side chain. The profile of hydrogen interactions and hydrophobicity of the FT1 binding site in C14DM are shown in Figure 14 C-D.

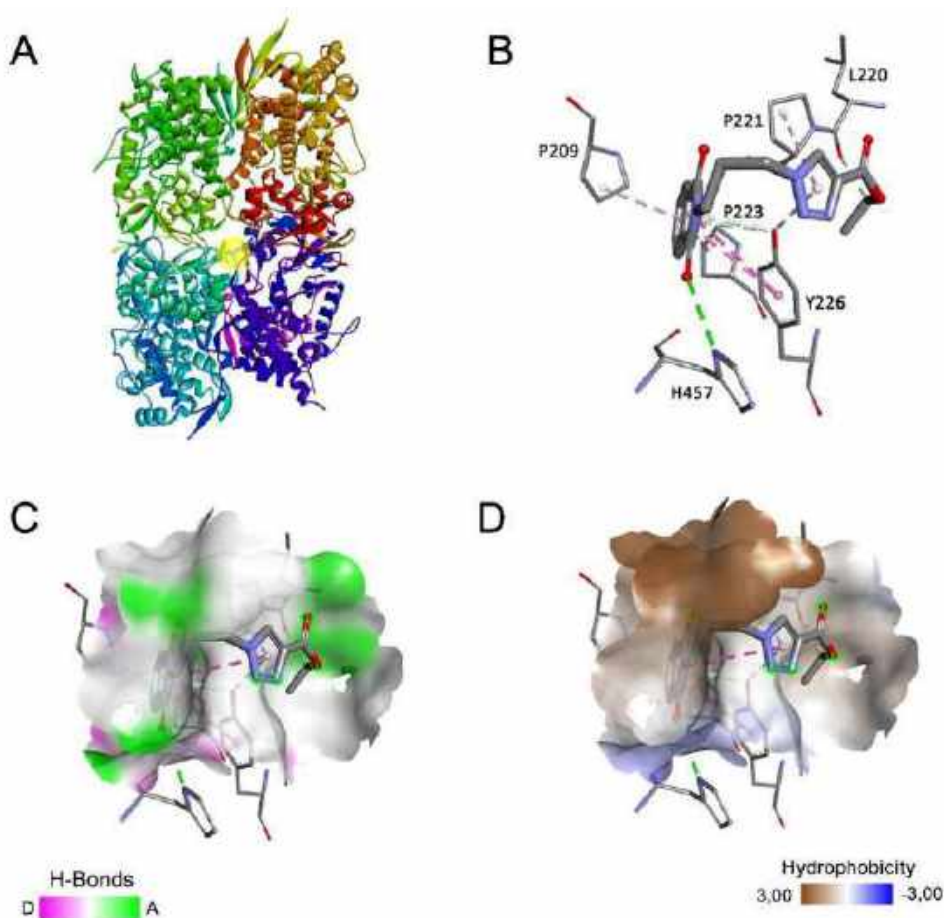
Figure 14 – The molecular docking of derivative FT1 with C14DM. (A) Anchoring region of the FT1 derivative in the C14DM. (B) Binding interaction of FT1 with amino acid residues and characterization of the binding site for hydrogen (C) and hydrophobicity (D) interactions.



The FT2 compound was bound in a region similar to that observed for its FT1 analog and also showed the same profile of intermolecular interactions (Figure 15A-B). Phthalimide group formed π -alkyl bonds with the pyrrolidine ring of two proline residues, P209 (D-chain) and P223 (C-chain) and also π - π stacked (4.90 Å)/ π -donor hydrogen bond interaction (3.20 Å), with tyrosine (Y226). This amino acid proved to be particularly important in the binding site occupied by the FT2 compound, as in addition to binding to phthalimide, the triazole nucleus also interacted, through donor hydrogen bond interaction (3.16 Å). In the side chain, a carbon-hydrogen bond interaction (3.03 Å) was observed. The presence of π - π T-shaped interaction between the phthalimide and 1,2,3-triazole groups, possibly related to the energetically more favorable conformation for the ligand at the C14DM site, did not come to our attention. The

hydrogen interactions and hydrophobicity profile of the FT2 compound binding site in C14DM are represented in figure 15 C-D.

Figure 15 – Molecular docking of derivative FT2 with C14DM. (A) Anchoring region of the FT2 derivative in the C14DM. (B) Binding interaction of FT2 with amino acid residues and characterization of the binding site for hydrogen (C) and hydrophobicity (D) interactions.

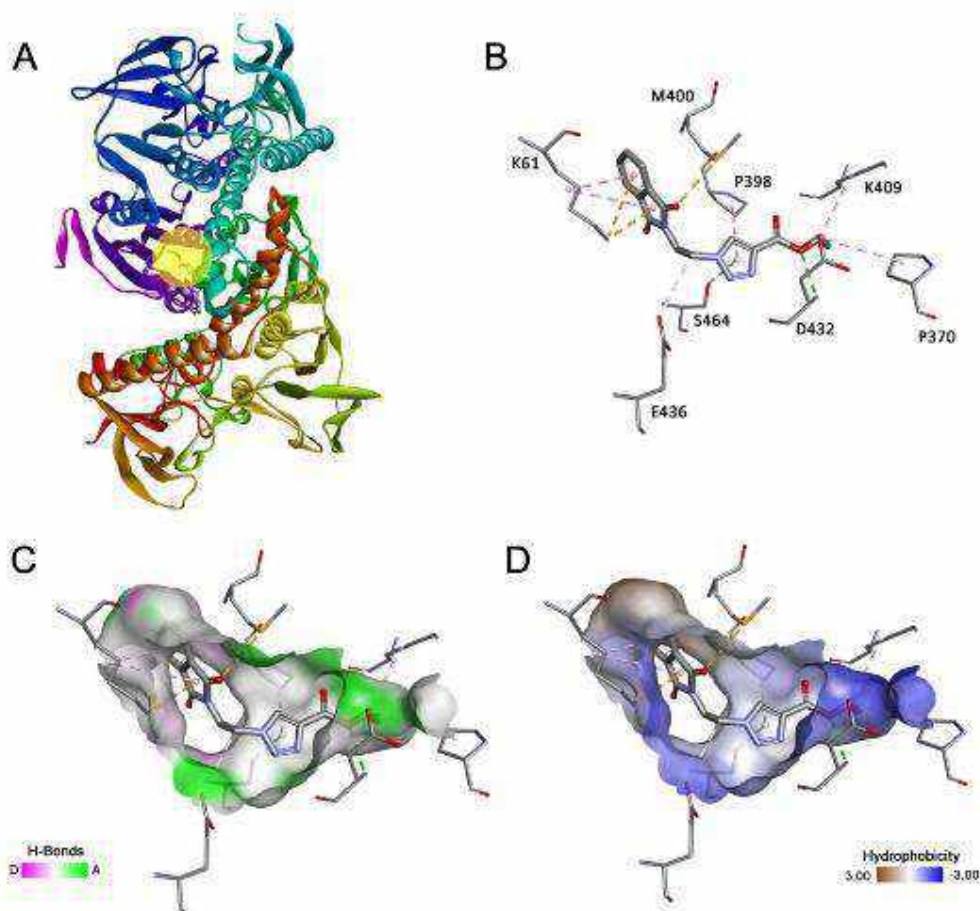


The sterol 14- α -demethylase (C14DM) is a key enzyme in sterol biosynthesis and the main target of azoles. In the *Leishmania* genus, genetic or chemical inactivation of C14DM leads to accumulation of 14-methylated sterol intermediates and plasma membrane abnormalities such as increased fluidity and failure to maintain orderly membrane microdomains (Emami *et al.*, 2017). Recent studies have pointed to a direct relationship between C14DM inhibition and the occurrence of damage to the *Leishmania* mitochondria, such as an alteration in the mitochondrial membrane

potential and an increase in reactive oxygen species (Mukherjee *et al.*, 2020). In this sense, the inhibitory potential of FT1 and FT2 derivatives on C14DM may be directly related to biochemical changes observed in flow cytometry analysis.

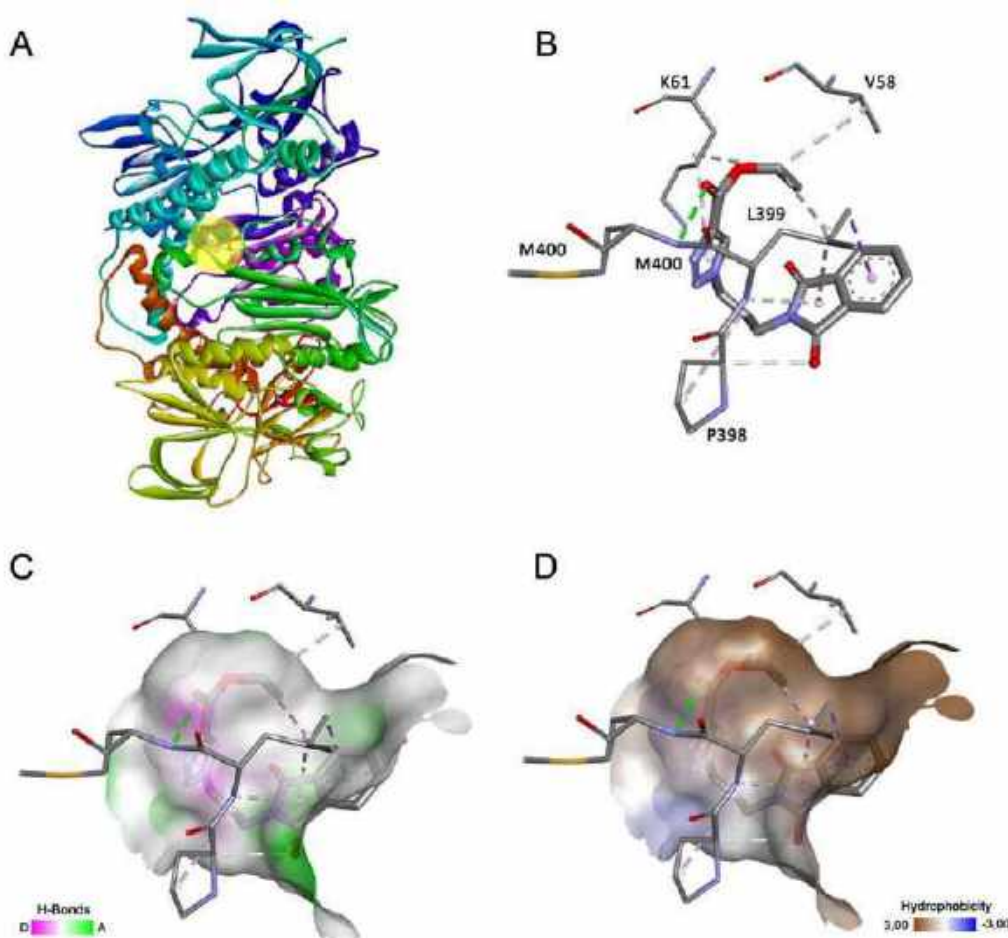
The profile of intermolecular interactions in the FT1-TryR and FT2-TryR complexes was also analyzed. The FT1 derivative interacted with a variety of TryR amino acid residues (Figure 16), in which the phthalimide nucleus formed sulfur-X bonds (3.25 Å) with M400 and π -cation interactions (3.52 Å) with K61. The triazole interacted with two residues, P398 (π -alkyl bond, 4.60 Å) and S464 (π -donor hydrogen bond interaction, 2.99 Å). The ester side chain formed a conventional hydrogen bond with D432 (2.84 Å) and alkyls interactions with K409 and P370. The profile of hydrogen interactions and the hydrophobicity of the FT1 compound binding site on TryR are depicted in Figure 16 C-D.

Figure 16 – The molecular docking of derivative FT1 with TryR. (A) Anchoring region of the FT1 derivative in the TryR. (B) Binding interaction of FT1 with amino acid residues and characterization of the binding site for hydrogen (C) and hydrophobicity (D) interactions.



The anchorage of the FT2 compound occurred at the binding site close to what was observed for FT1, including interactions with some same amino acids. The phthalimide ring of FT2 interacted L399 through a π - σ bond (3.32 Å), with the chiral center of P398, through hydrogen interactions (3.32 Å). This same residue also formed an alkyl bond with the aliphatic chain of FT2. The 1,2,3-triazole group showed π -alkyl (4.95 Å) interaction with K61, which also bound the ester side chain. Residue M400, previously described in the FT1 only anchorage site, also formed an interaction with FT2, starting from a conventional hydrogen bond (2.73 Å). The profile of hydrogen interactions and the hydrophobicity of the FT2 compound binding site on TryR are shown in Figure 17C-D.

Figure 17 – Molecular docking of derivative FT2 with TryR. (A) Anchoring region of the FT2 derivative in the TryR. (B) Binding interaction of FT2 with amino acid residues and characterization of the binding site for hydrogen (C) and hydrophobicity (D) interactions.



Trypanothione reductase, a flavoprotein oxidoreductase, unique to the thiol redox system, is involved in the NADPH-dependent reduction of trypanothione disulfide to trypanothione. This enzyme is considered an important target in the development of anti-*Leishmania* drugs (Kumar *et al.*, 2017). The inhibition constant prediction values for both FT1 and FT2 derivatives demonstrate the potential of these substances to inhibit TryR, which could occur simultaneously with the inhibition of C14DM. The increase in reactive oxygen species observed through molecular labeling with MitoSOX may be directly related to an eventual inhibition of TryR, since this protein acts in the process of neutralizing these substances, as in the case of hydrogen peroxide (Turcano *et al.*, 2018).

Studying the chemical interactions of the lead compounds in this study, FT1, and FT2, on drug target enzymes in *Leishmania* is useful to provide important data for use in further studies. Membrane changes observed in electron microscopy, as well as biochemical disorders evidenced by cytometric analysis, suggest that both compounds can act on common targets, such as C14DM and TryR, in addition to other pathways not investigated. These support the description previously made for the mechanism of action of a phthalimide-triazole derivative on *L. amazonensis* and *L. braziliensis*, which makes us believe that the redox balance and lipid synthesis may be sensitive metabolic pathways to the action of molecules hybrids of triazole and phthalimide (Holanda *et al.*, 2020).

CONCLUSION

Our results showed that triazole-bound phthalimide and naphthoquinone derivatives synthesized by click chemistry in this study have an excellent physicochemical, pharmacokinetic, and toxicological profile. The replacement of the phthalimide group by naphthoquinone considerably increased the anti-*Leishmania* and cytotoxicity effect, which led to a low selectivity index for fibroblasts. The FT1 and FT2 compounds, derived from phthalimide-triazole with an ester side chain, showed a leishmanicidal effect on the evolutionary forms of *L. amazonensis* and *L. braziliensis*, with higher selectivity indices than the other derivatives formed by the same groups. These molecules did not cause hemolysis of human red blood cells, even in treatment with the highest concentrations. The mechanism of action of both phthalimide-triazole derivatives is related to mitochondrial damage, through membrane depolarization and

increased reactive oxygen species. The increase in acidic compartments may indicate the occurrence of cellular events related to irreversible damage caused by treatment with FT1 and FT2. These events are related to *in silico* molecular anchoring data, which pointed to the potential of the leading compounds in this study to act as inhibitors of C14DM and TryR, enzymes essential for homeostasis in *Leishmania*. In this context, our results suggest the continuation of the study of the leishmanicidal effect of FT1 and FT2 molecules *in vivo* models of leishmaniasis and point to the promising potential of these compounds as therapeutic agents for oral use in the treatment of leishmaniasis, alone or in combination.

ACKNOWLEDGMENTS

This work was supported by the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE, BFT-109-2.08/18), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, PQ-400749/2019-0 and 312675/2018-6), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES code 001), Instituto Aggeu Magalhães and Inova-Fiocruz Program (VPPCB-007-FIO-18-2-85). Vanderlan Nogueira Holanda would like to thank FACEPE for the graduate scholarship (IBPG-0333-2.08/18).

REFERENCES

- Aliança, A. S., Oliveira, A. R., Feitosa, A. P. S., Ribeiro, K. R. C., de Castro, M. C. A. B., Leite, A. C. L., & Brayner, F. A. (2017). In vitro evaluation of cytotoxicity and leishmanicidal activity of phthalimido-thiazole derivatives. ***European Journal of Pharmaceutical Sciences***, 105, 1-10. DOI:10.1016/j.ejps.2017.05.005
- Alzate, J. F., Arias, A. A., Moreno-Mateos, D., Álvarez-Barrientos, A., & Jiménez-Ruiz, A. (2007). Mitochondrial superoxide mediates heat-induced apoptotic-like death in *Leishmania infantum*. ***Molecular and Biochemical Parasitology***, 152(2), 192-202. DOI: 10.1016/j.molbiopara.2007.01.006
- Assis, S. P. D. O., Silva, M. T. D., de Oliveira, R. N., & Lima, V. L. D. M. (2012). Synthesis and anti-inflammatory activity of new alkyl-substituted phthalimide 1H-1, 2,

3-triazole derivatives. **The Scientific World Journal**, 2012. DOI: 10.1100/2012/925925

Assolini, J. P., da Silva, T. P., da Silva Bortoleti, B. T., Goncalves, M. D., Tomiotto-Pellissier, F., Sahd, C. S. & Pavanelli, W. R. (2020). 4-nitrochalcone exerts leishmanicidal effect on *L. amazonensis* promastigotes and intracellular amastigotes, and the 4-nitrochalcone encapsulation in beeswax copaiba oil nanoparticles reduces macrophages cytotoxicity. **European Journal of Pharmacology**, 884, 173392. DOI: 10.1016/j.ejphar.2020.173392

Aulner, N., Danckaert, A., Rouault-Hardoin, E., Desrivot, J., Helynck, O., Commere, P. H. & Prina, E. (2013). High content analysis of primary macrophages hosting proliferating *Leishmania* amastigotes: application to anti-leishmanial drug discovery. **PLoS Neglected Tropical Diseases**, 7(4), e2154. DOI: 10.1371/journal.pntd.0002154

Babior, B. M., Kipnes, R. S., & Curnutte, J. T. (1973). Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. **The Journal of Clinical Investigation**, 52(3), 741-744.

Banerjee, P., Eckert, A. O., Schrey, A. K., & Preissner, R. (2018). ProTox-II: a webserver for the prediction of toxicity of chemicals. **Nucleic Acids Research**, 46(W1), W257-W263. DOI: 10.1093/nar/gky318

Bernardes, L. S. C., Zani, C. L., & Carvalho, I. (2013). Trypanosomatidae diseases: from the current therapy to the efficacious role of trypanothione reductase in drug discovery. **Current Medicinal Chemistry**, 20(21), 2673-2696.

Bogdan, C. (2020). Macrophages as host, effector and immunoregulatory cells in leishmaniasis: impact of tissue micro-environment and metabolism. **Cytokine: X**, 100041. DOI: 10.1016/j.cyttox.2020.100041

Bonandi, E., Christodoulou, M. S., Fumagalli, G., Perdicchia, D., Rastelli, G., & Passarella, D. (2017). The 1, 2, 3-triazole ring as a bioisostere in medicinal

chemistry. ***Drug Discovery Today***, 22(10), 1572-1581. DOI: 10.1016/j.drudis.2017.05.014

Borba, H. H. L., Steimbach, L. M., Riveros, B. S., Tonin, F. S., Ferreira, V. L., Bagatim, B. A. D. Q. & Wiens, A. (2018). Cost-effectiveness of amphotericin B formulations in the treatment of systemic fungal infections. ***Mycoses***, 61(10), 754-763. DOI: 10.1111/myc.12801

Borsari, C., Santarem, N., Torrado, J., Olías, A. I., Corral, M. J., Baptista, C. & Costi, M. P. (2017). Methoxylated 2'-hydroxychalcones as antiparasitic hit compounds. ***European Journal of Medicinal Chemistry***, 126, 1129-1135. DOI: 10.1016/j.ejmech.2016.12.017

Bozorov, K., Zhao, J., & Aisa, H. A. (2019). 1, 2, 3-Triazole-containing hybrids as leads in medicinal chemistry: A recent overview. ***Bioorganic & Medicinal Chemistry***, 27(16), 3511-3531. DOI: 10.1016/j.bmc.2019.07.005

Campos, V. R., dos Santos, E. A., Ferreira, V. F., Montenegro, R. C., de Souza, M. C., Costa-Lotufo, L. V. & Cunha, A. C. (2012). Synthesis of carbohydrate-based naphthoquinones and their substituted phenylhydrazono derivatives as anticancer agents. ***RSC Advances***, 2(30), 11438-11448. DOI: 10.1039/c2ra21514d

Coelho, A. C., Trinconi, C. T., Costa, C. H., & Uliana, S. R. (2014). In vitro and in vivo miltefosine susceptibility of a *Leishmania amazonensis* isolate from a patient with diffuse cutaneous leishmaniasis. ***PLoS Neglected Tropical Diseases***, 8(7), e2999. DOI: 10.1371/journal.pntd.0002999

Coser, E. M., Ferreira, B. A., Yamashiro-Kanashiro, E. H., Lindoso, J. A. L., & Coelho, A. C. (2021). Susceptibility to paromomycin in clinical isolates and reference strains of *Leishmania* species responsible for tegumentary leishmaniasis in Brazil. ***Acta Tropica***, 215, 105806. DOI: 10.1016/j.actatropica.2020.105806

Croft, S. L., Sundar, S., & Fairlamb, A. H. (2006). Drug resistance in leishmaniasis. *Clinical Microbiology Reviews*, 19(1), 111-126. DOI: 10.1128/CMR.19.1.111-126.2006

Barbosa, D. C., Holanda, V. N., de Assis, C. R. D., de Oliveira Farias, J. C. R., Henrique do Nascimento, P., da Silva, W. V., Lima, Navarro, D. M. A. F., Silva, M. V., V. L. M. & dos Santos Correia, M. T. (2020). Chemical composition and acetylcholinesterase inhibitory potential, in silico, of Myrciaria floribunda (H. West ex Willd.) O. Berg fruit peel essential oil. ***Industrial Crops and Products***, 151, 112372. DOI: 10.1016/j.indcrop.2020.112372

Silva, I. A., Morato, C. I., Quixabeira, V. B. L., Pereira, L. I. D. A., Dorta, M. L., de Oliveira, M. A. P. & Ribeiro-Dias, F. (2015). In vitro metacyclogenesis of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* clinical field isolates, as evaluated by morphology, complement resistance, and infectivity to human macrophages. ***BioMed Research International***, 2015. DOI: 10.1155/2015/393049

Assis, S. P. D. O., Silva, M. T. D., Silva, F. T. D., Sant'Anna, M. P., Tenório, C. D. A., Santos, C. F., Seabra, G., Lima, V. L. M & Oliveira, R. D. (2019). Design and Synthesis of Triazole-Phthalimide Hybrids with Anti-inflammatory Activity. ***Chemical & Pharmaceutical Bulletin***, 67(2), 96-105. DOI: 10.1248/cpb.c18-00607

Silva, T. A. M., Morais, M. H. F., Lopes, H. M. D. O. R., Gonçalves, S. A., Magalhães, F. D. C., Amâncio, F. F. & Carneiro, M. (2020). Prognostic factors associated with death from visceral leishmaniasis: A case-control study in Brazil. ***Transactions of The Royal Society of Tropical Medicine and Hygiene***, 114(5), 346-354. DOI: 10.1093/trstmh/traa010

Dantas-Pereira, L., Cunha-Junior, E. F., Andrade-Neto, V. V., Bower, J. F., Jardim, G. A., da Silva Júnior, E. N. & Menna-Barreto, R. F. (2021). Naphthoquinones and Derivatives for Chemotherapy: Perspectives and Limitations of their Anti-trypanosomatids Activities. ***Current Pharmaceutical Design***, 27(15), 1807-1824. DOI: 10.2174/1381612826666201109111802

Souza, W., Attias, M., & Rodrigues, J. C. (2009). Particularities of mitochondrial structure in parasitic protists (Apicomplexa and Kinetoplastida). ***The International Journal of Biochemistry & Cell Biology***, 41(10), 2069-2080. DOI: 10.1016/j.biocel.2009.04.007

Deeb, O., & Goodarzi, M. (2012). *In silico* quantitative structure toxicity relationship of chemical compounds: some case studies. ***Current Drug Safety***, 7(4), 289-297. DOI: 10.2174/157488612804096533

Dheer, D., Singh, V., & Shankar, R. (2017). Medicinal attributes of 1, 2, 3-triazoles: Current developments. ***Bioorganic Chemistry***, 71, 30-54. DOI: 10.1016/j.bioorg.2017.01.010

Dias-Lopes, G., Zabala-Peñafiel, A., de Albuquerque-Melo, B. C., Souza-Silva, F., do Canto, L. M., Cysne-Finkelstein, L., & Alves, C. R. (2021). Axenic amastigotes of *Leishmania* species as a suitable model for in vitro studies. ***Acta Tropica***, 105956. DOI: 10.1016/j.actatropica.2021.105956

Dias, C. N., de Lima Nunes, T. A., de Sousa, J. M. S., Costa, L. H., Rodrigues, R. R. L., Araújo, A. J. & da Franca Rodrigues, K. A. (2020). Methyl gallate: selective antileishmanial activity correlates with host-cell directed effects. ***Chemico-Biological Interactions***, 320, 109026. DOI: 10.1016/j.cbi.2020.109026

Elmeliegy, M., Vourvahis, M., Guo, C., & Wang, D. D. (2020). Effect of P-glycoprotein (P-gp) inducers on exposure of P-gp substrates: review of clinical drug–drug interaction studies. ***Clinical Pharmacokinetics***, 59(6), 699-714. DOI: 10.1007/s40262-020-00867-1

Emami, S., Tavangar, P., & Keighobadi, M. (2017). An overview of azoles targeting sterol 14 α -demethylase for antileishmanial therapy. *European journal of medicinal chemistry*, 135, 241-259. DOI: 10.1016/j.ejmech.2017.04.044

Erasmus, C., Aucamp, J., Smit, F. J., Seldon, R., Jordaan, A., Warner, D. F., & David, D. D. (2021). Synthesis and comparison of in vitro dual anti-infective activities of novel

naphthoquinone hybrids and atovaquone. **Bioorganic Chemistry**, 114, 105118. DOI: 10.1016/j.bioorg.2021.105118

Fidalgo, L. M., & Gille, L. (2011). Mitochondria and trypanosomatids: targets and drugs. **Pharmaceutical Research**, 28(11), 2758-2770. DOI: 10.1007/s11095-011-0586-3

Gomes, C. L., Sales, V. D. A. W., de Melo, C. G., da Silva, R. M. F., Nishimura, R. H. V., Rolim, L. A., & Neto, P. J. R. (2021). Beta-lapachone: Natural occurrence, physicochemical properties, biological activities, toxicity and synthesis. **Phytochemistry**, 186, 112713. DOI: 10.1016/j.phytochem.2021.112713

Guimarães, T. T., Maria do Carmo, F. R., Lanza, J. S., Melo, M. N., Rubens, L., Melo, I. M., Diogo, E. B. T., Ferreira, V. F., Camara, C. A., Valença, W. O., Oliveira, R. N., Frézard, F. Silva Júnior, E. N. (2013). Potent naphthoquinones against antimony-sensitive and-resistant Leishmania parasites: Synthesis of novel α -and nor- α -lapachone-based 1, 2, 3-triazoles by copper-catalyzed azide–alkyne cycloaddition. **European Journal of Medicinal Chemistry**, 63, 523-530. DOI: 10.1016/j.ejmech.2013.02.038

Gupta, N., Goyal, N., & Rastogi, A. K. (2001). In vitro cultivation and characterization of axenic amastigotes of Leishmania. **Trends in Parasitology**, 17(3), 150-153. DOI: 10.1016/S1471-4922(00)01811-0

Holanda, V. N., da Silva, W. V., do Nascimento, P. H., Silva, S. R. B., Cabral Filho, P. E., de Oliveira Assis, S. P., Oliveira, R. N., Figueiredo, R. C. B. Q. & de Menezes Lima, V. L. (2020). Antileishmanial activity of 4-phenyl-1-[2-(phthalimido-2-yl) ethyl]-1H-1, 2, 3-triazole (PT4) derivative on *Leishmania amazonensis* and *Leishmania braziliensis*: *In silico* ADMET, in vitro activity, docking and molecular dynamic simulations. **Bioorganic Chemistry**, 105, 104437. DOI: 10.1016/j.bioorg.2020.104437

Huang, S. Y., & Zou, X. (2006). An iterative knowledge-based scoring function to predict protein–ligand interactions: II. Validation of the scoring function. *Journal of Computational Chemistry*, 27(15), 1876-1882. DOI: 10.1002/jcc.20505

Lepesheva, G., & R Waterman, M. (2011). Sterol 14 α -demethylase (CYP51) as a therapeutic target for human trypanosomiasis and leishmaniasis. ***Current Topics in Medicinal Chemistry***, 11(16), 2060-2071. DOI: 10.2174/156802611796575902

Jana, K., Bandyopadhyay, T., & Ganguly, B. (2018). Stereoselective Metabolism of omeprazole by cytochrome P450 2C19 and 3A4: Mechanistic Insights from DFT Study. ***The Journal of Physical Chemistry B***, 122(22), 5765-5775. DOI: 10.1021/acs.jpcc.8b01179

Jeswani, G., Alexander, A., Saraf, S., Saraf, S., & Qureshi, A. (2015). Recent approaches for reducing hemolytic activity of chemotherapeutic agents. ***Journal of Controlled Release***, 211, 10-21. DOI: 10.1016/j.jconrel.2015.06.001

Jia, C. Y., Li, J. Y., Hao, G. F., & Yang, G. F. (2020). A drug-likeness toolbox facilitates ADMET study in drug discovery. ***Drug Discovery Today***, 25(1), 248-258. DOI: 10.1016/j.drudis.2019.10.014

Knopik-Skrocka, A. G. N. I. E. S. Z. K. A., & Bielawski, J. (2002). The mechanism of the hemolytic activity of polyene antibiotics. ***Cellular and Molecular Biology Letters***, 7(1), 31-48.

Krauth-Siegel, R. L., Meiering, S. K., & Schmidt, H. (2003). The parasite-specific trypanothione metabolism of *Trypanosoma* and *Leishmania*. *Biological Chemistry*. DOI: 10.1515/BC.2003.062

Kumar, A., Saha, B., & Singh, S. (2017). Dataset generated for dissection of mechanisms of trypanothione reductase and tryparedoxin peroxidase through dynamic network analysis and simulations in leishmaniasis. ***Data in Brief***, 15, 757-769. DOI: 10.1016/j.dib.2017.10.031

Laskowski, r. A., furnham, n., & thornton, j. M. (2013). The Ramachandran plot and protein structure validation. In ***Biomolecular Forms and Functions: A Celebration of 50 Years of the Ramachandran Map*** (pp. 62-75).

Lipinski, C. A. (2004). Lead-and drug-like compounds: the rule-of-five revolution. ***Drug Discovery Today: Technologies***, 1(4), 337-341. DOI: 10.1016/j.ddtec.2004.11.007

Luk, S., Atayee, R. S., Ma, J. D., & Best, B. M. (2014). Urinary diazepam metabolite distribution in a chronic pain population. ***Journal of Analytical Toxicology***, 38(3), 135-142. DOI: 10.1093/jat/bku001

Manikandan, P., & Nagini, S. (2018). Cytochrome P450 structure, function and clinical significance: a review. ***Current Drug Targets***, 19(1), 38-54. DOI: 10.2174/1389450118666170125144557

Meinel, R. S., das Chagas Almeida, A., Stroppa, P. H. F., Glanzmann, N., Coimbra, E. S., & da Silva, A. D. (2020). Novel functionalized 1, 2, 3-triazole derivatives exhibit antileishmanial activity, increase in total and mitochondrial-ROS and depolarization of mitochondrial membrane potential of *Leishmania amazonensis*. ***Chemico-Biological Interactions***, 315, 108850. DOI: 10.1016/j.cbi.2019.108850

Molognoni, L., Motta, G. E., Daguer, H., & Lindner, J. D. D. (2020). Microbial biotransformation of N-nitro-, C-nitro-, and C-nitrous-type mutagens by *Lactobacillus delbrueckii* subsp. *bulgaricus* in meat products. ***Food and Chemical Toxicology***, 136, 110964. DOI: /10.1016/j.fct.2019.110964

Morris, G. M., & Lim-Wilby, M. (2008). Molecular docking. In ***Molecular modeling of Proteins*** (pp. 365-382). Humana Press. DOI: 10.1007/978-1-59745-177-2_19

Mozafari, O., Shorofi, S. A., & Yousefi, S. S. (2016). First Report on Infant Acute Urticaria after Mother's Parenteral Use of Meglumine Antimoniate (Glucantime): A Case Report. ***Iranian Journal of Public Health***, 45(9), 1217.

Mukherjee, S., Moitra, S., Xu, W., Hernandez, V., & Zhang, K. (2020). Sterol 14- α -demethylase is vital for mitochondrial functions and stress tolerance in *Leishmania major*. **PLoS Pathogens**, 16(8), e1008810. DOI: 10.1371/journal.ppat.1008810

Mwenechanya, R., Kovářová, J., Dickens, N. J., Mudaliar, M., Herzyk, P., Vincent, I. M., ... & Barrett, M. P. (2017). Sterol 14 α -demethylase mutation leads to amphotericin B resistance in *Leishmania mexicana*. **PLoS Neglected Tropical Diseases**, 11(6), e0005649. DOI:10.1371/journal.pntd.0005649

Nare, B., Hardy, L. W., & Beverley, S. M. (1997). The roles of pteridine reductase 1 and dihydrofolate reductase-thymidylate synthase in pteridine metabolism in the protozoan parasite *Leishmania major*. **Journal of Biological Chemistry**, 272(21), 13883-13891. DOI: 10.1074/jbc.272.21.13883

Nascimento, W. S., Camara, C. A., & de Oliveira, R. N. (2011). Synthesis of 2-(1H-1, 2, 3-Triazol-1-yl)-1, 4-naphthoquinones from 2-Azido-1, 4-naphthoquinone and Terminal Alkynes. **Synthesis**, 2011(20), 3220-3224. DOI: 10.1055/s-0030-1260172.

Peixoto, J. F., Oliveira, A. D. S., Monteiro, P. Q., Gonçalves-Oliveira, L. F., Andrade-Neto, V. V., Ferreira, V. F. & Alves, C. R. (2021). In Silico Insights into the Mechanism of Action of Epoxy- α -Lapachone and Epoxymethyl-Lawsone in *Leishmania* spp. **Molecules**, 26(12), 3537. DOI: 10.3390/molecules26123537

Pertino, M. W., Alexander, F., Schmeda-Hirschmann, G., Vega, C., Rolón, M., Coronel, C. & Valdez, E. V. (2020). Synthesis, trypanocidal and anti-leishmania activity of new triazole-lapachol and nor-lapachol hybrids. **Bioorganic Chemistry**, 103, 104122. DOI: 10.1016/j.bioorg.2020.104122

Petit, P. X., Ardilla-Osorio, H., Penalvia, L., & Rainey, N. E. (2020). Tafazzin mutation affecting cardiolipin leads to increased mitochondrial superoxide anions and mitophagy inhibition in Barth syndrome. **Cells**, 9(10), 2333. DOI: 10.3390/cells9102333

Protti, Í. F., Rodrigues, D. R., Fonseca, S. K., Alves, R. J., de Oliveira, R. B., & Maltarollo, V. G. (2021). Do Drug-likeness Rules Apply to Oral Prodrugs? **ChemMedChem**, 16(9), 1446-1456. DOI: 10.1002/cmdc.202000805

Rani, A., Singh, G., Singh, A., Maqbool, U., Kaur, G., & Singh, J. (2020). CuAAC-ensembled 1, 2, 3-triazole-linked isosteres as pharmacophores in drug discovery. **RSC Advances**, 10(10), 5610-5635. DOI: 10.1039/C9RA09510A

Riaz, M. T., Yaqub, M., Shafiq, Z., Ashraf, A., Khalid, M., Taslimi, P. & Gulçin, İ. (2021). Synthesis, biological activity and docking calculations of bis-naphthoquinone derivatives from Lawsone. **Bioorganic Chemistry**, 114, 105069. DOI: 10.1016/j.bioorg.2021.105069

Roatt, B. M., de Oliveira Cardoso, J. M., De Brito, R. C. F., Coura-Vital, W., de Oliveira Aguiar-Soares, R. D., & Reis, A. B. (2020). Recent advances and new strategies on leishmaniasis treatment. **Applied Microbiology and Biotechnology**, 1-13. DOI: 10.1007/s00253-020-10856-w

Saldanha, M. G., Pagliari, C., Queiroz, A., Machado, P. R. L., Carvalho, L., Scott, P., Arruda, S. (2020). Tissue damage in human cutaneous leishmaniasis: correlations between inflammatory cells and molecule expression. **Frontiers in Cellular and Infection Microbiology**, 10. DOI: 10.3389/fcimb.2020.00355

Sandes, J. M., Fontes, A., Regis-da-Silva, C. G., de Castro, M. C. B., Lima-Junior, C. G., Silva, F. P. & Figueiredo, R. C. (2014). Trypanosoma cruzi cell death induced by the Morita-Baylis-Hillman adduct 3-Hydroxy-2-methylene-3-(4-nitrophenyl)propanenitrile). **PLoS One**, 9(4), e93936. DOI: 10.1371/journal.pone.0093936

Sandoval, P. J., Zorn, K. M., Clark, A. M., Ekins, S., & Wright, S. H. (2018). Assessment of substrate-dependent ligand interactions at the organic cation transporter OCT2 using six model substrates. **Molecular Pharmacology**, 94(3), 1057-1068. DOI: 10.1124/mol.117.111443

Santos, S. S., de Araújo, R. V., Giarolla, J., El Seoud, O., & Ferreira, E. I. (2020). Searching for drugs for Chagas disease, leishmaniasis and schistosomiasis: a review. *International Journal of Antimicrobial Agents*, 55(4), 105906. DOI: 10.1016/j.ijantimicag.2020.105906

Saunders, E. C., Ng, W. W., Kloehn, J., Chambers, J. M., Ng, M., & McConville, M. J. (2014). Induction of a stringent metabolic response in intracellular stages of *Leishmania mexicana* leads to increased dependence on mitochondrial metabolism. *PLoS Pathogens*, 10(1), e1003888. DOI: 10.1371/journal.ppat.1003888

Scariot, D. B., Britta, E. A., Moreira, A. L., Falzirolli, H., Silva, C. C., Ueda-Nakamura, T., ... & Nakamura, C. V. (2017). Induction of early autophagic process on *Leishmania amazonensis* by synergistic effect of miltefosine and innovative semi-synthetic thiosemicarbazone. *Frontiers in Microbiology*, 8, 255. DOI: 10.3389/fmicb.2017.00255

Sereno, D., Da Silva, A. C., Mathieu-Daude, F., & Ouaisi, A. (2007). Advances and perspectives in *Leishmania* cell-based drug-screening procedures. *Parasitology International*, 56(1), 3-7. DOI: 10.1016/j.parint.2006.09.001

Sharma, U., Kumar, P., Kumar, N., & Singh, B. (2010). Recent advances in the chemistry of phthalimide analogues and their therapeutic potential. *Mini reviews in Medicinal Chemistry*, 10(8), 678-704. DOI: doi.org/10.2174/138955710791572442

Silva, M. T. D., Oliveira, R. N. D., Valença, W. O., Barbosa, F. C., Silva, M. G. D., & Camara, C. A. (2012). Synthesis of N-substituted phthalimidoalkyl 1H-1, 2, 3-triazoles: a molecular diversity combining click chemistry and ultrasound irradiation. *Journal of the Brazilian Chemical Society*, 23, 1839-1843. DOI: 10.1590/S0103-50532012005000053

Singh, G., Pritam, M., Banerjee, M., Singh, A. K., & Singh, S. P. (2020). Designing of precise vaccine construct against visceral leishmaniasis through predicted epitope ensemble: a contemporary approach. *Computational Biology and Chemistry*, 86, 107259. DOI: 10.1016/j.compbiolchem.2020.107259

Song, I. S., Lee, D. Y., Shin, M. H., Kim, H., Ahn, Y. G., Park, I., ... & Liu, K. H. (2012). Pharmacogenetics meets metabolomics: discovery of tryptophan as a new endogenous OCT2 substrate related to metformin disposition. ***PloS one***, 7(5), e36637. DOI: 10.1371/journal.pone.0036637

Staderini, M., Piquero, M., Abengózar, M. Á., Nacher-Vazquez, M., Romanelli, G., López-Alvarado, P. & Menéndez, J. C. (2019). Structure-activity relationships and mechanistic studies of novel mitochondria-targeted, leishmanicidal derivatives of the 4-aminostyrylquinoline scaffold. ***European Journal of Medicinal Chemistry***, 171, 38-53. DOI: 10.1016/j.ejmech.2019.03.007

Sundar, S., Mehta, H., Suresh, A. V., Singh, S. P., Madhukar, R., & Murray, H. W. (2004). Amphotericin B treatment for Indian visceral leishmaniasis: conventional versus lipid formulations. *Clinical infectious diseases*, 38(3), 377-383. DOI: doi.org/10.1086/380971

Teixeira, S., Visbal, G., Lima Prado Godinho, J., Urbina, J. A., de Souza, W., & Cola Fernandes Rodrigues, J. (2018). In vitro antileishmanial activity of ravuconazole, a triazole antifungal drug, as a potential treatment for leishmaniasis. ***Journal of Antimicrobial Chemotherapy***, 73(9), 2360-2373. DOI: doi.org/10.1093/jac/dky229

Tellez, J., Echeverry, M. C., Romero, I., Guatibonza, A., Santos Ramos, G., Borges De Oliveira, A. C. & Demicheli, C. (2021). Use of liposomal nanoformulations in antileishmania therapy: Challenges and perspectives. ***Journal of Liposome Research***, 31(2), 169-176. DOI: 10.1080/08982104.2020.1749067

Turcano, L., Torrente, E., Missineo, A., Andreini, M., Gramiccia, M., Di Muccio, T., ... & Ilari, A. (2018). Identification and binding mode of a novel Leishmania Trypanothione reductase inhibitor from high throughput screening. ***PLoS Neglected Tropical Diseases***, 12(11), e0006969. DOI: 10.1371/journal.pntd.0006969

Ursu, O., Rayan, A., Goldblum, A., & Oprea, T. I. (2011). Understanding drug-likeness. *Wiley Interdisciplinary Reviews: Computational Molecular Science*, 1(5), 760-781. DOI: 10.1002/wcms.52

Valença, W. O., de Freitas, J. J. R., & Oliveira, R. N. D. (2012). Synthesis of aryl-1H-1, 2, 3-triazoles via 1, 3-dipolar cycloaddition. *Orbital: The Electronic Journal of Chemistry*, 4(1), 31-32.

Veber, D. F., Johnson, S. R., Cheng, H. Y., Smith, B. R., Ward, K. W., & Kopple, K. D. (2002). Molecular properties that influence the oral bioavailability of drug candidates. *Journal of Medicinal Chemistry*, 45(12), 2615-2623. DOI: 10.1021/jm020017n

Wos, M., Miazga-Karska, M., Kaczor, A. A., Klimek, K., Karczmarzyk, Z., Kowalczyk, D., Pitucha, M. (2017). Novel thiosemicarbazide derivatives with 4-nitrophenyl group as multi-target drugs: α -glucosidase inhibitors with antibacterial and antiproliferative activity. *Biomedicine & Pharmacotherapy*, 93, 1269-1276. DOI: 10.1016/j.biopha.2017.07.049

Yu, L., Shi, X., Tian, S., Gao, S., & Li, L. (2017). Classification of cytochrome P450 1A2 Inhibitors and noninhibitors based on deep belief network. *International Journal of Computational Intelligence and Applications*, 16(01), 1750002. DOI: 10.1142/S146902681750002X

Zilberstein, D., & Koren, R. N. (2019). Host-free systems for differentiation of axenic Leishmania. In *Leishmania* (pp. 1-8). Humana Press, New York, NY. DOI: 10.1007/978-1-4939-9210-2

5.2 ARTIGO 2

O artigo “Antileishmanial activity of 4-phenyl-1-[2-(phthalimido-2-yl)ethyl]-1h-1,2,3-triazole (pt4) derivative on leishmania amazonensis and leishmania braziliensis: in silico admet, in vitro activity, docking and molecular dynamic simulations” foi publicado no periódico Bioorganic Chemistry, 105 (2020), link para acesso: <https://doi.org/10.1016/j.bioorg.2020.104437>

Bioorganic Chemistry 105 (2020) 104437

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Antileishmanial activity of 4-phenyl-1-[2-(phthalimido-2-yl)ethyl]-1-H-1,2,3-triazole (PT4) derivative on *Leishmania amazonensis* and *Leishmania braziliensis*: In silico ADMET, in vitro activity, docking and molecular dynamic simulations

Vanderlan Nogueira Holanda^{a,b}, Welson Vicente da Silva^b, Pedro Henrique do Nascimento^b, Sérgio Ruschi Bergamachi Silva^c, Paulo Euzébio Cabral Filho^d, Shalom Porto de Oliveira Assis^e, César Augusto da Silva^f, Ronaldo Nascimento de Oliveira^g, Regina Celia Bressan Queiroz de Figueiredo^{h,i}, Vera Lucia de Menezes Lima^{a,i,*}

^a Laboratório de Lipídios e Aplicação de Biotecnologia em Doenças Prevalentes e Negligenciadas, Departamento de Bioquímica, Centro de Biotecnologia, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, 1235, 50070-901 Recife, PE, Brazil

^b Laboratório de Biologia Celular de Doenças, Instituto Agnir, Maranhão, Departamento de Microbiologia, Avenida Professor Moraes Rego, 1235, 50070-901 Recife, PE, Brazil

^c Instituto de Química, Universidade Federal do Rio Grande do Norte, Av. Nogueira de Gouveia, 2155 - Monte Brasil, 59050-400 Natal, RN, Brazil

^d Departamento de Biologia e Biotecnologia, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, 1235, 50070-901 Recife, PE, Brazil

^e Núcleo de Pesquisa em Ciências Exatas e Biotecnologia, Universidade Católica de Pernambuco, Rua do Príncipe, 526, 50050-900 Recife, PE, Brazil

^f Coleção de Medicinas, Universidade Federal da Vale do São Francisco, Avenida José de Sá Menezes, s/n - Campus Universitário, 56309-205 Petrolina, PE, Brazil

^g Laboratório de Mitocondria e Doenças, Departamento de Química, Universidade Federal Rural de Pernambuco, Rua Frei Manuel de Medeiros, s/n - Dois Irmãos, 52071-000 Recife, PE, Brazil

ARTICLE INFO

Keywords:
Leishmaniasis
Chemotherapy
Phthalimide
1,2,3-triazole
Click chemistry

ABSTRACT

Organic compounds obtained by click chemistry reactions have demonstrated a broad spectrum of biological activities being widely applied for the development of molecules against pathogens of medical and veterinary importance. Cutaneous leishmaniasis (CL), caused by protozoan parasite of genus *Leishmania*, encompasses a complex of clinical manifestations that affect the skin and mucous membranes. The available drugs for the treatment are toxic and costly, with long periods of treatment, and the emergence of resistant strains has been reported. In this study we investigated the in vitro effects of a phthalimide 1,2,3-triazole derivative, the 4-Phenyl-1-[2-(phthalimido-2-yl)ethyl]-1H-1,2,3-triazole (PT4) obtained by click chemistry, on mammalian cells and on *L. amazonensis* and *L. braziliensis*, the causative agents of CL in Brazil. In silico ADMET evaluation of PT4 showed that this molecule has good pharmacokinetic properties with no violation of Lipinski's rules. The in vitro assays showed that PT4 was more selective for both *Leishmania* species than to mammalian cells. This compound also presented low cytotoxicity to mammalian cells with $CC_{50} > 500 \mu\text{M}$. Treatment of promastigote forms with different concentrations of PT4 resulted in ultrastructural alterations, such as plasma membrane swelling, shortening of cell body, increased cell volume and cell rupture. The molecular dynamic simulations showed that PT4 interacts with Lanosterol 14 α -demethylase from *Leishmania*, an essential enzyme of lipid synthesis pathway in this parasite. Our results demonstrated PT4 was effective against both species of *Leishmania*. PT4 caused a decrease of mitochondrial membrane potential and increased production of reactive oxygen species, which may lead to parasite death. Taken together, our results pointed PT4 as promising therapeutic agent against CL.

^{*} Corresponding author at: Laboratório de Lipídios e Aplicação de Biotecnologia em Doenças Prevalentes e Negligenciadas, Departamento de Bioquímica, Centro de Biotecnologia, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, 1235, 50070-901 Recife, PE, Brazil.
E-mail address: lima.vem@ufpe.br (V.L.M. Lima).

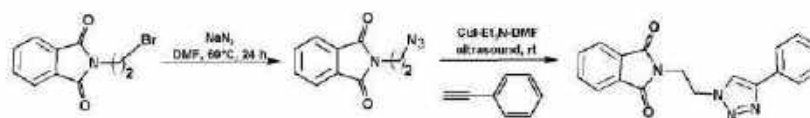
[†] Both authors contributed equally to this work.

<https://doi.org/10.1016/j.bioorg.2020.104437>

Received 22 July 2020; Received in revised form 30 September 2020; Accepted 26 October 2020

Available online 24 October 2020

0045-2068/© 2020 Elsevier Inc. All rights reserved.



Scheme 1. Click reaction for PT4 synthesis.

1. Introduction

Leishmaniasis is a complex of prevalent and neglected diseases caused by protozoa of the genus *Leishmania*. The clinical manifestations of leishmaniasis range from potential fatal visceral leishmaniasis [1] to the most common cutaneous leishmaniasis (CL), which is characterized by the appearance of skin and mucous membrane lesions [2]. CL occurs in several countries of Latin America, in the Mediterranean, Middle East, and Central Asia. This disease is characterized by lesions that can lead to anatomical disfigurement of the nasal septum and ears, depending on the immune status of patient and on the *Leishmania* species [3,4]. Furthermore, an intense inflammatory response, characteristic of CL, may lead to tissue damage, ulcer formation, scarring, which ultimately result in social stigmatization [5].

Regardless of the clinical manifestations of Leishmaniasis, pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate have been used since the early twentieth century as the first choice for the treatment of this illness [6]. Despite of their efficacy, these drugs require long term parenteral administration, are expensive and have adverse reactions, such as cardiotoxicity and acute renal failure [7]. In addition, the appearance of parasite resistance has also been reported [8]. Due to the limitations related to their pharmacokinetics and bioavailability, these drugs are not used in topical formulations. Pentamidine and amphotericin B are introduced as second-choice drugs against leishmaniasis therapy for the treatment of refractory cases. However, these drugs also have high cost and toxicity, imposing important health concerns [9]. In this regard, the search for new safe and economically viable therapeutic agents against leishmaniasis is still necessary.

Triazoles and their derivatives have attracted the attention of the scientific community due to their interesting properties as: anti-inflammatory [10], antimicrobial [11], antifungal [12], anticancer [13] and antimalarial [14]. The heterocyclic rings of triazoles bind with high affinity to a variety of enzymes and receptors via non-covalent interactions [15]. These properties make 1,2,3-triazoles compounds very attractive, not only as bioactive compounds, but also as molecular blocks for organic synthesis and as functionalizes in materials science [16,17]. In the last decades, with the establishment of *click chemistry* reactions, the synthesis of 1,2,3-triazoles derivatives and hybrid molecules was substantially improved. The *click chemistry* refers to a group of reactions that are low cost, fast, simple to perform, versatile, regioselective, giving high product yield [18]. Furthermore, these reactions use solvents that are inoffensive and can be easily removed [19].

As the triazoles derivatives, heterocyclic molecules belonging to the phthalimide class have also been attracted attention due to their relevant biological activities such as: anticonvulsive [20], antioxidant [21], analgesic [22], antimicrobial [23], and anti-inflammatory [24,25]. Among the interesting chemical properties of phthalimides and their *N*-substituted derivatives, the presence of both hydrophobic aryl ring, (—CONH—), which acts as a hydrogen donor, and an electron donor system (C=N) contributes for the biological activities of these molecules [26].

Because the infection with *Leishmania* spp. lead to an exacerbated inflammatory response [27], the anti-inflammatory propriety of phthalimide and their derivatives, allied to its putative leishmanicidal potential, could be a useful strategy to combat leishmaniasis infection.

The combination of chemical groups having anti-inflammatory and

antiparasitic action, by *click chemistry* reactions, is promising for the prospection of molecules candidates for CL therapy. In this regard, previous study carried out by our group has identified a hybrid molecule phthalimide-1,2,3-triazole, 4-Phenyl-1-[2-(phthalimido-2-yl)ethyl]-1H-1,2,3-triazole (PT4) as having a potent *in vivo* anti-inflammatory activity [20]. Thus, in this work we selected this molecule to evaluate its leishmanicidal potential against *Leishmania amazonensis* and *Leishmania braziliensis*, the main etiologic agents of CL in Brazil.

2. Materials and methods

2.1. Synthesis of 2-[2-(4-phenyl-1H-1,2,3-triazol-1-yl)ethyl]-1H-isindole-1,3(2H)-dione (PT4)

The strategy for the synthesis of 2-[2-(4-phenyl-1H-1,2,3-triazol-1-yl)ethyl]-1H-isindole-1,3(2H)-dione (PT4) derivative was presented in Scheme 1. The compound was obtained as previously described [29]. The infrared spectra were recorded with an IFS66 Bruker spectrophotometer using KBr discs. ^1H and ^{13}C NMR were obtained on Varian Unity Plus 400 MHz spectrometer using $\text{DMSO}-d_6$ as solvent. The compound was purified by column chromatography on Merck silica gel 60 (70–230 mesh), with a system hexane-EtOAc (1:1) ratio. The purity of fractions was monitored by TLC analysis on a GF $_{254}$ plate. The azide and 1,2,3-triazole compounds were prepared according to Silva et al. [30].

2.2. *In silico* ADMET assay

The prediction of absorption, distribution, metabolism, excretion and toxicity (ADMET) parameters of PT4 and the reference drugs for leishmaniasis was performed using the online SwissADME (<http://www.swissadme.ch/>) platform of the Swiss Institute of Bioinformatics, PROTOX-II Server (http://tox.charite.de/protox_ii/index.php?die=home) and pKCSM server (<http://biosig.unimelb.edu.au/pkcsam/>). The physical-chemical parameters, the potential for oral bioavailability and the similarity with other compounds already described were also predicted using OSIRIS Data Explorer and DataWarrior version 4.7 software (<http://www.openmolecules.org/datawarrior/>) and compared with the descriptors obtained for the reference drugs.

2.3. Cytotoxicity assay in mammalian cells

The cytotoxic potential of PT4 on macrophages, obtained from Balb/c mice peritoneal exudate (mPEC) and from J774A.1 (ATCC® TIB-67™) lineage and fibroblasts (ATCC® PCS-201-018) were assayed by MTT, as established by Mosmann [31] with slight modifications. J774A.1 macrophages, mPEC and fibroblasts (5×10^3 cells / well) were plated in 96-well plates containing 100 μL of the RPMI (for macrophages) or DMEM (for fibroblast) medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C for 3 hours in a 5% CO_2 atmosphere. After this, the adhered cells were incubated in the absence or presence of PT4 (78.5–1,256.5 μM) in culture medium for 48 hours. After incubation time, the cells were washed with HBSS buffer and incubated in RPMI containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) for 3 hours at 37 °C in the absence of light. The medium was then discarded and 100 μL of an acidified isopropanol solution (HCl 0.04 N + absolute isopropanol) was added to each well to solubilize the formazan crystals.

The absorbance was read spectrophotometrically in a microplate spectrophotometer (Bio-Rad®, California, USA) at 540 nm. Each assay was performed in triplicate in three independent experiments. The cytotoxic concentration for 50% of cells (CC_{50}) was calculated by linear regression using the IBM SPSS Statistics 25.

2.4. Parasites

Promastigote forms of *Leishmania amazonensis* (LTB0016) and *Leishmania braziliensis* (LTB2903) were maintained in Schneider's medium (Sigma-Aldrich, USA) supplemented with 10% of foetal bovine serum and 100 IU penicillin and 0.01 mg/mL streptomycin, at 26 °C. Exponential-phase promastigotes were used in all experiments. Amastigote forms were obtained from Balb/c peritoneal macrophages infected with the stationary phase of promastigote forms.

2.5. In vitro effects of PT4 on promastigotes and amastigote of *L. Amazonensis* and *L. braziliensis*

L. amazonensis and *L. braziliensis* promastigotes (10^6 cells/mL) were incubated in Schneider's medium in the presence or the absence of PT4 (314.1–19.6 μ M) for 48 hours. The culture density was determined by direct counting in a Neubauer chamber. The concentration of PT4 compound capable of inhibiting the growth of the culture by 50% (IC_{50}) was determined by linear regression analysis using the IBM SPSS Statistics 25, after 48 hours of drug treatment. To investigate the effect of PT4 on intracellular amastigote forms, mPEC (10^6 cells/mL) were placed in 24-well plates and maintained at 37 °C for 3 hours in a 5% CO_2 atmosphere. Subsequently, the adhered cells were infected with promastigotes of *L. amazonensis* and *L. braziliensis*, at 15:1 parasite/cell ratio, at 37 °C for 14 hours. The non-internalized parasites were removed by washing and then the infected cells were incubated for 24 hours with PT4 at concentrations corresponding to $\frac{1}{2}$ IC_{50} , IC_{50} or $2 \times IC_{50}$ values, determined for promastigote forms of each species. Infected-cells cultured in drug-free medium were used as negative control. Cells infected and treated with pentamidine (30 μ M) were used as positive control. After the incubation time, treated and untreated cells were washed with PBS, fixed with methanol and stained with Giemsa. The percentage of infected cells was determined by counting 150 randomly chosen macrophages in duplicate. The survival index was determined by multiplying the number of infected cells by the mean number of amastigotes per cell. The concentration that inhibited amastigote (IC_{50} /amastigote) growth by 50% within the macrophages was determined by regression analysis as described above. These assays were performed in duplicate in three independent experiments.

2.6. Scanning electron microscopy

To identify putative morphological changes induced by PT4 treatment on the parasites, scanning electron microscopy analysis was performed. For this, promastigote forms of *L. amazonensis* and *L. braziliensis* treated or not, for 48 hours, with IC_{50} or $2 \times IC_{50}$ of PT4, were harvest by centrifugation, washed with 0.1 M phosphate buffer, pH 7.2, and then fixed for 2 hours with 2.5% glutaraldehyde/4% paraformaldehyde solution, in 0.1 M phosphate buffer, at pH 7.2. After the fixation, cells were allowed to adhere to coverslips previously coated with poly-L-lysine. The samples were then post-fixed with 1% osmium tetroxide/0.8% potassium ferricyanide/5 mM $CaCl_2$ in 0.1 M cacodylate buffer solution, at pH 7.2, for 1 hour in dark condition. After post-fixation, the samples were washed in the same buffer, dehydrated in graded ethanol series, critical-point-dried with CO_2 , coated with a 20 nm-thick gold layer and observed with a JEOL T-200 scanning electron microscope.

2.7. Docking and molecular dynamic (MD) simulations

The structure of PT4 was built using Avogadro software [39] and

optimized using the PM3 semi-empirical method [33] implemented in Orca 4.2 [34]. Molecular docking was performed based on the confrontation between optimized PT4 structure and the sterol 14- α -demethylase enzyme (CYP51) from *Leishmania infantum*. The docking analysis was carried out using Autodock Vina software [35]. The crystal structure of sterol 14- α -demethylase was obtained from Protein Data Bank (PDB) under the code 3LAD [36]. Water molecules were removed, and polar hydrogens were added. The best binding poses were assessed in a $30 \times 30 \times 30$ Å docking cube centered inside of the substrate binding cavity. The two most energetically favourable structures obtained from docking were selected as initial conformations for MD simulations. These calculations and analyses were performed for two binding poses, named PT4_A and PT4_B, using the GROMACS 2018.4 [37,38] software package with CHARMM36 force field [39]. The force field topology and parameters for PT4 were obtained from CHARMM-GUI module [40]. The systems were solvated by 17,879 SPC water molecules and counter ions Na^+ and Cl^- were added to neutralize the charge and maintain the system at a concentration of 150 mM. The protonation states of amino acid sidechains were verified by PROPKA 3.0 [41] software assuming a physiological pH. Each system was submitted to the energy minimization step using steepest descent algorithms and the equilibration period (EP) of 125 ps was made with NVT ensemble. The production runs were carried out in the NVT ensemble with a total time simulation of 100 ns for each system. The V-rescale thermostat [42] was used to maintain the system temperature at 310 K. Other MD parameters and covalent bonds involving hydrogen atoms in the protein and water molecules were restrained by LINCS and SETTLE algorithms, respectively [43,44]. The Leap-Frog algorithm [45] was applied to integrate the motion equation with a time step of 2.0 fs. The long-range interactions were treated using Particle-Mesh Ewald sum (PME) [46] with a 1.2 nm cutoff distance. The Interaction Potential Energy (IPE) and hydrogen bond evaluations were performed as previously described [47]. The two systems, PT4_A and PT4_B, presented the same number of particles and the only difference was the disposition of PT4 into the binding cavity.

2.8. Analysis of PT4 on mitochondrial membrane potential and reactive oxygen species production

To determine the effect of PT4 on mitochondrial membrane potential ($\Delta\psi_m$), *L. amazonensis* and *L. braziliensis* promastigotes were treated with IC_{50} or $2 \times IC_{50}$ of PT4 for 48 hours. After this, treated and control parasites were incubated with 10 μ g/mL of rhodamine 123 (Sigma Aldrich, St. Louis, USA) for 15 min. Changes in the mitochondrial membrane polarization were measured using the Index of Variation (IV), obtained by the equation $(MT-MC)/MC$ where, MC is the median fluorescence intensity of the control and MT the median fluorescence intensity of the treated cells. The mitochondrial reactive oxygen species production (ROS) production was assayed using the $O_2^{\cdot-}$ sensitive hydroethidine analogue mitochondrial targeted probe, MitoSOX 3,3'-phenanthridine diamine, 5-(6'-triphenylphosphoniumhexyl)-5,6 dihydro-6 phenyl (Invitrogen - Molecular Probes, USA). The cells were loaded with 2 mL of 5 μ M MitoSOX reagent and incubated for 10 min at 26 °C, protected from light. After incubation, the parasites were washed three times with HBSS buffer. H_2O_2 (10 μ M) was used as a positive control. For both analyses, the data collection was performed in a BD Accuri™ C6 Plus flow cytometer (BD Biosciences, USA), in a corresponding channel detection for each fluorescent probe. Twenty thousand events per sample were analysed.

2.9. Ethical considerations

The present research was carried out in accordance with the ethical principles adopted by Brazilian legislation 11,794/2008 and approved by the Instituto Aggeu Magalhães / Fundação Oswaldo Cruz Ethics Committee on Animal Research (No. 146/2019).

Table 1
Analysis of pharmacokinetic and toxicological parameters (ADMET) of PT4 and the reference drugs used in the treatment of CL.

Parameter	PT4	GLU	PEN	MIL	AMB
Lipinski Rules Violation	No	Yes	No	No	Yes
Physicochemical properties					
HBA (≤ 10)	4	9	4	4	10
HBD (≤ 5)	0	7	4	0	12
LogP (≤ 5)	2.23	-2.90	2.74	3.83	-0.39
MW (≤ 500) g/mol	318.33	365.95	340.42	407.57	324.08
n-ROTB (≤ 10)	4	6	10	20	3
TPSA ($\text{\AA}^2$)	68.09	167.55	118.20	58.40	319.61
Absorption					
BBB	Yes	No	No	No	No
GIA	High	Low	High	Low	Low
P-gp substrate	No	No	No	Yes	Yes
skin permeability (cm/s)	-6.85	-11.34	-6.56	-3.97	-11.34
Metabolism					
CYP450 2C9 inhibitor	Yes	No	Yes	No	No
CYP450 2D6 inhibitor	No	No	Yes	No	No
CYP450 2C19 inhibitor	Yes	No	No	No	No
CYP450 3A4 inhibitor	No	No	No	No	No
CYP450 1A2 inhibitor	Yes	No	No	No	No
Excretion					
Total Clearance (log ml/min/kg)	0.117	0.896	0.814	1.112	1.456
Renal OCT2 substrate	No	No	Yes	No	No
Toxicity					
LD ₅₀ (mg/kg)	4700	3500	500	246	100

PEN - Pentamidine; GLU - glucantime; MIL - Miltefosine; AMB - Amphotericin B. Physicochemical properties: HBA - Number of hydrogen bond acceptors; HBD - Number of hydrogen donors; LogP - Octanol-water partition coefficient (lipophilicity); MW - Molecular Weight; n-ROTB - Number of rotatable bonds; TPSA - polar surface; Absorption: BBB - blood-brain barrier; GIA - gastrointestinal absorption; P-gp - permeability glycoprotein; Metabolism: CYP450 - Cytochrome P450 Enzyme; Toxicity: 50%LD₅₀ - 50% lethal dose.

3. Results and discussion

The organic synthesis of new chemotherapeutic compounds against neglected diseases, including leishmaniasis, may be hindered when the methods used are complex, time-consuming, expensive and have low possibility of large-scale production. In a previous work we used *click chemistry* methodology to efficiently synthesize alkyl-substituted phthalimide 1H-1,2,3-triazole derivatives. Among them, the compound 2-[2-(4-phenyl-1H-1,2,3-triazol-1-yl)ethyl]-1H-isoindole-1,3(2H)-dione (PT4) derivative, presenting an important anti-inflammatory activity [26], was selected for the analysis of its leishmanicidal potential on the etiological agents of CL in Brazil. This hybrid phthalimide-1,2,3 triazole compound was obtained with 93% yield and presented the following characteristics: colorless solid; mp 187–189 °C (lit. 157–158 °C); Rf 0.4 (EtOAc/Hexane, 1:1); IR (KBr) ν_{max} 3126, 2952, 1774, 1716, 1464, 1432, 1396, 1231, 1076, 765, 720 cm^{-1} . The ^1H and ^{13}C NMR analyses are in accordance with the previously reported data [29].

In the search of new compounds against leishmaniasis the drug-likeness, absorption, distribution, metabolism and excretion (ADMET) properties of drug candidates should be considered [46]. *In silico* ADMET is a useful and low-cost method for accurate prediction of pharmacokinetic parameters and of possible interactions of drugs with cells, enzymes and biological membranes [49]. In this regard, *in silico* ADMET platforms were used to characterize the pharmacokinetics profile of PT4 comparing with the reference drugs Glucantime (GLU), Pentamidine (PEN), Miltefosine (MIL) and Amphotericin B (AMB) (Table 1).

The Lipinski's rule-of-five is an empirical rule of thumb which describes the physicochemical parameters to determine drug-likeness and filter hit molecules [50]. These rules state that an orally active drug does not have more than two violation of the following parameters: 1) No > 5

hydrogen bond donors; 2) No > 10 hydrogen bond acceptors; 3) a molecular mass < 500 Daltons; 4) an octanol-water partition coefficient LogP not > 5. According to our predictive analysis the reference drug Glucantime presented 1 violation (NH or OH > 5) and Amphotericin B had three violations (MW > 500, N or O > 10 and NH or OH > 5). On the other hand, PT4 fulfil all the requirements of Lipinski's rule of five as a candidate for development of oral formulation against CL. Octanol-water partition coefficient (LogP) is a measure of hydrophobicity of a given compound. The value of LogP for PT4 was 2.23 whereas the reference drug Miltefosine was the most lipophilic molecule with LogP value of 3.83. It well known that the hydrophobicity is essential for drug absorption, bioavailability, hydrophobic drug receptor interactions, metabolism and cytotoxicity [51]. PT4 presented a topological polar surface (TPSA) of 68.09 $\text{\AA}^2$, which favours its permeability and oral bioavailability. It has been assumed by various predictive studies that the bioavailability and oral permeability properties decrease as the value of TPSA increases [52]. For polarity, the optimal values of bioavailability should range between 20 and 130 $\text{\AA}^2$ [53]. Both reference drugs Glucantime and Amphotericin B presented higher TPSA, which impairs their passive diffusion through the BBB. The combination of the number of rotatable bond count with the TPSA reflects the flexibility of PT4. It has been demonstrated that higher TPSA/numbers of rotatable bonds can decrease the bioavailability of drugs on rat model [52]. Although PT4, Glucantime and Amphotericin B have low number of rotatable bonds, the higher values of TPSA found for the reference drugs, probably contribute for their low bioavailability profile. Additionally, in the case of CNS permeation by passive diffusion, the TPSA must be below 80 $\text{\AA}^2$ (Table 1).

Cytochrome P450 (CYP450) enzymes are essential for detoxification of foreign chemicals and drug metabolism in mammalian cells [54]. Our predicted results pointed PT4 as a potential inhibitor of three CYP450 enzymes (CYP450 2C9, CYP450 C19, and CYP450 1A2) whereas Pentamidine was found as a potential inhibitor of CYP450 2C9 and CYP450 2D6. None of CYP450 enzymes were predicted to be inhibited by Glucantime, Miltefosine and Amphotericin B. Drugs interact with the CYP450 system in several ways. The metabolic interaction between CYP450 and drugs or xenobiotics are referred to as either inhibitors or inducers [55]. It well documented that commercially available drugs containing triazole groups, such as Itraconazole and Posaconazole antifungals, are substrates and inhibitors of CYPs [56]. To varying extents, two enzymes in the CYP3A family catalyse the metabolism of all triazole-containing drugs. CYP3A4 and CYP3A5. Interestingly, PT4 was not predicted to inhibit CYP450 3A4, suggesting that this compound, as other triazoles derivatives can be efficiently metabolized by this enzyme. Although PT4 has been predicted as inhibitors of some CYP450 enzymes, the extent by which PT4 affects these enzymes may be influenced by factors as its ability to bind the enzyme (reversibly or irreversibly), the dose and the CYP450 polymorphism. Therapeutically, further studies are needed to better understand the nature of PT4 interactions with human CYPs enzymes and other drugs.

When compared with the reference drugs for leishmaniasis, PT4 has a reduced risk of toxicity. The predicted 50% lethal dose for murine model (LD₅₀) of 4,700 mg/kg, was at least 1.34 and 470 times higher than those found for Glucantime (3,500 mg/kg) and Amphotericin B (100 mg/kg), the less and most toxic among the reference drugs tested. Overall, our *in silico* ADMET prediction showed that PT4 has a desirable drug-like properties and favourable safety profile.

Our *in silico* ADMET results prompted us to further investigate the *in vitro* effects of this molecule on *Leishmania amazonensis* and *Leishmania braziliensis*, as well as its cytotoxic potential on mammalian cells. For the analysis of cytotoxicity of PT4 on mammalian cells, two cell types were chosen: Macrophages (peritoneal and J774A.1) and fibroblast cells. *Leishmania* spp. is a mandatory intracellular pathogen, which requires macrophages for its survival and multiplication [57]. Like macrophages, fibroblasts shows a high degree of injury during the development of cutaneous and/or mucosal lesions in cutaneous leishmaniasis [58]. Our

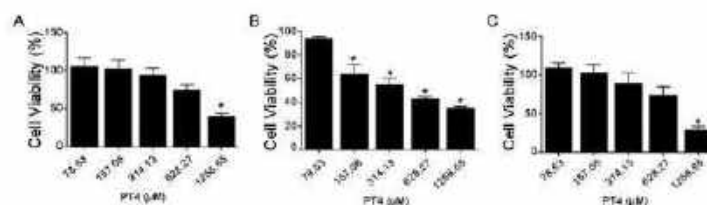


Fig. 1. Effect of PT4 on mammalian cells: (a) mPEEC, (b) J774A.1 macrophages and (c) fibroblasts. The values represent the mean $\pm$ standard deviation of three independent experiments in triplicate. * significant differences at $p < 0.05$, compared to untreated control.

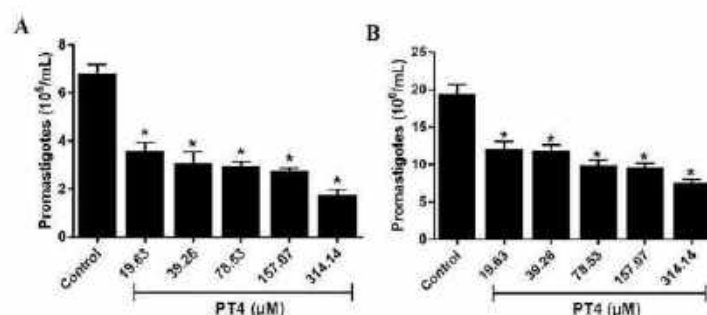


Fig. 2. Effect of PT4 on (a) promastigote forms of *L. amazonensis* and (b) *Leishmania braziliensis*. The values represent the mean $\pm$ standard deviation of three independent experiments in triplicate. * significant differences at $p < 0.05$, compared to untreated control.

results showed that PT4 has an inhibitory effect on the viability of all mammalian cells tested. However, only at the higher concentration, PT4 was able to significantly decrease the percentage of viable peritoneal macrophages and fibroblasts. None of PT4 concentrations tested was able to inhibit the viability of treated cells by 100% (Fig. 1). The peritoneal macrophages were the most resistant to PT4 treatment, whereas J774A.1 cells were the most susceptible. The CC_{50} /48 hours was 981.37, 521.47 and 895.17 μ M for peritoneal macrophages, J774A.1 cells and fibroblasts, respectively.

The PT4 significantly inhibited the growth of promastigote forms of both *Leishmania* species tested, in a dose-dependent manner (Fig. 2). *L. amazonensis* was more susceptible to PT4 treatment than *L. braziliensis* with an estimated IC_{50} of 70.46 and 181.73 μ M, respectively. The biochemical/molecular differences in the susceptibility to drugs between *Leishmania* species are well documented in the literature [59] and can be partially explained by their distinct division rate, differential permeability, difference in plasma membrane composition and intracellular targets [60].

The PT4 also significantly inhibited the amastigote survival inside macrophages at all concentrations tested (Fig. 3), with IC_{50} values of 125.18 and 253.18 μ M for *L. amazonensis* and *L. braziliensis*, respectively. As observed for promastigotes, amastigotes of *L. amazonensis* were more susceptible to the PT4 treatment. In both species, however, amastigotes were more resistant to this compound than promastigotes. Although PT4 significantly inhibited the survival of amastigote inside the macrophages, only at the higher concentration (314.14 μ M) this molecule was able to exhibit an inhibitory activity similar to the reference drug pentamidine (PMD) at 30 μ M (Fig. 3). However, it is important to emphasize that pentamidine has serious collateral effects and decreasing efficacy in prolonged treatment [61].

The main drawback of the chemotherapy leishmaniasis is the lack of specificity of currently available drugs used for the treatment, which often results in high toxicity to mammalian cells [61]. Thus, the selectivity index (SI), i.e., the ratio of CC_{50} for the host cells to IC_{50} value for

the parasite, is an important parameter to be taken in account in the search of new leishmanicidal agents [62]. Our results showed that PT4 was selective against both evolutive forms of the parasite. The SI values were 13.94 and 5.40 (for promastigotes) and 7.84 and 4.21 (for amastigotes) of *L. amazonensis* and *L. braziliensis*, respectively (Table 2).

To investigate the effects of PT4 on the ultrastructure of *L. amazonensis* and *L. braziliensis* promastigote forms, scanning electron microscopy (SEM) was performed (Fig. 4). Control cells had a smooth plasma membrane, spindle-shaped body and elongated flagellum (Fig. 4A,B). Drastic morphological changes could be observed in PT4-treated cells as compared to control cells. The level of cell injuries was strongly dependent on the concentration of PT4 tested, in both *Leishmania amazonensis* (Fig. 4B, D and Fig. 5F) and *Leishmania braziliensis* (Fig. 4A, 4C and 4E). The most prominent changes observed were the increase of cell volume (Fig. 5F) with shortening and rounding of cell body (Fig. 4C). An interesting finding was the increased presence of extracellular vesicles (EVs) of different sizes budding from the cell body and flagellum in treated cells (Fig. 4C and E). EVs are a population of lipid-enclosed spherical vesicles containing bioactive components such as soluble proteins, carbohydrates, lipids and genetic material from parental cells [63]. Commonly, EVs are classified based on their biogenesis and size into exosomes (30–150 nm in diameter), microvesicles (100–1000 nm in diameter) and apoptotic bodies (100–5000 nm in diameter). Microvesicles and exosomes have been shown to be constitutively release by several protozoan parasites, including *Leishmania* species and *T. cruzi*, as a mechanism of both parasite-host and parasite-parasite communication [63].

The increase in the EVs resembling microvesicles and exosomes in cells treated with PT4 suggests that besides their role in parasite communication EVs may also participate in the response of parasite to drugs. The large EVs, also observed budding from PT4-treated parasites, could correspond to apoptotic bodies induced by PT4 treatment [64]. PT4-treated parasites also presented incomplete division, with abnormal septation and short flagellum. These results suggest that PT4 may also

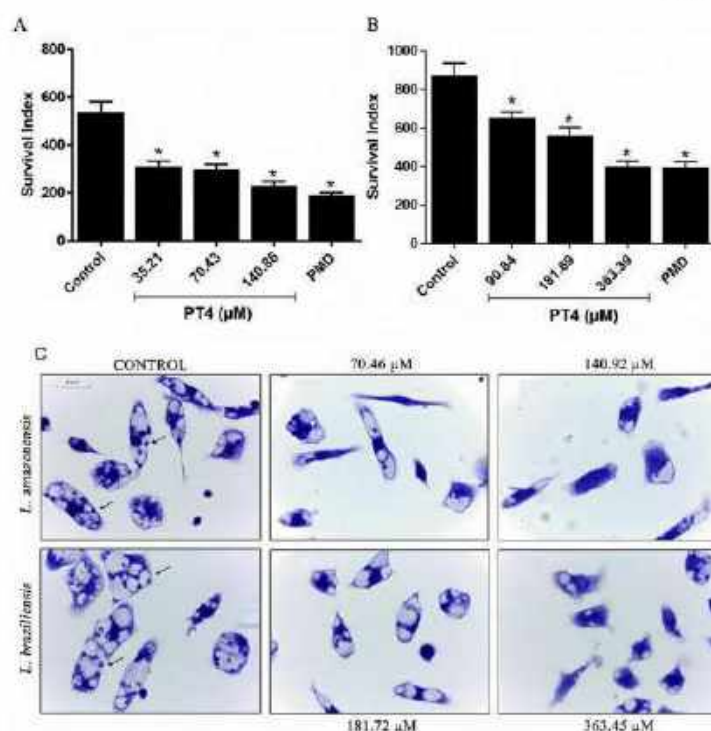


Fig. 3. Effects of PT4 on intracellular amastigote forms of (A) *L. amazonensis* and (B) *L. braziliensis*. (C) Representative image of Giemsa staining showing the presence of intracellular amastigotes (black arrow) in macrophages treated or not with PT4. The values represent the mean $\pm$ standard deviation of three independent experiments in duplicate. * significant difference $p < 0.05$, compared to untreated control.

Table 2
Evaluation of the leishmanicidal effect and cytotoxicity of PT4.

Cell Type	CC ₅₀ μ M	IC ₅₀ (50%) μ M	IC ₅₀ (90%) μ M	Selectivity Index			
				Pre		Amast	
				L.a	L.b	L.a	L.b
mPFC	902.43 $\pm$ 5.6	–	–	13.9	5.4	7.8	4.2
IT74A.1	521.67 $\pm$ 1.2	–	–	7.4	2.8	4.3	2.2
Fib	896.17 $\pm$ 1.2	–	–	12.2	4.9	7.1	3.8
Go	–	78.46 $\pm$ 3.9	125.65 $\pm$ 3.3	–	–	–	–
Th	–	181.72 $\pm$ 5.5	223.18 $\pm$ 3.7	–	–	–	–

mPFC = Peritoneal macrophages; Fib = Fibroblasts; CC₅₀ = cytotoxic concentration to 50% of cells; IC₅₀ = inhibitory concentration to 50% of parasites; Pre = promastigotes; Amast = amastigotes; L.a = *Leishmania amazonensis* and L.b = *Leishmania braziliensis*.

have a cytostatic effect on both species of *Leishmania*.

Ultrastructural changes similar to those found in our study were also observed in *Leishmania* spp. treated with triazole inhibitors of ergosterol biosynthesis [65]. Trypanosomatids and fungi are strongly dependent on ergosterol and other 24-alkyl sterols that were not found in mammalian cells [66,67]. The Cytochrome P450 (CYP) is the

superfamily of protoheme containing monooxygenases, found in a large number of organisms. Among P450 enzymes, sterol 14 α -demethylase (CYP51), an essential enzyme in sterol biosynthesis, has been pointed as an important drug target in trypanosomatids [68]. It has been demonstrated that commercially available anti-fungal azoles, as posaconazole and fluconazole, that inhibit the ergosterol biosynthesis in fungi, are also effective against *Trypanosoma cruzi* and *Leishmania* parasites [69]. To investigate whether PT4 could also be an inhibitor of *Leishmania* CYP51 *in silico* docking of PT4 against this enzyme was performed.

Because the crystal structure of CYP51 of *L. amazonensis* and *L. braziliensis* was not available on the protein data bank (PDB) we used the CYP51 of *L. infantum* (LiCYP51, Protein data bank entry: 3L4D) as a template for molecular docking assays and MD simulations. This protein presented 97% and 95%, of amino acid sequence similarity with two hypothetical orthologue proteins of *L. amazonensis* and *L. braziliensis*, respectively. The sequence similarity reaches 100% if we compare only the residues from the active site [36]. Previous study revealed that CYP51 is highly conserved between kinetoplastids. Chen et al. [69] reported that a TcCYP51 from *T. cruzi* and TbCYP51 from *Trypanosoma brucei* share 53% sequence identity. Furthermore, *Leishmania* CYP51 is 72–78% identical to those of *T. cruzi* and *T. brucei*. The high level of conservation of CYP51 in these parasites makes possible to extrapolate structural features that can be modelled to facilitate drug discovery and development [69].

By using LiCYP51 as receptor and the models generated from docking analysis, the MD simulations were carried out to identify the PT4 intermolecular interaction profile. The interaction potential energy

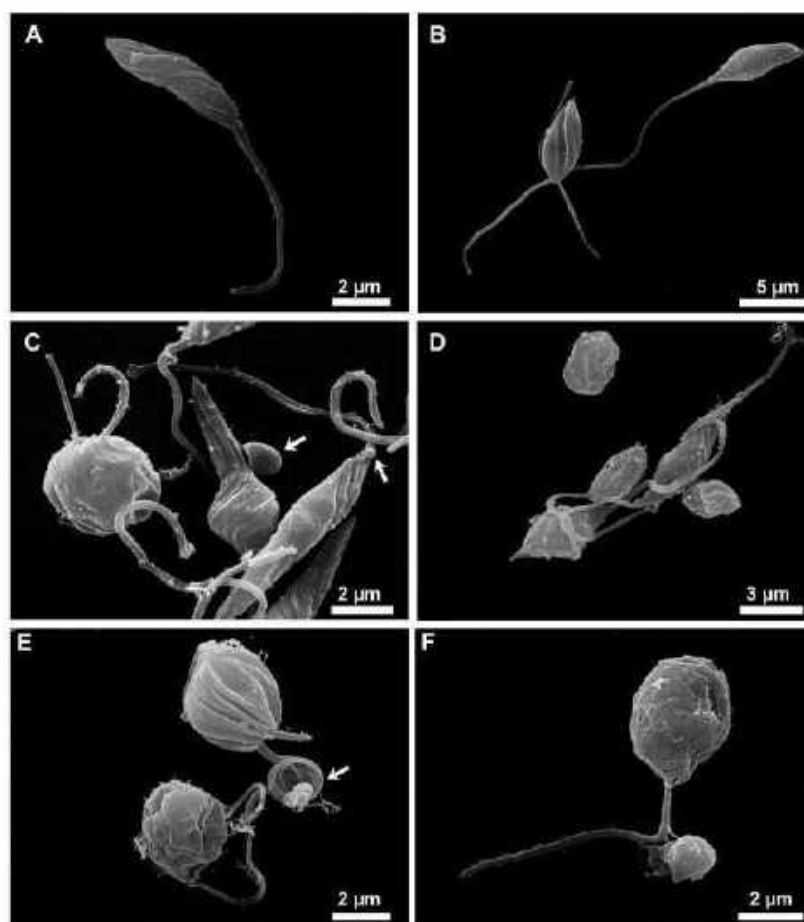


Fig. 4. Ultrastructural changes induced by PT4 on *L. brasiliensis* (A, C and E) and *L. amazonensis* (B, D and F) promastigotes. Control cells (A–B), cells treated with IC₅₀ (C–D) and 2x (E–F).

(IPE) calculations were performed to evaluate the contribution of each residue of the binding cavity and channel entrance of CYP51 receptor with the ligand. By using non-hydrogen atoms of PT4 as reference, the RMSD analysis showed (Fig. 5A) a structural stabilization around 70 ns, hence, the IPE and hydrogen bond analysis were made in the last 50 ns for both systems. The highest variation on RMSD values evidenced the motions of inhibitor inside the binding cavity and consequent settle down. The independent MD simulations taken in account two possible entering modes of PT4 molecule to an internal region of the cavity. These modes were obtained from docking analysis and indicated the first approaching of 1,2,3-triazole or phthalimide moiety to the enzyme cavity channel, named respectively PT4_A and PT4_B systems, as shown in Fig. 5B.

An 8 Å sphere centered on PT4 molecule was used for IPE calculation over time. This volume encompasses 62 neighbouring (average) residues during the simulated time for PT4_A and PT4_B, although most of them interact with an energy value very close to zero. Applying an arbitrary cutoff of IPE lower than -0.5 kcal/mol, the number of residues interacting with PT4 was 17 and 16 for PT4_A and PT4_B, respectively. This

total includes the heme group. All these most relevant residues identified by IPE calculations were presented in Table 3. They constitute the substrate channel entrance and the binding cavity described previously for the sterol 14 α -demethylase receptor of *L. infantum* [36].

The analysis of IPE values revealed that PT4 interacts with CYP51 residues in a similar way to other antifungal azoles [70]. As PT4, these nitrogenated heterocyclic compounds, contain a basic atom, which easily coordinates with heme iron (Fig. 3C). The calculated IPE were -8.59 ± 2.13 kcal/mol and -14.71 ± 3.87 kcal/mol for PT4_A and PT4_B. For PT4_A orientation, the interaction occurs through triazole nitrogen with an average distance of 4.60 ± 0.39 Å and for PT4_B, the nitrogen of phthalimide moiety interacts with an average distance of 4.52 ± 0.38 Å. This strong interaction with the heme group plays a key role in replacing the sterol substrate becoming unfeasible for the iron reduction and consequent oxidative removal of the 14 α -methyl group from the sterol core.

Among other interacting residues presented in Table 1, Tyr102, Phe109, Tyr115 (helix B', B'/C loop), Leu126 (helix C), Ala206, Phe239, Ala290 (helix D), Leu355, Met357, Met359 (β 4 strand 1–4) are

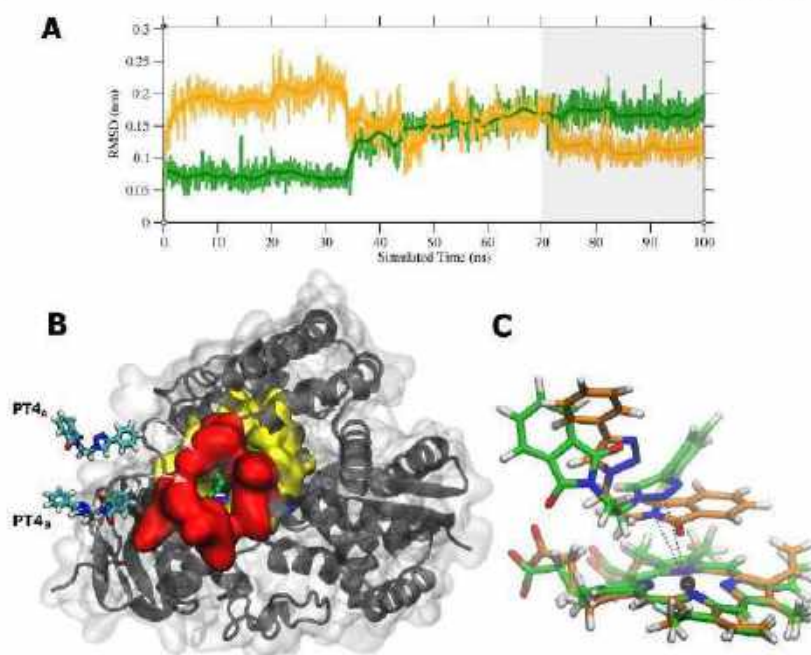


Fig. 5. MD simulation analysis and graphical representations. A) Root Mean Square Deviations (RMSD) for PT4_A (green) and PT4_B (orange) and one-millisecond running averages of the deviations versus time. B) Graphical representation of the simulated system with PT4_A and PT4_B orientation (tubes), substrate channel entrance (red surface), substrate binding cavity (yellow surface), PT4 model inside the cavity (green tubes), heme group (blue surface) and chitin A tertiary structure of sterol 14-demethylase (CYF51) (gray cartoon). C) Superimposed view of PT4-heme group interactions. PT4_A and PT4_B were differentiated through carbon coloring green and orange tubes respectively. Iron, oxygen, nitrogen and hydrogen atoms were represented by black sphere, red, blue and white tubes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Average (standard deviation) of Interaction Potential Energy (IPE) for each residue of *LCYF51* interacting with PT4_A and PT4_B systems over time.

Residue	IPE (kcal/mol)	
	PT4 _A	PT4 _B
Tyr102	-2.84 (1.26)	-5.45 (0.96)
Phe104	-1.48 (0.59)	-2.65 (0.61)
Met105	-1.59 (0.55)	-1.92 (0.46)
Phe109	-0.61 (0.38)	-0.96 (0.36)
Tyr115	-4.16 (1.25)	-5.54 (2.14)
Leu126	-0.53 (0.30)	-0.54 (0.41)
Val212	-0.79 (0.60)	-1.19 (0.46)
Ala286	-0.65 (0.46)	-0.88 (0.54)
Phe289	-1.25 (0.60)	-0.99 (0.61)
Ala290	-2.81 (0.82)	-4.80 (0.54)
Leu335	-1.62 (0.61)	-2.42 (0.96)
Met352	-2.41 (0.96)	-3.29 (2.06)
Met359	-1.11 (0.53)	-1.10 (0.76)
Met459	-2.91 (1.58)	-1.88 (0.79)
Val460	-1.45 (0.40)	-0.86 (0.42)
Heme	-8.59 (2.33)	-14.71 (3.87)

constituents of substrate binding cavity (SBC) while Val212 (helix F''), Met459, Val460 (β4 hairpin) belong to substrate channel entrance (SCE), as shown in Fig. 5B. Taken together, the PT4_A and PT4_B orientations showed similar interactions with SBC and SCE residues. Besides heme interaction, the most energetically favourable interactions take place between PT4 and a tyrosine pair belonging to the binding cavity,

with -2.84 ± 1.26 kcal/mol and -4.16 ± 1.26 kcal/mol for PT4_A-Tyr102 and PT4_A-Tyr115 respectively, and -5.45 ± 0.96 kcal/mol and -5.54 ± 2.14 kcal/mol for PT4_B-Tyr102 and PT4_B-Tyr115. Together with Arg360, His420 and Arg123, both Tyr102 and Tyr115 are conserved heme-contacting residues that formed hydrogen bonds with heme propionates through their hydroxyl side chains at least 99.4% over simulated time. The conserved position of these tyrosine residues near to heme group and consequent proximity with heme-inhibitors are important for the stabilization of cavity binding via side-chain interactions through benzene ring or hydroxyl moiety. The IPE values with the other SBC residues showed their extended wrap regarding PT4 presence. In addition to helix B', B'/G loop contacts, β4 strand 1–4 and helix I structures form noncovalent interactions with benzene-like moieties of PT4 whatever entering orientation. It is noteworthy the interactions with Ala286, Phe289, Ala290 (helix I) because the high proximity with this region could be related to the better activity of heme-coordinated inhibitors. This helix acts as an anchoring point for ligand avoiding their replacement in the active site by the substrate [71].

Concerning to the PT4 orientation inside the binding cavity, no significant differences were found on the spatial features and energetic values (Fig. 5C). The total IPE between PT4 and all surrounding residues showed similar values, -43.9 ± 3.99 kcal/mol and -49.3 ± 5.06 kcal/mol for PT4_A and PT4_B, respectively. Due to methodological limitations, a bond breaking/forming cannot be assessed, hence, the Fe_{heme}-N_{tyr} bond formation has not been described. This information could be properly evaluated by using a quantum method in further investigations. It has been already demonstrated that inactivation of the CYF51 in

Table 4
Effect of PT4 on the mitochondrial membrane potential of *Leishmania amazonensis* and *Leishmania braziliensis*.

<i>Leishmania amazonensis</i> Treatment	Rho 123 (MFI)	VI
Control	18,635.5	–
PT4 (70.46 μ M)	7,381.0	–0.61
PT4 (125.05 μ M)	7,255.5	–0.61
<i>Leishmania braziliensis</i> Treatment	Rho 123 (MFI)	VI
Control	26,985.0	–
PT4 (181.72 μ M)	5,969.0	–0.77
PT4 (303.04 μ M)	7,675.5	–0.72

IV: Index of Variation = $MF - MC/MC$, where MF is the median of fluorescence intensity in treated cells; MC is the mean of fluorescence intensity in untreated cells; MFI – median of fluorescence intensity.

Leishmania parasites can interfere with membrane stability, increasing its fluidity, causing the collapse of membrane lipid rafts and altered morphology [72].

Previous work has shown that inhibition of sterol biosynthesis by triazole derivatives, alone or in combination, affects the mitochondrial function of *L. amazonensis* promastigotes by inducing the dissipation of mitochondrial membrane potential and increased ROS production [65]. To investigate whether PT4 was able to interfere with the physiology of *L. braziliensis* and *L. amazonensis* single mitochondrion, promastigote forms were treated or not with IC_{50} and 2X IC_{50} of PT4 and submitted to staining with the fluorescent probes, Rhodamine 123 (for evaluation of mitochondrial membrane potential) and MitoSOX (for evaluation of mitochondrial ROS production). The treatment of parasites with PT4 caused a decrease in the fluorescence intensity for Rho 123 in both species and concentration tested (Table 4). Consistently, the treatment of parasites with PT4 also lead to mitochondrial membrane depolarization as indicated by the negative values of IV (Table 4).

Drug-induced alterations in the mitochondrial membrane potential in *Leishmania* species, have been associated with the increase of ROS, which may rupture the electron transport chain leading to mitochondrial collapse and, consequently, to cell death [72]. Thus, we investigated if the loss of mitochondrial membrane potential, induced by PT4 treatment, was followed by a shift in the mitochondrial ROS production by the promastigote forms of *L. amazonensis* and *L. braziliensis* (Fig. 6).

Our results showed an increased in the intensity of MitoSOX fluorescent signal in both *Leishmania* species treated with PT4, as observed by the displacement of fluorescence intensity curves to right in treated parasites, compared to control without treatment (negative control).

Our results suggested that PT4 induces mitochondrial dysfunction with marked reduction of $\Delta\Psi_m$ and increased ROS production. The increased ROS production in the single mitochondrion of *Leishmania* species, associated with mitochondrial membrane potential dissipation can lead the parasites to death [74]. Previous studies showed that molecules containing 1,2,3-triazole and phthalimide groups have a high potential to interfere with mitochondrial physiology by altering membrane potential and increasing ROS production [75].

4. Conclusions

Our results showed that the compound 2-[2-(4-phenyl-1H-1,2,3-triazol-1-yl)ethyl]-1H-isindole-1,3(2H)-dione (PT4), synthesized by click chemistry has good predicted pharmacokinetic properties which can be useful for the development of an oral formulation for the treatment of leishmaniasis. We also demonstrated that this compound was highly selective for the parasites inhibiting the growth and survival of promastigote and amastigote forms of *L. amazonensis* and *L. braziliensis*. The ultrastructural changes induced by PT4 corroborate the deleterious effects of this compound on the parasite membrane in both *Leishmania* species. Our molecular docking and molecular dynamic analysis pointed PT4 as a putative inhibitor of parasite sterol 14 α -demethylase, mainly by interacting with heme groups of this enzyme. The PT4 also caused dissipation of mitochondrial potential and increased production of mitochondrial ROS in *L. amazonensis* and *L. braziliensis*. Altogether, our results support further investigation of PT4 as a promising agent for the treatment of CL.

CRediT authorship contribution statement

Vanderlan Nogueira Holanda: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. Welson Vicente Silva: Investigation, Formal analysis. Pedro Henrique Nascimento: Investigation, Formal analysis. Sérgio Ruschi Bergamachi Silva: Resources, Investigation. Paulo Euzébio Gabriel Filho: Resources, Investigation. Shalom Porto Oliveira Assis: Resources, Investigation. César Augusto Silva: Resources, Investigation. Ronaldo Nascimento Oliveira: Resources, Investigation. Regina Celia Bressan Queiroz Figueiredo: Conceptualization, Methodology, Writing - review & editing, Visualization, Project administration, Funding acquisition. Vera Lucia Menezes Lima: Conceptualization, Methodology, Writing - review & editing, Visualization.

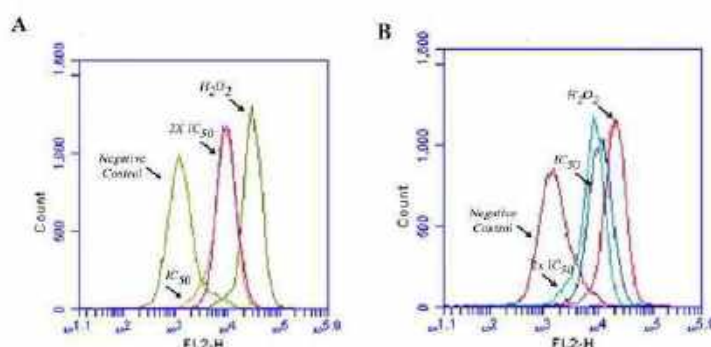


Fig. 6. Representative flow cytometric histograms of the effects of PT4 on ROS production in *L. amazonensis* (A) and *L. braziliensis* (B) promastigote forms, labelled with MitoSOX (FL2-H). Cells without treatment and treated with H_2O_2 were considered as negative and positive control respectively. FL2 - emission band pass filter (585/40 nm).

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.

Acknowledgments

This work was supported by the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Instituto Aggeu Magalhães/FIOCRUZ-PE. We would like to thank Prof. Dr. Norberto Monteiro (Federal University of Ceará - Brazil) for providing the computational resources used for MD simulations and analysis, Dr. Karina Lidianna Alcântara Sarriva and Dr. Antonio Pereira das Neves Neto for technical support, the Program for Technical Development of Health Inputs (PDIS/FIOCRUZ) for the use of its facilities and Dr. Adriana Fantes (Biomedical Nanotechnology research group - UFPE) for the use of flow cytometer.

Funding

This work was supported by the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE, BFT-109-2.05/18) Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, PQ-400/49/2019-0 and 31.2575/2018-6), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES code 001), Instituto Aggeu Magalhães and Inova-Fiocruz Program (VPPCB-007-MO-18-2-85). Vanderlan Nogueira Holanda would like to thank FACEPE for the graduate scholarship (IBPG-0333-2.05/18).

References

- [1] E.A. Le Rute, E.E. Zilberstein, S.A. Vilela, Post-Ebola Acute Dermal Leishmaniasis as a Reservoir for Visceral Leishmaniasis Transmission, *Trends Parasitol.* 35 (8) (2019) 590–592, <https://doi.org/10.1016/j.pt.2019.06.007>.
- [2] J. Blum, et al., *Leishmaniasis: Recommendations for Treatment of Cutaneous and Mucosal Leishmaniasis in Travelers*, 2019, J. Travel Med. 21 (2) (2013) 116–129, <https://doi.org/10.1111/jtms.12089>.
- [3] D.V. Costa, et al., American tegumentary leishmaniasis: a case of therapeutic challenge, *Anais Brasileiros de Dermatologia* 89 (2014) 924–926, <https://doi.org/10.1590/s0006-7081.2014.00025>.
- [4] Berthel, T. R. N. et al. Pentavalent Antimonials Combined with Other Therapeutic Alternatives for the Treatment of Cutaneous and Mucocutaneous Leishmaniasis: A Systematic Review. *Dermatology Research and Practice*, v. 2018, p. 9014726, 2018/12/24 2018. doi: <https://doi.org/10.1155/2018/9014726>.
- [5] N. Maspi, A. Abdoli, F. Ghaffarfar, Pro- and anti-inflammatory cytokines in cutaneous leishmaniasis: a review, *Pathogens Global Health* 119 (6) (2015) 247–260, <https://doi.org/10.1080/20447724.2015.1222942>.
- [6] S. Souda, J. Chakraborty, An update on pharmacotherapy for leishmaniasis, *Expert Opin. Pharmacother.* 16 (2) (2015) 237–252, <https://doi.org/10.1586/14737466.2015.1077060>.
- [7] S.M. Shamsian, S. Akhavan, Electrocardiographic and biochemical adverse effects of meglumine antimoniate (MA) during treatment of Spina cutaneous leishmaniasis patients, *J. Pakistan Assoc. Dermatol.* 22 (4) (2016) 412–417.
- [8] L.F. Geronzi, G. Gonçalves-Oliveira, E.E. Almeida-Amaral, 2'-Hydroxyflavone activity in vitro and in vivo against wild-type and antimony resistant *Leishmania* sensu stricto, *PLoS Negl Trop Dis.* 12 (12) (2018), e0006520, <https://doi.org/10.1371/journal.pntd.0006520>.
- [9] L.F. Oliveira, et al., Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World, *Asia Trop.* 118 (2) (2011) 87–96, <https://doi.org/10.1016/j.astrop.2011.02.007>.
- [10] S. Terzi, et al., 1,2,4-Triazole-based benzothiazole/benzoxazole derivatives: Design, synthesis, p38 MAP kinase inhibition, anti-inflammatory activity and molecular docking studies, *Bioorg. Chem.* 81 (2018/12/01/ 2018) 639–641, <https://doi.org/10.1016/j.bioorg.2018.09.015>.
- [11] S. Zhang, Comprehensive review on the anti-bacterial activity of 1,2,3 triazole hybrids, *Eur. J. Med. Chem.* 168 (2019) 357–372, <https://doi.org/10.1016/j.ejmech.2019.04.025>.
- [12] F. Xie, et al., Design, synthesis, and in vitro evaluation of novel triazined triazoles, *Bioorg. Med. Chem. Lett.* 27 (10) (2017) 2171–2173, <https://doi.org/10.1016/j.bmcl.2017.03.042>.
- [13] M. Ghobadipour, et al., Click chemistry-assisted synthesis of novel anticonvulsants 1,2,3-triazole hybrids and investigation of their cytotoxicity and enzyme cell cycle alterations, *Bioorg. Chem.* 88 (2019) 102967, <https://doi.org/10.1016/j.bioorg.2019.102967>.
- [14] X. M. Chu, et al., Triazole derivatives and their antileishmanial and antimicrobial activities, *Eur. J. Med. Chem.* 166 (2019) 206–228, <https://doi.org/10.1016/j.ejmech.2019.01.047>.
- [15] C. Xiao, et al., Acid-base-Joindly Promoted Copper(I) Catalyzed azide-alkyne Cycloaddition, *J. Org. Chem.* 76 (16) (2011) 6932–6936, <https://doi.org/10.1021/jo200663a>.
- [16] D. Dheer, V. Singh, R. Shandur, Medicinal antibodies of 1,2,3-triazoles Current developments, *Bioorg. Chem.* 71 (2017) 30–54, <https://doi.org/10.1016/j.bioorg.2017.01.010>.
- [17] Alves, D. et al. Copper Catalyzed and Organocatalysis Showing the Way: Synthesis of Selenium-Containing Highly Functionalized 1,2,3-Triazoles, *The Chemical Record*, v. 18, n. 5, p. 527–542, 2018/05/01 2018. ISSN 1527-8999. doi: <https://doi.org/10.1002/cr.201700038>.
- [18] C.D. Hoyle, X.M. Liu, D. Wang, Click Chemistry, A Powerful Tool for Pharmaceutical Research, *Pharm. Res.* 25 (10) (2008) 2216–2236, <https://doi.org/10.1007/s11095-008-9016-1>.
- [19] H.C. Killo, M.G. Flinn, E.B. Sharpless, Click Chemistry: Diverse Chemical Function from a Few Good Reactions, *Angew. Chem.* 113 (40) (11) (2001) 2004–2021, <https://doi.org/10.1002/ange.200100613>.
- [20] K. Kaminski, et al., Synthesis and anticonvulsant properties of new acetamide derivatives of phthalimide, and its saturated cyclohexane and norbornene analogs, *Eur. J. Med. Chem.* 46 (9) (2011) 4634–4641, <https://doi.org/10.1016/j.ejmech.2011.07.043>.
- [21] C.S. Kanchik, L. Mallesha, P. Mallu, Investigation of antioxidant properties of phthalimide derivatives, *Canadian Chem. Trans.* 3 (2015) 179–206, <https://doi.org/10.1371/journal.pone.0150319>.
- [22] Alami, A. M. et al. Structure-based design of phthalimide derivatives as potential cyclooxygenase 2 (COX-2) inhibitors: Anti-inflammatory and analgesic activities. *European Journal of Medicinal Chemistry*, v. 92, p. 115–123, 2015/03/06/ 2015. ISSN 0263-5724. doi: <https://doi.org/10.1016/j.ejmech.2014.11.039>.
- [23] L. Peng, et al., Synthesis of N-substituted phthalimides and their antifungal activity against *Alternaria solani* and *Botrytis cinerea*, *Microb. Pathog.* 95 (2016) 186–192, <https://doi.org/10.1016/j.micpath.2016.04.012>.
- [24] N. Baccouch, et al., N-ethyl-2-ethoxycarbonylphthalimide: Design, Synthesis and Evaluation as Analgesic and Anti-inflammatory Agent, *Mol. Rev. Med. Chem.* 10 (8) (2019) 679–687, <https://doi.org/10.2174/15680267186608031010094>.
- [25] C.R.A. Batista, et al., The phthalimide analogues N-3-hydroxypropylphthalimide and N-ethoxycarbonyl-3-methylphthalimide exhibit activity in experimental models of inflammatory and neuropathic pain, *Pharmacol. Rep.* 71 (6) (2019) 1177–1183, <https://doi.org/10.1016/j.pharep.2019.08.001>.
- [26] M. Kuchwaha, D. Kanchik, Recent advances and future prospects of phthalimide derivatives, *J. Appl. Pharm. Sci.* 6 (03) (2012) 159–171, <https://doi.org/10.7324/JAPS.2012.60303>.
- [27] L.A. Rodriguez, et al., Natural Products: Insights into Leishmaniasis Inflammatory Response, *Medicines Inflamm.* 2015 (2015), 835911, <https://doi.org/10.1155/2015/835911>.
- [28] Aires, S. P. G. et al. Design and synthesis of triazole-phthalimide hybrids with anti-inflammatory activity. *Chemical and Pharmaceutical Bulletin*, v. 67, n. 2, p. 96–105, 2019. ISSN 0009-2363. doi: <https://doi.org/10.1246/cpb.18-0067>.
- [29] U. Senet, et al., Azide-alkyne click for quick preparation of 1,4-disubstituted 1,2,3-triazoles, *Bull. Korean Chem. Soc.* 31 (7) (2010) 1843, <https://doi.org/10.1002/bkcs.2010.31.7.1843>.
- [30] M.T.D. Silva, et al., Synthesis of N-substituted phthalimide(ide)s: III-1,2,3-triazoles: a molecular diversity combining click chemistry and ultrasonic irradiation, *J. Braz. Chem. Soc.* 23 (2012) 1839–1853, <https://doi.org/10.1590/s0100-0662011006000043>.
- [31] T. Morozumi, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods* 65 (1–2) (1980) 55–63.
- [32] M.G. Hamel, et al., Averaging an enhanced immune chemical editor, classification, and analysis platform, *J. Cheminf.* 4 (1) (2012) 15.
- [33] J.J.P. Stewart, Optimization of parameters for semiempirical methods I Method, *J. Comput. Chem.* 10 (1989) 229–239, <https://doi.org/10.1002/jcc.550010230>.
- [34] P. Nesse, The ORCA program system, *Wiley Interdisciplinary Rev. Comput. Mol. Sci.* 2 (1) (2012) 73–78, <https://doi.org/10.1002/wcms.801>.
- [35] O. Trott, A.J. Olson, AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, *J. Comput. Chem.* 31 (2) (2010) 455–461, <https://doi.org/10.1002/jcc.21334>.
- [36] T.Y. Hughes, et al., Substrate preferences and catalytic parameters determined by structural characteristics of steryl 14 α -dehydrogenase (CYF31) from *Leishmania infantum*, *J. Biol. Chem.* 285 (30) (2011) 26836–26848, <https://doi.org/10.1074/jbc.M111.257095>.
- [37] M.J. Abraham, et al., GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers, *SoftwareX* 1 (2015) 19–25, <https://doi.org/10.1016/j.softx.2015.06.001>.
- [38] S. Pronk, et al., GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation tool, *Bioinformatics* 29 (7) (2013) 845–854, <https://doi.org/10.1093/bioinformatics/btt055>.
- [39] J. Huang, et al., CHARMM36m: an improved force field for folded and intrinsically disordered proteins, *Nat. Meth.* 14 (1) (2017) 71–73, <https://doi.org/10.1038/nmeth.4067>.

- [40] S. Kien, et al., CHARMM-GUI ligand reader and module for CHARMM free-energy generation of small molecules, *J. Comput. Chem.* 38 (21) (2017) 1879–1886, <https://doi.org/10.1002/jcc.24826>.
- [41] M.H.M. Olson, et al., PROPKA3: consistent treatment of internal and surface residues in empirical pKa predictions, *J. Chem. Theory Comput.* 7 (2) (2011) 529–537, <https://doi.org/10.1021/j100037a009>.
- [42] G. Inesi, D. Dorazio, M. Parrinello, Canonical sampling through velocity rescaling, *J. Chem. Phys.* 126 (1) (2007) 014101, <https://doi.org/10.1063/1.2408420>.
- [43] S. Miyazawa, P.A. Kollman, Scatter An analytical version of the SHAKE and RATTLE algorithm for rigid water models, *J. Comput. Chem.* 13 (8) (1992) 952–962, <https://doi.org/10.1002/jcc.10063>.
- [44] B. Hess, et al., LINCS: a linear constraint solver for molecular simulations, *J. Comput. Chem.* 18 (12) (1997) 1463–1472, <https://doi.org/10.1002/jcc.10012> (1998) 0673 (199709)18:12<1463::AID-JCC10012>3.0.CO;2-1.
- [45] W.J. Van Gunsteren, H.J.C. Berendsen, A leap-frog algorithm for stochastic dynamics, *Mol. Simul.* 1 (3) (1998) 173–187, <https://doi.org/10.1080/09027028008000941>.
- [46] T. Darden, D. York, L. Pedersen, Particle mesh Ewald: An N log (N) method for Ewald sums in large systems, *J. Chem. Phys.* 98 (12) (1993) 10089–10092, <https://doi.org/10.1063/1.464397>.
- [47] S.B. Silva, et al., The role of local residue environmental changes in the catalytic mechanism of the GHM1 xylanase from *Sacchara subtilis*, *Int. J. Biol. Macromol.* 97 (2017) 574–584, <https://doi.org/10.1016/j.ibioma.2017.01.054>.
- [48] T. Leising, et al., Synthesis and antileishmanial activity of fluorinated rhodocytine analogues: The “fluorine walk” analysis, *Bioorg. Med. Chem.* 28 (1) (2020), 115187, <https://doi.org/10.1016/j.biomc.2019.115187>.
- [49] S. Vijayakumar, et al., A pharmacoinformatic approach on G-protein-coupled receptor 2 (GPCR2) and different small molecules: Homology modelling, molecular docking, MD simulations, drug designing and ADMET analysis, *Comput. Biol. Chem.* 79 (2019) 95–107, <https://doi.org/10.1016/j.compbiolchem.2018.11.013>.
- [50] C.A. Lipinski, et al., Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, *Mol. Drug Deliv. Rev.* 23 (1) (1997) 3–25, [https://doi.org/10.1016/S0169-4098\(96\)00152-1](https://doi.org/10.1016/S0169-4098(96)00152-1).
- [51] Khan, A. A. et al. Advanced drug delivery to the lymphatic system: lipid based nanocarriers. *International Journal of Nanomedicine*, v. 8, p. 2713–2744, 2013. ISSN 1178-2013. 1178-9114. doi: 10.2147/IJN.S41521.
- [52] D. Laguerre, et al., Computational analysis of calculated photochemical and ADMET properties of protein-protein interaction inhibitors, *Sci. Rep.* 7 (1) (2017) 46277, <https://doi.org/10.1038/s41598-017-06277-7>.
- [53] A. Dalin, O. Micheliello, V. Zorzi, SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules, *Sci. Rep.* 7 (2017) 42512, <https://doi.org/10.1038/s41598-017-07373-7>.
- [54] Mani-Kandola, P.; Nagai, S. Cyclochrome P450: structure, function and clinical significance: a review. *Current drug targets*, v. 19, n. 1, p. 38–54, 2018. ISSN 1389-4501. doi: <https://doi.org/10.2174/1389450118056170125144577>.
- [55] Lynch, T.; Friet, A. L. The effect of cyclochrome P450 metabolism on drug response, interactions, and adverse effects. *American family physician*, v. 76, n. 3, p. 394–396, 2007. ISSN 0002-095X.
- [56] Anandak, J. R.; Gutierrez, P. G. Pharmacogenomics of triazole antifungal agents: implications for safety, tolerability and efficacy. *Expert Opinion on Drug Metabolism & Toxicology*, v. 13, n. 11, p. 1135–1146. doi: <https://doi.org/10.1080/17445019.2017.1394213>.
- [57] A.A. Elvir, et al., Infection with *Leishmania major* induces a cellular stress response in macrophages, *PLoS ONE* 9 (1) (2014), e85715, <https://doi.org/10.1371/journal.pone.0085715>.
- [58] C. Bagdas, et al., Fibroblasts as Host Cells in Latent Leishmaniasis, *J. Exp. Med.* 191 (12) (2000) 2122–2130, <https://doi.org/10.1084/jem.191.12.2122>.
- [59] Croft, S. L.; Yardley, V.; Skentzel, H. Drug sensitivity of *Leishmania* species: some unresolved problems. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, v. 96, p. 8127–8129, 2002/04/01/ 2002. ISSN 0950-2688. doi: [https://doi.org/10.1016/S0950-2688\(02\)90063-5](https://doi.org/10.1016/S0950-2688(02)90063-5).
- [60] A.C. Coelho, et al., In vitro and in vivo antileishmanial susceptibility of a *Leishmania amazonensis* isolate from a patient with diffuse cutaneous leishmaniasis follow-up, *PLoS Negl. Trop. Dis.* 10 (7) (2016), e0004720, <https://doi.org/10.1371/journal.pntd.0004720>.
- [61] Nagle, A. S. et al. Recent Developments in Drug Discovery for Leishmaniasis and Human African Trypanosomiasis. *Chemical Reviews*, v. 114, n. 22, p. 11309–11347, 2014/11/26 2014. ISSN 0007-2665. doi: <https://doi.org/10.1021/cr500565f>.
- [62] M.V. Papadopoulos, et al., Novel nitro-*crinazole* (imidazole)-based heterocyclic amides/ sulfonamides as potential antitrypanosomal agents, *Eur. J. Med. Chem.* 87 (2014) 79–88, <https://doi.org/10.1016/j.ejmech.2014.09.045>.
- [63] Morfitt, M.; Borop, A.; Nejkun, P. A new level of complexity in parasite host interaction: The role of extracellular vesicles. In: ROULINSON, D. & STOFFARD, J. R. (Eds.), *Advances in Parasitology*. Academic Press, v.104, p.39–112, 2019. doi: <https://doi.org/10.1016/b978-0-12-042003-0>.
- [64] M.A. Khajeh, V.A. Liguori, Involvement of Membrane Blebbing in Immunological Disorders and Cancer, *Med. Principles Pract.* 25 (2016) 16–27, <https://doi.org/10.1159/000441844>.
- [65] S.T. Macedo-Silva, et al., Fomea In Vitro Antiproliferative Synergism of Combination of Ergosterol Biosynthesis Inhibitors against *Leishmania amazonensis*, *Antimicrob. Agents Chemother.* 59 (10) (2015) 6402, <https://doi.org/10.1128/AAC.01301-15>.
- [66] L. L. McCall, et al., Targeting Ergosterol Biosynthesis in *Leishmania donovani*: Essentiality of Sterol 14alpha-demethylase, *PLoS Negl. Trop. Dis.* 9 (3) (2015), e0003388, <https://doi.org/10.1371/journal.pntd.0003388>.
- [67] S. Enami, P. Tavangar, M. Keighobadi, An overview of azoles targeting sterol 14 α -demethylase for antileishmanial therapy, *Eur. J. Med. Chem.* 135 (2017) 241–259, <https://doi.org/10.1016/j.ejmech.2017.04.014>.
- [68] G.I. Lepeshova, F. Villalba, M.B. Waterman, Targeting Trypanosoma cruzi sterol 14 α -demethylase (CYP51), In: *Advances in parasitology*, Academic Press, 2013, pp. 65–87, <https://doi.org/10.1016/B978-0-12-085823-3.00009-6>.
- [69] C.R. Chen, et al., Structural characterization of CYP51 from *Trypanosoma cruzi* and *Trypanosoma brucei* bound to the antifungal drugs posaconazole and fluconazole, *PLoS Negl. Trop. Dis.* 4 (4) (2010), e501, <https://doi.org/10.1371/journal.pntd.0000501>.
- [70] J.A. Urbina, et al., In vitro antiproliferative effects and mechanism of action of the new triazole derivative UT-9525 against the protozoan parasite *Trypanosoma (Setiocyprum) cruzi*, *Antimicrob. Agents Chemother.* 44 (9) (2000) 2496–2502, <https://doi.org/10.1128/AAC.44.9.2496-2502.2000>.
- [71] Lepeshova, G.; Waterman, M. Sterol 14 α -demethylase (CYP51) as a therapeutic target for human trypanosomiasis and leishmaniasis, *Current Topics in Medicinal Chemistry*, v. 11, n. 16, p. 2069–2071, 2011. doi: <https://doi.org/10.2174/156802611796077902>.
- [72] W. Xu, et al., Sterol Biosynthesis Is Required for Host Resistance but Not Extracellular Survival in *Leishmania*, *PLoS Pathog.* 10 (10) (2014), e1004427, <https://doi.org/10.1371/journal.ppat.1004427>.
- [73] R.F.S. Menno-Berrio, S.L. Carras, The Double-Edged Sword in *Trypanosoma*: The Phoral Role of Mitochondria in Oxidative Stress and Bioregulation, *Biochem. Res. Int.* 2014 (2014), e14014, <https://doi.org/10.1155/2014/14014>.
- [74] R.S. Michel, et al., Novel functionalized 1,2,3-triazole derivatives exhibit antileishmanial activity, increase in total and mitochondrial ROS and depolarization of mitochondrial membrane potential of *Leishmania amazonensis*, *Chem. Biol. Interact.* 313 (2020), 108850, <https://doi.org/10.1016/j.cb.2019.108850>.
- [75] Alloups, A. S. D. S. et al. In vitro evaluation of cytotoxicity and leishmanicidal activity of polihydroxy-triazole derivatives. *European Journal of Pharmaceutical Sciences*, v. 106, p. 1–10, 2017/07/16/ 2017. ISSN 0928-0987. doi: <https://doi.org/10.1016/j.ejps.2017.05.005>.

5.3 PATENTE DE INVENÇÃO

Foi realizado o pedido nacional de invenção, modelo de utilidade, certificado de adição de invenção e entrada na fase nacional junto ao Instituto Nacional de Propriedade Industrial (INPI). O referido pedido está registrado sob o número BR 10 2020 026525 3.



Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2020 026525 3

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: FUNDAÇÃO OSWALDO CRUZ

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 33781055000135

Nacionalidade: Brasileira

Qualificação Jurídica: Instituição de Ensino e Pesquisa

Endereço: AV. BRASIL, 4365, MANGUINHOS

Cidade: Rio de Janeiro

Estado: RJ

CEP: 21045-900

País: Brasil

Telefone: 0000-0000

Fax:

Email:

**PETICIONAMENTO
ELETRÔNICO**

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 23/12/2020 às 12:16, Petição 870200160790

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): DERIVADOS FTALIMÍDICOS-TRIAZÓLICOS ÚTEIS NO TRATAMENTO DE LEISHMANIOSE, COMPOSIÇÃO FARMACÊUTICA, USO DA COMPOSIÇÃO FARMACÊUTICA E MÉTODO DE TRATAMENTO DE LEISHMANIOSE

Resumo: A invenção descreve a aplicação terapêutica do composto da fórmula FT1 ou FT2 ou um sal farmacêuticamente aceitável do mesmo, no tratamento da leishmaniose. Composições farmacêuticas compreendendo os referidos compostos ou um sal farmacêuticamente aceitável do mesmo, e uso destas são também contempladas no presente pedido.

Figura a publicar: 1

Dados do Procurador**Procurador:**

Nome ou Razão Social: João Luis D'Orey Facco Vianna

Numero OAB:

Numero API: 256

CPF/CNPJ: 60674164768

Endereço: Rua Teófilo Otoni, 63 - 5º ao 8º andar

Cidade: Rio de Janeiro

Estado: RJ

CEP: 20090-080

Telefone: (21) 2113-1919

Fax: (21) 2113-1920

Email: joao.vianna@kasznarleonardos.com

Escritório:

Nome ou Razão Social: KASZNAR LEONARDOS PROPRIEDADE INTELECTUAL

CPF/CNPJ: 15305456000137

**PETICIONAMENTO
ELETRÔNICO**

Esta solicitação foi enviada pelo sistema Petição Eletrônica em 23/12/2020 às 12:16, Petição 870200160790

Dados do Inventor (72)

Inventor 1 de 5

Nome: REGINA CELIA BRESSAN QUEIROZ DE FIGUEIREDO

CPF: 00618117776

Nacionalidade: Brasileira

Qualificação Física: Biólogo, biomédico e afins

Endereço: Rua Setúbal 278, apt 201, Boa Viagem

Cidade: Recife

Estado: PE

CEP: 51030-010

País: BRASIL

Telefone:

Fax:

Email:

Inventor 2 de 5

Nome: WELSON VICENTE DA SILVA

CPF: 12650975423

Nacionalidade: Brasileira

Qualificação Física: Estudante de Graduação

Endereço: Praça Joaquim Nabuco, 10, Centro

Cidade: Recife

Estado: PE

CEP: 55050-000

País: BRASIL

Telefone:

Fax:

Email:

Inventor 3 de 5

**PETICIONAMENTO
ELETRÔNICO**

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 23/12/2020 às 12:16, Petição 870200160790

Nome: VANDERLAN NOGUEIRA HOLANDA

CPF: 05693627330

Nacionalidade: Brasileira

Qualificação Física: Biólogo, biomédico e afins

Endereço: Rua Emetério Maciel, 150, apt 204, Varzea

Cidade: Recife

Estado: PE

CEP: 50750-120

País: BRASIL

Telefone:

Fax:

Email:

Inventor 4 de 5

Nome: VERA LÚCIA DE MENEZES LIMA

CPF: 09419357449

Nacionalidade: Brasileira

Qualificação Física: Biólogo, biomédico e afins

Endereço: Rua Severino Meira, 20, Barro

Cidade: Recife

Estado: PE

CEP: 50900-240

País: BRASIL

Telefone:

Fax:

Email:

Inventor 5 de 5

**PETICIONAMENTO
ELETRÔNICO**

Esta solicitação foi enviada pelo sistema Petição Eletrônica em 23/12/2020 às 12:16, Petição 870200160790

Nome: RONALDO NASCIMENTO DE OLIVEIRA

CPF: 49664867420

Nacionalidade: Brasileira

Qualificação Física: Físico, químico, meteorologista, geólogo, oceanógrafo e afins

Endereço: Av. Rui Barbosa, 579, apt 603, Espinheiro

Cidade: Recife

Estado: PE

CEP: 52011-040

País: BRASIL

Telefone:

Fax:

Email:

Documentos anexados

Tipo Anexo	Nome
Comprovante de pagamento de GRU 200	P142748 GRU.pdf
Procuração	P142748 PROCURAÇÃO.pdf
Procuração	SUBSTABELECIMENTO.pdf
Relatório Descritivo	P142748 RELATÓRIO.pdf
Reivindicação	P142748 REIVINDICAÇÕES.pdf
Desenho	P142748 DESENHOS.pdf
Resumo	P142748 RESUMO.pdf

Acesso ao Patrimônio Genético

- ☒ Declaração Positiva de Acesso - Declaro que o objeto do presente pedido de patente de invenção foi obtido em decorrência de acesso à amostra de componente do Patrimônio Genético Brasileiro, realizado a partir de 30 de junho de 2000, e que foram cumpridas as determinações da Lei 13.123 de 20 de maio de 2015, informando ainda:

Número da Autorização de Acesso: A046FC9

Acesso:

Data da Autorização de Acesso: 26/03/2020

Origem do material genético e do conhecimento tradicional associado, quando for o caso

Trypanosoma cruzi

Leishmania spp.

Desenvolvimento de novos fármacos contra patógenos de interesse médico e veterinário

PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Petição Eletrônica em 23/12/2020 às 12:16, Petição 870200160790

Declaração de veracidade

☒ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.

**PETICIONAMENTO
ELETRÔNICO**

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 23/12/2020 às 12:16, Petição 870200160790

Kasznar
Leonardos

Rua Teófilo Ottoni, 63 - 5º ao 8º andares - 20090-080 - Rio de Janeiro - RJ - Brasil
Av. Nove de Julho, 3.147 - 7º e 11º andares - 01407-000 - São Paulo - SP - Brasil

PROCURAÇÃO GERAL – OUTORGANTES ESTABELECIDOS NO BRASIL
Patentes, Desenhos Industriais e Marcas

Outorgante: FUNDAÇÃO OSWALDO CRUZ

Endereço: AV. BRASIL, 4365, MANGUINHOS, 21045-900, RIO DE JANEIRO

pelo presente mandato outorga a

(i) Kasznar Leonardos Barbosa Colonna Rosman Vianna Agentes da Propriedade Industrial também denominada **KASZNAR LEONARDOS PROPRIEDADE INTELECTUAL**, CNPJ nº 15.305.456/0001-37, sociedade simples, Agente da Propriedade Industrial ("API") inscrita no Instituto Nacional da Propriedade Industrial - INPI sob nº 2297/2012, (ii) **KASZNAR LEONARDOS ADVOGADOS**, CNPJ nº 15.272.612/0001-00, sociedade de advogados inscrita na OAB, (iii) **Eduardo Colonna Rosman**, CPF 494.507.437-20, API nº 252, OAB/RJ 100.855; (iv) **Elisabeth Kasznar Fekete**, CPF 736.284.147-00, API nº 1295, OAB/SP 77.706; (v) **Filipe da Cunha Leonardos**, CPF 887.191.117-20, API nº 441, OAB/RJ 57.882; (vi) **Gabriel Francisco Leonardos**, CPF 002.515.997-60, API nº 251, OAB/RJ 64.537; (vii) **Gustavo José Ferreira Barbosa**, CPF 628.331.207-10, API nº 253, OAB/RJ 93.469; e (viii) **João Luis d'Orey Facco Vianna**, CPF 606.741.647-68, API nº 256, OAB/RJ 98.892, todos estabelecidos nos endereços acima, isoladamente, independentemente da ordem de nomeação, poderes para: (a) requerer registros de propriedade industrial, tais como os de marca, títulos de estabelecimento, indicações geográficas e de desenhos industriais; patentes, e certificados de adição; (b) requerer registros de programas de computador e de contratos relativos a programas de computador; (c) pagar quaisquer taxas de manutenção, bem como promover as prorrogações ou renovações cabíveis; (d) apresentar protestos, oposições, impugnações, pedidos de reconsideração e de nulidade administrativa, recursos, réplicas e defesas escritas ou orais para defesa ativa e passiva dos interesses do mandante; (e) requerer: buscas e certidões, a anotação de mudanças de nome ou sede, de transferências, de cessões de domínio e de gravame ou penhor, bem como a averbação e o registro de contratos de licença ou cessão de uso de quaisquer dos direitos acima mencionados, assim como de contratos de transferência de tecnologia, prestação de serviços de assistência técnica, franquia e assemelhados; (f) requerer devoluções de taxas ou retribuições, receber e dar quitação relativamente às mesmas; (g) retirar, desistir e transigir; (h) substabelecer no todo ou em parte os poderes aqui conferidos; (i) representar o Outorgante administrativamente; e (j) praticar tudo mais que for necessário ao bom e fiel cumprimento deste mandato. São aqui ratificados os atos eventualmente já praticados pelos outorgados em prol do Outorgante. Os poderes aqui conferidos poderão ser exercidos perante qualquer Repartição Pública ou Autárquica, Federal, Estadual ou Municipal, do Brasil, inclusive o INPI, a Agência Nacional de Vigilância Sanitária – ANVISA, o Banco Central do Brasil, os Bancos Comerciais e as Corretoras de Valores autorizadas a realizar o Registro Declaratório Eletrônico - RDE de contratos de transferência de tecnologia e licenciamento.

ESTA PROCURAÇÃO É VÁLIDA POR 03 ANOS CONTADO(S) DA DATA DE ASSINATURA.

Local e Data:

03/04/2018

Nome do Signatário:

Função ou Título:

Assinatura:
Nina Verônica Trindade Lima
Presidente

*Este modelo é adequado para outorgantes estabelecidos no Brasil.
O reconhecimento de firma não é necessário.*

SUBSTABELECIMENTO

KASZNAR LEONARDOS DALE BARBOSA COLONNA ROSMAN VIANNA – AGENTES DA PROPRIEDADE INDUSTRIAL, Sociedade Simples Pura, também operando sob a denominação KASZNAR LEONARDOS PROPRIEDADE INTELECTUAL, com sede nesta Cidade, na Rua Teófilo Otoni, nº 63, 6º andar, inscrita no CNPJ/MF sob o nº 15.305.456/0001-37, matriculada como Agente da Propriedade Industrial (API) sob o nº 2297/12 ("Substabeecedora"), neste ato representada por seu sócio-administrador, **GUSTAVO JOSÉ FERREIRA BARBOSA**, brasileiro, casado, portador da C.J. RG nº 3.414.686, expedida pelo IFP/RJ, inscrito como API sob o nº 253/98 e no CPF/MF sob o nº 628.331.207-10, residente e domiciliado nesta cidade, na Rua Eurico Melo, 35, Barra da Tijuca, e por seu sócio, **FILIPE DA CUNHA LEONARDOS**, brasileiro, Agente da Propriedade Industrial (API), casado, portador da carteira de identidade nº 4396782, expedida pelo IFP/RJ, inscrito no CPF/MF sob o nº 887.191.117-20, inscrito como API sob o nº 441/1998, residente e domiciliado na Cidade e Estado do Rio de Janeiro, à Rua Timóteo da Costa nº 1.033, bloco 2, apto. 1.004, Leblon, por este instrumento substabelece os poderes recebidos mediante procuração constante nos autos, ou que se encontra aqui anexa, em favor dos seguintes Substabelecidos:

Nome	Carteira de Identidade	Expedida por	CPF	API
ADRIANA LOUREIRO JOSÉ	072.88465-3	IFP/RJ	008.357.417-45	444/98
ALICE RAYOL RAMOS SANDES	10.094.561-7	IFP/RJ	081.000.137-30	2113/11
ANA LUCIA PEREIRA ROQUE	10.463.786-3	IFP/RJ	070.658.337-00	2119/11
ANDRÉ BASTOS VENTURINI	011.871.386-6	DIC/RJ	055.639.127-60	2120/11
ANDRÉ LUIS DE FARIA	09323362-5	IFP/RJ	011.733.987-32	436/98
ANTONIO CARLOS RAMOS	708255	SSP/MG	204.110.907-63	438/98
CLAUDIO ROBERTO BARBOSA	19219485	SSP/SP	138.453.388-57	1594/01
DENISE LEITE DE OLIVEIRA DALE	04.112.597-2	IFP/RJ	606.592.117-34	440/98
EDSON PAULA DE SOUZA	09.861.055-3	IFP/RJ	018.466.457-86	2148/11
EDUARDO COLONNA ROSMAN	02.762.505-2	IFP/RJ	494.507.437-20	252/98
ELISABETH KASZNAR PERETE	3.381.313	IFP/RJ	756.284.147-00	1295/98
FABIO DE ARAUJO OTTONI FERREIRA	11.749.313-0	IFP/RJ	081.759.257-10	1813/05
FILIPE DA CUNHA LEONARDOS	04.396.782	IFP/RJ	887.191.117-20	441/98
FLAVIA BENZATTI TREMURA POLLI RODRIGUES	27.583.583-1	SSP/SP	288.352.138-78	2165/11
GABRIEL FRANCISCO LEONARDOS	04.697.909-2	IFP/RJ	002.515.997-60	251/98
GISELA RIBEIRO GUSSMANN	10530143-6	IFP/RJ	069.629.147-92	1975/07
GUSTAVO JOSÉ FERREIRA BARBOSA	3.414.686	IFP/RJ	628.331.207-10	253/98
JOÃO LUIS D'OREY FACCO VIANNA	05.199.476-2	IFP/RJ	606.741.647-68	256/98
LIZ CARNEIRO LEÃO STARLING	04.785.262-9	IFP/RJ	721.634.537-15	254/98
MARCELO CANELLAS LEITE	06.387.929-0	IFP/RJ	776.435.427-91	1369/99
MARIA CLAUDIA SABATINI	10.313.943-2	IFP/RJ	068.489.467-02	2212/11
MAURICIO LEONARDOS	1.233.204	IFP/RJ	008.357.907-97	750/98
MONICA DE CASSIA PEREIRA DA MOTTA	07.039.382-2	IFP/RJ	899.192.897-87	439/98
PATRICIA SCHNEIDER JORGE	10.626.017-7	IFP/RJ	073.938.137-77	1844/05
PRESCILA MAYUMI KASHIWABARA	28.997.419-7	SSP/RJ	284.025.168-07	2237/13
RICARDO CARDOSO COSTA BOCLIN	08050715-5	IFP/RJ	976.131.857-53	1857/05
ROBERTA DE OLIVEIRA MENDES	94014013175	IIICE/CE	618.885.833-04	2246/11
RONALDO MAGALHÃES VARELLA GOMES	4.496.124	IFP/RJ	777.613.677-87	1394/99
SONIS DE MORAES SOUZA	1.590.576	IFP/RJ	097.703.997-87	1374/99
TATIANA ALMEIDA SILVEIRA	09444762-0	IFP/RJ	032.276.897-74	1677/03
THEREZA GONÇALVES CURI ABRANCHES	08808913-1	IFP/RJ	035.340.537-02	046/98
TOMAZ FRANCISCO LEONARDOS	1.029.718	IFP/RJ	008.359.007-20	249/98
WALTER LUIS	04.870.305-2	IFP/RJ	690.807.807-82	435/98

todos brasileiros, com escritório nesta Cidade do Rio de Janeiro, na Rua Teófilo Otoni nº 63, 6º andar, a quem substabelece poderes para serem exercidos **ISOLADAMENTE**, independente da ordem de nomeação, podendo os Substabelecidos representar clientes da Substabeecedora na assinatura de quaisquer documentos, requerimentos, petições, bem como

substabelecer, e, em especial, (i) requerer e obter registros propriedade industrial, tais como os de marcas de indústria, de comércio e de serviços, títulos de estabelecimento, expressões ou sinais de propaganda, indicações geográficas, patentes de privilégio de invenção, modelos de utilidade, certificados de adição e registros de desenhos industriais; (ii) requerer e obter registros de programas de computador e de contratos relativos a programas de computador; (iii) promover perante a autoridade competente a apresentação de provas de uso e o pagamento das anuidades, quinquênios ou quaisquer outras taxas de manutenção, bem como as prorrogações ou renovações cabíveis; (iv) apresentar protestos, oposições, impugnações, pedidos de reconsideração e de nulidade administrativa, recursos, réplicas e defesas escritas ou orais para defesa ativa e passiva dos interesses da mandante; (v) requerer e obter buscas e certidões, a anotação de mudanças de nome ou sede, de transferências, de cessões de domínio e de gravame ou penhor, bem como a averbação e o registro de contratos de licença para fabricação ou cessão de uso de quaisquer dos direitos acima mencionados e de contratos de transferência de tecnologia, prestação de serviços de assistência técnica, franquias e assemelhados; (vi) requerer devoluções de taxas ou retribuições, receber e dar quitação relativamente a tais taxas ou retribuições; (vii) desistir e transigir; e (viii) praticar tudo o mais que for necessário ao bom e fiel cumprimento deste mandato. São aqui ratificados os atos eventualmente já praticados pelos Substabelecidos, ficando certo, ainda, que os poderes ora enunciados poderão ser exercidos perante qualquer Repartição Pública ou Autárquica, Federal, Estadual ou Municipal do Brasil, inclusive o Instituto Nacional da Propriedade Industrial – INPI, a Agência Nacional de Vigilância Sanitária – ANVISA, o Conselho Nacional de Combate à Pirataria e Delitos Contra a Propriedade Intelectual – CNCP, e ainda perante o Banco Central do Brasil – BACEN. Para os devidos fins, este substabelecimento é aplicável a todas as procurações já outorgadas no passado, ou que venham a ser futuramente outorgadas à Substabelecidora, cessando automaticamente seus efeitos em relação ao Substabelecido que não mais prestar serviços à Substabelecidora.

Rio de Janeiro, 04 de fevereiro de 2013.

KASZNAR LEONARDOS PROPRIEDADE INTELECTUAL


Gustavo José Ferreira Barbosa


Filipe da Cunha Leonardos

180 Ofício de Notas - Japicião Luis Vitoriano Vieira Teixeira
Av. Presidente Vargas, 433 22. andar - RJ - Tel. 2597-6131 - Nº 966139
Reconhecido por semelhança af(1) firma(s): GUSTAVO JOSÉ FERREIRA BARBOSA#
0-98/14-SLT28556, FILIPE DA CUNHA LEONARDOS-91/4-SLT28557, #=====
Rio de Janeiro, 04 de Fevereiro de 2013 às 15:09:42
2- Em Testemunha da verdade,
FERNANDO RICHARTE DUARTE - Autorizado - JRG - 1487
Firma 0,76 + Bócos 3,40 + TEL 0,71 + Fundos 0,52 = R\$12,22



1 / 26

DERIVADOS FTALIMÍDICOS-TRIAZÓLICOS ÚTEIS NO
TRATAMENTO DE LEISHMANIOSE, COMPOSIÇÃO
FARMACÊUTICA, USO DA COMPOSIÇÃO FARMACÊUTICA E
MÉTODO DE TRATAMENTO DE LEISHMANIOSE

CAMPO DA INVENÇÃO

[001] A invenção pertence ao campo da farmacologia, com particular aplicação ao tratamento das leishmanioses.

ANTECEDENTES DA INVENÇÃO

[002] As leishmanioses são antropozoonoses causadas por protozoários do gênero *Leishmania*. São transmitidas por insetos vetores – flebotomíneos – do gênero *Lutzomyia* no novo mundo e do gênero *Phlebotomus* no velho mundo. O complexo das diferentes formas de leishmaniose afeta 12 milhões de pessoas no planeta (DESJEUX, 2004.).

[003] Segundo a Organização Mundial da Saúde (OMS, 2010), 90% dos casos de Leishmaniose Visceral ocorrem em Bangladesh, Brasil, Índia, Nepal e Sudão e 90% dos casos da forma cutânea ocorrem no Afeganistão, Brasil, Irã, Peru, Arábia Saudita e Síria. Por sua vez, a forma mucocutânea está presente em países como a Bolívia, Brasil e Peru (MS, 2017; WHO/OMS, 2010 e 2011). A OMS enquadra as leishmanioses entre as seis doenças infecto-parasitárias endêmicas de maior relevância, pelo seu alto coeficiente de detecção e capacidade de produzir deformidades (CHAPPUIS et al., 2007; WHO/OMS/TRS, 1990).

[004] As leishmanioses se manifestam em duas formas clínicas principais: leishmaniose visceral (I), que pode ser fatal se deixada sem tratamento, e tegumentar (II), cujas formas mucocutânea e cutânea causam lesões que geralmente se curam espontaneamente, mas podem deixar cicatrizes ou deformidades, o que levam essa doença infecciosa a ser classificada como uma das seis mais importantes do planeta dada a sua gravidade e prevalência (MARZOCHI e MARZOCHI, 1994; WHO, 2011;

MARZOCHI et al., 2014)

[005] A forma visceral é considerada a mais grave dentre as leishmanioses. Como o seu nome sugere, acomete as vísceras, afetando principalmente órgãos com alta concentração de leucócitos com destaque para medula óssea, fígado, baço e linfonodos levando frequentemente a anomalias no tamanho desses órgãos. Esta doença pode levar a óbito até 90% das pessoas acometidas pela doença se deixadas sem tratamento (CHAPPUIS et al., 2007; WHO/OMS, 2010 e 2011).

[006] A forma cutânea apresenta lesões clássicas que correspondem à ulceração de bordas elevadas, endurecidas e de fundo com tecido de granulação, que pode evoluir para a forma mucosa por disseminação hematogênica ou linfática do parasito (MARZOCHI, 1992; REY, 2008).

[007] O parasito causador da Leishmaniose é um protozoário da família Trypanosomatidae. Atualmente, as leishmanias que afetam o homem são classificadas em dois complexos fenotípicos agrupados em dois subgêneros, englobando várias espécies cada um deles. O subgênero *Viannia* que inclui os complexos *braziliensis*, *guianensis*, *lainsoni*, *naifi* e o *Leishmania* que inclui *L. mexicana*, *L. infantum*, *L. amazonensis*, *L. enrieti* e *L. hertigi* (MARZOCHI, 1992).

[008] Morfologicamente, o parasito pode apresentar três formas distintas: amastigota, promastigotas e paramastigota.

[009] A forma amastigota é obrigatoriamente intracelular, habitando o interior das células do sistema fagocítico mononuclear de hospedeiros mamíferos. Possui forma arredondada ou ovalada, com cinetoplasto em forma de bastão e flagelo restrito a bolsa flagelar (MARZOCHI, 1992).

[0010] A forma promastigota é alongada, apresenta flagelo livre, é móvel com núcleo localizado na região central da célula, o cinetoplasto entre a região anterior e o núcleo apresenta granulações e pequenos vacúolos. Esta forma habita o trato digestório dos hospedeiros invertebrados (MARZOCHI,

3 / 26

1992; MICHALICK e RIBEIRO, 2011).

[0011] As formas paramastigotas apresentam forma ovaladas ou arredondadas semelhantemente às amastigotas, com cinetoplasto margeando o núcleo ou posterior a este, pequeno flagelo livre e são encontradas aderidas ao trato digestório do vetor.

[0012] O ciclo de vida das Leishmanias se inicia quando a fêmea do flebotomíneo realiza o repasto sanguíneo em um hospedeiro mamífero infectado e ingere formas amastigotas do parasito, livres na circulação do hospedeiro ou no interior de macrófagos. Se o sangue ingerido durante esse processo possuir parasitos, ele vai para o intestino médio do inseto e as amastigotas transformam-se em promastigotas procíclicas, após cinco dias transformam-se em promastigotas metacíclicas, voltando a ser infectante aos hospedeiros mamíferos.

[0013] As promastigotas metacíclicas migram para a probóscide do vetor e durante o hematofagismo são inoculadas no hospedeiro vertebrado (BATES, 1994). A presença de moléculas como a lipopolisacaridase e a proteína GP63 fazem com que estas formas resistam à ação lítica do complemento permitindo rápida fagocitose por células do sistema imune como neutrófilos, células dendríticas e, principalmente, macrófagos que foram atraídos até o local da inoculação iniciando uma reação inflamatória à infecção (GUEIRARD et al., 2008).

[0014] Após o processo de internalização e formação do vacúolo parasitóforo, as formas promastigotas deste parasito diferenciam-se em formas amastigotas, aptas a se multiplicarem. Essas formas multiplicam-se em divisão binária até a ruptura da célula liberando na circulação as amastigotas que poderão infectar novas células vizinhas e novos vetores que venham a realizar o repasto neste hospedeiro infectado, podendo inclusive infectar animais domésticos que funcionarão como reservatórios da doença (SILVEIRA, 2004).

4 / 26

[0015] Dentre as espécies relacionadas à ocorrência da Leishmaniose Visceral, a *Leishmania infantum* é comumente identificada como agente causador da infecção no Brasil. O parasita possui duas formas evolutivas, a promastigota, que se desenvolve no hospedeiro invertebrado (vetor) e a amastigota, forma de desenvolvimento em hospedeiros vertebrados, incluindo o ser humano (STUART et al., 2008).

[0016] Por não existir uma vacina eficaz que impeça a infecção por *Leishmania*, o combate à doença é pautado em medidas sanitárias e, principalmente, no tratamento. Os tratamentos para as Leishmanioses podem ser tópicos ou sistêmicos dependendo da espécie de *Leishmania* envolvida na infecção, região geográfica e apresentação clínica dos sintomas (MONGE-MAILLO e LÓPEZ-VÉLEZ, 2013).

[0017] O tratamento de primeira escolha para leishmaniose visceral são os antimoniais pentavalentes. Em casos mais graves, ou em pacientes que não respondem adequadamente ao tratamento com os antimoniais, a anfotericina B é a segunda escolha. Em último caso, o fármaco de escolha é a pentamidina. Todos esses tratamentos apresentam inadequações como baixa taxa de cura, efeitos adversos tais como nefrotoxicidade, esplenotoxicidade, pancreatotoxicidade, cardiotoxicidade, que sugerem a urgência na descoberta ou desenvolvimento de novas medicações ou alternativas para aumentar a eficiência e diminuir a toxicidade ao paciente (CROFT et al., 2006; BRASIL, 2017).

[0018] De modo geral, os fármacos aplicados ao tratamento sistêmico das leishmanioses são os antimoniais pentavalentes – estibogluconato de sódio e antimoniato de meglumina – e outros agentes antileishmaniais como anfotericina B, a pentamidina, a miltefosina e a paromomicina. No entanto, tais opções terapêuticas apresentam elevada toxicidade para vários órgãos e sistemas, limitando a ampla utilização e subsidiando o desenvolvimento de novas abordagens farmacológicas com maior eficácia e menos efeitos

adversos (PETERSEN, 2012; TIUMAN, 2011).

[0019] Compostos heterocíclicos nitrogenados, obtidos através de rotas sintéticas, vêm ganhando destaque pelo seu potencial antiparasitário e a sua baixa toxicidade (YANG et al., 2010). Em particular, derivados heterocíclicos derivados da classe ftalimidas têm sido estudados no desenvolvimento de fármacos por suas amplas atividades biológicas tais como: ação antimicrobiana (AKGÜN et al., 2012), anti-inflamatória (BACH et al., 2017; DA SILVA et al., 2019), antidiurética (EL-AZAB et al., 2016), antifúngica (PAN et al., 2016). As ftalimidas são derivadas do ácido ftálico, com característica estrutural – CO-N(R)-CO e um anel imida (grupo funcional constituído por 2 grupamentos carbonil ligados ao nitrogênio). Estas moléculas são hidrofóbicas, neutras podendo atravessar facilmente as membranas biológicas atingindo alvos específicos nos patógenos.

[0020] Outra classe de heterocíclicos nitrogenados de grande importância para a química medicinal é a dos compostos triazólicos (1,2,4-triazol e 1,2,3-triazol). Estas moléculas são utilizadas como ligantes de dois ou mais grupos farmacofóricos e desde o aprimoramento das técnicas de síntese orgânica, os grupos triazólicos têm sido utilizados na obtenção de novos compostos bioativos (EFTHYMIU et al., 2012; WANG et al., 2016). Esta classe de compostos possui propriedades químicas relevantes para sua interação com alvos moleculares com alta afinidade e solubilidade facilitada como por exemplo a formação de ligações do tipo pontes de hidrogênio (HAIDER et al., 2014). Estas propriedades tornam os compostos triazólicos interessantes, não só como compostos bioativos, por si só, mas também como blocos moleculares na síntese orgânica (KUMAR et al., 2017). Recentemente foi ainda observado que, além de ser utilizado para hibridação molecular e unir grupos farmacofóricos, o 1,2,3-triazol também exerce efeito farmacológico sobre células e tecidos biológicos, se tornando um excelente arcabouço na produção de medicamentos (BOZOROV et al., 2019).

6 / 26

[0021] No contexto das leishmanioses, compostos que aliem o efeito leishmanicida dos triazóis com a atividade anti-inflamatória das ftalimidas se apresentam como benéficos no tratamento desta doença, pois estes organismos normalmente causam uma intensa reação inflamatória agravando ainda mais a saúde dos pacientes acometidos.

[0022] Moléculas contendo grupos 1,2,3,- triazol ou ftalimida isoladamente já foram testadas contra tripanosomatídeos (GOMES et al., 2016; MEINEL et al., 2020). Compostos híbridos que combinam os núcleos triazol e ftalimida já foram divulgados (SILVA, 2018), mas tais derivados ftalimida-triazol, que incluem também um anel 1,4-naftoquinônico, apresentaram baixa seletividade às espécies tratadas, baixa atividade e/ou toxicidade elevada em células de macrófagos.

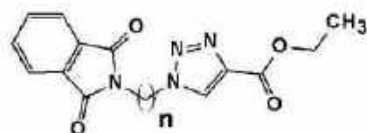
[0023] Portanto, há necessidade de prover moléculas que se mostrem eficazes contra as diferentes espécies de protozoários causadores das leishmanioses, seletivas aos parasitos e que não apresentam citotoxicidade.

[0024] Neste contexto, o objeto desta invenção representa uma alternativa quimioterápica às abordagens farmacológicas disponíveis para tratamento de leishmaniose. Objetivamente, descreve-se a atividade leishmanicida dos derivados ftalimídicos-triazólicos 1- [2- (1,3-dioxo-1,3-di-hidro-2H-isoindol-2-il) etil] -2,3-di-hidro-1H-1,2,3-triazol-4-carboxilato de etila (FT1) e 1- [3- (1,3-dioxo-1,3-di-hidro-2H-isoindol-2-il) propil] -1H-1,2,3-triazol-4-carboxilato de etila (FT2), ou sais farmaceuticamente aceitáveis do mesmo e sua potencial aplicação no tratamento de leishmaniose.

SUMÁRIO DA INVENÇÃO

[0019] A invenção propõe o uso do composto da fórmula FT1 ou FT2

7/26



FT1 = n: 2

FT2 = n: 3

ou um sal farmacêuticamente aceitável do mesmo, para preparar um medicamento para tratamento de leishmaniose.

[0020] O conceito inventivo que se apresenta também compreende o referido composto de fórmula FT1 ou FT2, ou um sal farmacêuticamente aceitável do mesmo, para uso no tratamento de leishmaniose.

[0021] São também aqui divulgadas composições farmacêuticas contendo o composto de fórmula FT1 ou FT2, ou um sal farmacêuticamente aceitável do mesmo, e pelo menos um excipiente farmacêuticamente aceitável. Ainda, outros aspectos da invenção envolvem uma composição farmacêutica que contém outros ingredientes ativos com atividade leishmanicida além do composto de fórmula FT1 ou FT2, ou um sal farmacêuticamente aceitável do mesmo.

[0022] Consequentemente, as composições farmacêuticas da invenção também podem ser usadas para preparar um medicamento útil no tratamento de leishmaniose.

[0023] Ainda, a invenção prevê um método de tratamento de leishmaniose que compreende a administração de uma quantidade terapeuticamente eficaz do composto de fórmula FT1 ou FT2, ou de um sal farmacêuticamente aceitável do mesmo, a um indivíduo que sofre de leishmaniose.

BREVE DESCRIÇÃO DAS FIGURAS

Figura 1 - Avaliação do efeito dos compostos das fórmulas FT1 e FT2 sobre a ultraestrutura de formas promastigotas *L. amazonensis* e *L. infantum*

8 / 26

[0024] As formas promastigotas de *L. amazonensis* (La) e *L. infantum* (Li) foram incubadas na presença de 1x e 2x a concentração IC₅₀ dos compostos das fórmulas FT1 e FT2.

Figura 2 - Avaliação do efeito do composto de fórmula FT1 sobre a ultraestrutura sobre promastigotas de *L. infantum*

[0025] As células a-b correspondem às células de controle e as células c-d correspondem àquelas tratadas com o composto de fórmula FT1, com 0,5x (c-d), 1x (e-f) e 2x (g-h) o valor da IC₅₀. A seta branca fina indica o enrugamento de membrana plasmática; e a seta preta com bordas branca aponta para a perda/ausência do flagelo.

Figura 3 - Avaliação do efeito do composto de fórmula FT2 sobre a ultraestrutura sobre promastigotas de *L. infantum*.

[0026] Células a-b correspondem às células de controle e as células c-h são aquelas tratadas com o composto de fórmula FT2, com 0,5x (c-d), 1x (e-f) e 2x o valor da IC₅₀ (g-h). A seta branca fina (D) indica a presença de vesículas exocíticas; a seta branca fina (F) aponta para o enrugamento de membrana plasmática; a seta preta com bordas branca mostra a perda/ausência do flagelo; e a seta preta com bordas cinza indica a depressão no corpo celular.

DESCRIÇÃO DETALHADA DA INVENÇÃO

[0026] A presente invenção aborda o uso do composto 1- [2- (1,3-dioxo-1,3-di-hidro-2H-isoindol-2-il) etil] -2,3-di-hidro-1H-1,2,3-triazol-4-carboxilato de etila ou 1- [3- (1,3-dioxo-1,3-di-hidro-2H-isoindol-2-il) propil] -1H-1,2,3-triazol-4-carboxilato de etila – também designado ao longo do texto por composto de fórmula FT1 ou FT2, respectivamente - ou de um sal farmaceuticamente aceitável do mesmo, para preparar um medicamento para tratar leishmaniose. O invento tem base na identificação inédita da atividade leishmanicida do composto de fórmula FT1 ou FT2, conforme está ilustrado nos exemplos.

[0027] Quando aqui usado, o termo “sal farmaceuticamente aceitável” refere-se a sais dos compostos de fórmula FT1 ou FT2 que são substancialmente não tóxicos para organismos vivos. Sais farmaceuticamente aceitáveis dos compostos de fórmula FT1 e FT2 podem ser formados com uma base inorgânica ou orgânica que produza um cátion farmaceuticamente aceitável. Tais sais com bases inorgânicas ou orgânicas incluem, por exemplo, um sal de metal alcalino, tal como um sal de sódio ou potássio, um sal de metal alcalino terroso tal como um sal de cálcio ou magnésio, um sal de amônio ou, por exemplo, um sal com metilamina, dimetilamina, trimetilamina, piperidina, morfolina ou tris-(2-hidroxietil) amina. Sais farmaceuticamente aceitáveis e métodos comuns para preparar os mesmos são bem conhecidos na técnica - vide, por exemplo, PUDIPEDDI ET AL, 2002; BERGE ET AL, 1977.

[0022] O composto de fórmula FT1 ou FT2 para uso no tratamento de leishmaniose, composições farmacêuticas contendo uma das referidas substâncias de fórmula FT1 ou FT2, o uso destas composições para preparar medicamento para tratar leishmaniose e métodos de tratamento de leishmaniose também estão compreendidos no escopo da invenção, que é descrita detalhadamente a seguir.

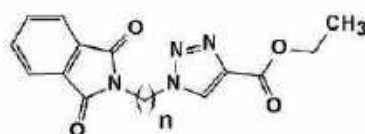
[0023] A não ser que seja definido de maneira distinta, todos os termos técnicos e científicos aqui utilizados têm o mesmo significado entendido por um técnico no assunto ao qual a presente invenção pertence. O relatório descritivo também provê definições de termos para auxiliar na interpretação daquilo que é aqui descrito e das reivindicações. A terminologia utilizada na descrição da invenção tem finalidade de descrever concretizações preferenciais somente, sem qualquer intenção de limitar o escopo de seus ensinamentos.

[0024] Salvo quando indicado em contrário, os parâmetros numéricos mostrados no relatório descritivo são aproximações que podem variar

10/26

dependendo das propriedades a serem obtidas.

[0025] O cerne da invenção compreende o uso do composto de fórmula FT1 ou FT2



FT1 = n: 2

FT2 = n: 3

ou um sal farmaceuticamente aceitável do mesmo, para preparar um medicamento para tratamento de leishmaniose. Em um aspecto particular da invenção, o medicamento preparado com o composto de fórmula FT1 ou FT2, ou um sal farmaceuticamente aceitável do mesmo, é aplicado ao tratamento de leishmaniose tegumentar e visceral, mucocutânea e cutânea. Particularmente, o referido medicamento é para uso no tratamento de leishmaniose cutânea ou visceral. Mais particularmente, o referido medicamento é útil no tratamento de leishmaniose causada pela espécie *L. amazonensis*, *L. braziliensis* ou *L. infantum*.

[0026] Em uma modalidade da invenção, o composto de fórmula FT1 ou FT2, ou um sal farmaceuticamente aceitável do mesmo, é para uso no tratamento de leishmaniose. Em um aspecto, a leishmaniose é leishmaniose tegumentar e visceral, mucocutânea e cutânea. Particularmente, o composto de fórmula FT1 ou FT2 é para uso no tratamento de leishmaniose cutânea ou visceral. Mais particularmente, o composto de fórmula FT1 ou FT2 é para o tratamento de leishmaniose causada pela espécie *L. amazonensis*, *L. braziliensis* ou *L. infantum*.

[0027] Outra modalidade da invenção compreende composições farmacêuticas que contêm o composto de fórmula FT1 ou FT2, ou um sal farmaceuticamente aceitável do mesmo. As composições farmacêuticas da invenção podem ser preparadas e formuladas de acordo com os métodos

11/26

convencionais, tais como divulgados, por exemplo, nas Farmacopeias Britânicas, Europeias e dos Estados Unidos e em Remington: a ciência e prática da farmácia (GENNARO, A. R. Remington, a ciência e a prática da farmácia. Guanabara Koogan, 2004).

[0028] No contexto da invenção, as composições farmacêuticas podem compreender, além do composto de fórmula FT1 ou FT2, ou um sal farmaceuticamente aceitável do mesmo, um ou mais excipientes farmaceuticamente aceitáveis e um veículo.

[0029] O termo “excipiente farmaceuticamente aceitável” se refere a substâncias que não são o ingrediente ativo de uma composição farmacêutica, mas são necessárias à sua formulação. De maneira geral, são considerados seguros para uso médico e veterinário de acordo com os padrões governamentais estabelecidos. São exemplos não exaustivos de excipientes farmaceuticamente aceitáveis: agentes dispersantes, desintegradores, aglutinantes, preenchedores, lubrificantes, agentes surfactantes e conservantes.

[0030] Os veículos podem ser, por exemplo, água, tampões (por exemplo, solução salina tamponada com fosfato), solventes orgânicos, tais como álcoois ou glicerina, glicóis, álcoois alifáticos, óleo mineral, óleo vegetal e dimetil sulfóxido; misturas de água e solventes orgânicos e misturas de solventes orgânicos, tais como álcool glicerina; materiais à base de lipídios, como ácidos graxos, acilgliceróis, fosfoglicerídeos, esfingolípidos e ceras; materiais à base de proteínas, como colágeno e gelatina; materiais à base de silicone (voláteis e não voláteis); e materiais à base de hidrocarbonetos, como microcomponentes e matrizes poliméricas.

[0031] As referidas composições farmacêuticas podem ser formuladas para qualquer via de administração, incluindo, por exemplo, administração tópica, oral, nasal, retal ou parenteral. O termo parenteral, como aqui utilizado, inclui injeção subcutânea, injeção intradérmica, injeção

12 / 26

intravascular (por exemplo, intravenosa), injeção intramuscular, injeção espinhal, injeção intracraniana, injeção intratecal e injeção intraperitoneal, bem como qualquer técnica semelhante de injeção ou infusão.

[0032] As composições farmacêuticas podem ser formuladas para serem liberadas a uma taxa predeterminada. A liberação instantânea pode ser obtida, por exemplo, por administração sublingual (isto é, administração pela boca de tal maneira que o(s) ingrediente(s) ativo(s) é/são rapidamente absorvidos pelos vasos sanguíneos do plexo sublingual).

[0033] A taxa de liberação pode ser variada usando métodos bem conhecidos na técnica, incluindo (a) variação da espessura da composição do revestimento, (b) alteração da quantidade da maneira de adição de plastificante em um revestimento (c) inclusão de ingredientes adicionais, tais como agentes que modificam a liberação, (d) alteração da composição, tamanho das partículas ou formato das partículas da matriz e (e) fornecimento de uma ou mais passagens através do revestimento. A quantidade de modulador contido em uma formulação de liberação sustentada depende, por exemplo, do método de administração (por exemplo, a localização do implante), a taxa e a duração da liberação esperada e a natureza da condição a ser tratada ou impedido.

[0034] Em um aspecto da invenção, as composições podem compreender, além do composto de fórmula FT1 ou FT2, ou um sal farmaceuticamente aceitável do mesmo, outros ingredientes ativos com atividade leishmanicida.

[0035] O termo “leishmanicida” designa uma substância capaz de promover a morte de parasitos pertencentes a qualquer uma das espécies do gênero *Leishmania*, tanto *in vitro* quanto *in vivo*.

[0036] Em concretizações particulares destas composições, a substância com ação leishmanicida adicional pode ser selecionada de compostos antimoniais pentavalentes, pentamidina e anfotericina B.

13 / 26

[0037] Uma modalidade adicional da invenção compreende o uso de qualquer uma das possíveis concretizações das referidas composições farmacêuticas para preparar um medicamento para tratar leishmanioses. Em um aspecto particular da invenção, o medicamento preparado com as referidas composições farmacêuticas é aplicado ao tratamento de leishmaniose tegumentar e visceral, mucocutânea e cutânea. Particularmente, o referido medicamento é para o tratamento de leishmaniose cutânea ou visceral. Mais particularmente, o referido medicamento é útil no tratamento de leishmaniose causada pela espécie *L. amazonensis*, *L. braziliensis* ou *L. infantum*.

[0038] Ainda, outra modalidade da invenção compreende um método de tratamento de leishmaniose que compreende administrar uma quantidade terapêuticamente eficaz do composto de fórmula FT1 ou FT2, ou um sal farmacêuticamente aceitável do mesmo, a um indivíduo infectado com qualquer um dos parasitos pertencentes a qualquer uma das espécies do gênero *Leishmania*. Em um aspecto particular, o indivíduo está acometido com leishmaniose tegumentar e visceral, mucocutânea e cutânea, mais particularmente leishmaniose cutânea ou visceral. Mais particularmente, o referido indivíduo está infectado com a espécie *L. amazonensis*, *L. braziliensis* ou *L. infantum*.

[0039] A expressão “terapeuticamente eficaz”, conforme usada aqui, depende da condição de um indivíduo e do composto específico administrado. O termo refere-se a uma quantidade eficaz para alcançar um efeito clínico desejado. Uma quantidade terapêuticamente eficaz varia de acordo com a natureza da condição a ser tratada, o período temporal de cobertura da atividade desejada, a idade e a condição do indivíduo e, em última análise, é determinada pelo profissional de saúde.

[0040] Embora várias formas de realização que incorporam os ensinamentos da invenção tenham sido aqui mostradas e descritas em detalhes, àqueles versados na técnica será possível vislumbrar prontamente

14 / 26

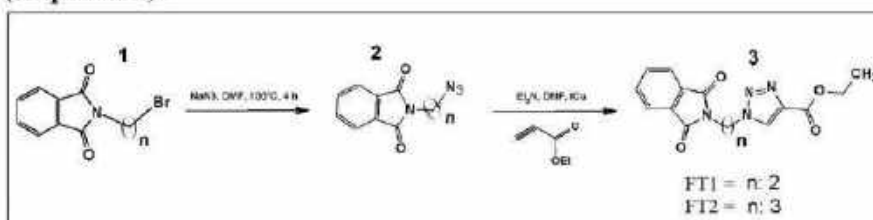
muitas outras formas de realização variadas que possam ainda incorporar esses ensinamentos. Quaisquer aspectos equivalentes se destinam a estar dentro do escopo desta invenção. De fato, várias modificações da invenção adicionalmente àquelas mostradas e descritas aqui se tornarão aparentes aos peritos na técnica a partir da descrição precedente. Tais modificações também se destinam a estar dentro do escopo das reivindicações a seguir.

[0041] A invenção será agora descrita por meio de exemplos, os quais pretendem apenas ilustrar as possíveis concretizações englobadas pelo conceito inventivo aqui descrito, não sendo, de modo algum, limitativos quanto ao escopo e a abrangência da presente invenção.

EXEMPLOS

EXEMPLO 1: OBTENÇÃO E SOLUBILIZAÇÃO DOS COMPOSTOS SINTÉTICOS 1- [2- (1,3-DIOXO-1,3-DI-HIDRO-2H-ISOINDOL-2-IL) ETIL] -2,3-DI-HIDRO-1H-1,2,3-TRIAZOL-4-CARBOXILATO DE ETILA (FT1) OU 1- [3- (1,3-DIOXO-1,3-DI-HIDRO-2H-ISOINDOL-2-IL) PROPIL] -1H-1,2,3-TRIAZOL-4-CARBOXILATO DE ETILA (FT2)

[0042] A síntese e caracterização dos compostos de fórmulas FT1 e FT2 foram realizadas de acordo com o procedimento previamente descrito na literatura (SILVA et al., 2012), cujo esquema simplificado se encontra abaixo (Esquema 1).

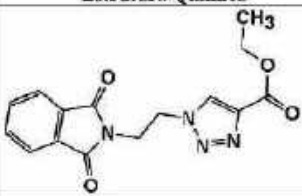
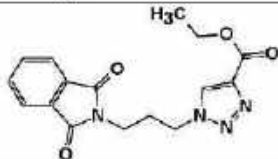


Esquema 1: Esquema simplificado da síntese dos compostos de fórmula FT1 e FT2.

[0043] A estrutura, nomenclatura química e massa molecular dos compostos de fórmulas FT1 e FT2 encontram-se na **Tabela 1** a seguir.

15 / 26

Tabela 1: Estrutura química, nomenclatura e peso molecular dos compostos de fórmulas FT1 e FT2

Código	Estrutura Química	Nomenclatura Química	Massa Molecular
FT1		1- [2- (1,3-dioxo-1,3-di-hidro-2H-isolindol-2-il) etil] -2,3-di-hidro-1H-1,2,3-triazol-4-carboxilato de etila	314,3 g/mol
FT2		1- [3- (1,3-dioxo-1,3-di-hidro-2H-isolindol-2-il) propil] -1H-1,2,3-triazol-4-carboxilato de etila	328,3 g/mol

[0044] Para concretizar os exemplos a seguir, os compostos foram solubilizados em DMSO e obtido uma concentração de 50 mg/mL, seguido de um aquecimento por 5 min a 70 °C. Após a total solubilização completa, os compostos foram dissolvidos em diferentes concentrações em meio de cultura de modo que a porcentagem de DMSO nas maiores concentrações testadas foi inferior a 1%, concentração não citotóxica para as células. As soluções estoque foram mantidas a temperatura ambiente e protegidas da luz até o uso.

EXEMPLO 2: CITOTOXICIDADE E ATIVIDADE LEISHMANICIDA *IN VITRO*

Material e Métodos

Análise Estatística

[0045] Os dados dos experimentos foram obtidos por meio de pelo menos três ensaios independentes feitos em triplicata. As análises de regressão linear foram feitas no programa SPSS 18.0 (IBM Co., New York, USA) para Windows. As análises de significância, considerado significativo valores $p < 0.05$, foram realizadas através do teste ANOVA e pós-tese de Dunnet no programa GraphPad Prism 5.0 (Graphpad, California, USA) para Windows.

Cultivo dos parasitas

[0046] Formas promastigotas de *L. amazonensis* (LTB0016), *L.*

16 / 26

braziliensis (LTB2903) e *L. infantum* (BH46) foram mantidas a 26°C em meio Schneider suplementado com 10% de soro fetal bovino. Cultura de promastigotas contendo 2×10^6 parasitas/mL foram incubadas a 26°C na ausência e presença de diferentes concentrações dos compostos descritos acima. O crescimento celular foi avaliado nos tempos de 24, 48 e 72h por contagem direta em câmara de Neubauer. Alternativamente, foram quantificados a produção de ATP, utilizando a bioluminescência através do kit Molecular ATP determination (Thermo Fisher, USA) seguindo a determinação do fabricante. O efeito dos compostos sobre o crescimento foi estimado através da IC_{50} (concentração da droga capaz de inibir em 50% a proliferação dos parasitas) após 48h de tratamento. Foram realizados dois ensaios independentes em duplicata. Formas promastigotas não tratadas foram consideradas como controle negativo.

Avaliação do potencial citotóxico sobre células de linhagem de mamíferos

[0047] Macrófagos J774A.1 ATCC® e Fibroblastos ATCC® PCS-201-012™ foram incubadas na presença ou ausência (controle) de várias concentrações dos compostos teste por 48 horas. Após a incubação com as drogas as células foram submetidas a técnica de MTT (MOSMANN, 1983). As amostras foram lidas por espectrofotometria 570 nm. Alternativamente, também foi utilizado o método de quantificação de ATP como descrito acima. A concentração citotóxica para 50% das células (CC_{50}) foi determinada através de regressão linear, tanto para o MTT quanto para o kit de ATP. A citotoxicidade dos compostos foi comparada com a atividade leishmanicida, através da determinação do índice de seletividade, pela fórmula $IS = CC_{50} \text{ célula hospedeira} / IC_{50} \text{ promastigota}$. Os compostos de fórmula FT1 e FT2 apresentaram $IS \geq 10$, apresentando-se como promissores para investigações (IOSET, 2009) e, então, submetidos à análise ultraestrutural.

Análise Estrutural

[0048] Para a análise do efeito dos compostos de fórmula FT1 e FT2

17 / 26

sobre a ultraestrutura dos parasitas por microscopia eletrônica de varredura (MEV), formas promastigotas de *L. amazonensis* e *L. infantum*, submetidas ou não (controle) ao tratamento com 1x e 2 x a IC_{50} dos compostos de fórmula FT1 e FT2 (**Figura 1**) e formas promastigotas de *L. infantum* submetidas ou não (controle) ao tratamento com 0,5x, 1x e 2x a IC_{50} dos compostos de fórmula FT1 e FT2 (**Figuras 2 e 3**) foram fixadas com uma solução de 2,5% glutaraldeído 2,5%, paraformaldeído 4%, em cacodilato de sódio 0,1 M, pH 7,2 durante 60 min a 4°C. As amostras foram pós-fixadas em uma solução contendo 1% OsO_4 , 0,8% ferricianeto de potássio, 5 mM $CaCl_2$ em tampão cacodilato 0,1M, e então desidratadas em baterias crescentes de etanol, metalizados com ouro para observação no microscópio SEM 510 Jeol.

[0049] As células de controle apresentavam uma morfologia usual, com o corpo celular fusiforme, flagelo preservado e membrana intacta.

Avaliação da toxicidade

[0063] Na avaliação da citotoxicidade, postula-se que compostos com CC_{50} superior que 500 $\mu g/mL$ são considerados com baixa citotoxicidade e CC_{50} entre 100 a 500 $\mu g/mL$ são considerados moderadamente tóxicos (RÍOS et al., 2008).

Resultados

Citotoxicidade em células de mamíferos e efeito leishmanicida em promastigotas de *L. amazonensis*, *L. braziliensis* e *L. infantum*

[0064] A análise do potencial citotóxico em células de mamíferos (macrófagos J774A.1 ATCC® e Fibroblastos ATCC® PCS-201-012™) a atividade leishmanicida dos compostos das fórmulas FT1 e FT2 demonstraram que estes compostos foram eficazes em inibir as formas promastigotas de *L. amazonensis*, *L. braziliensis* e *L. infantum*, apresentaram baixa toxicidade em células de mamíferos e foram muito mais seletivos para os parasitos em relação as células do hospedeiro (**Tabela 2**).

Tabela 2. Efeito leishmanicida e citotoxicidade dos compostos das fórmulas

18 / 26

FT1 e FT2 sobre células de mamíferos.

Comp.	La	Lb	Li	J774	Fib	Índice de Seletividade		
	IC ₅₀ (µM) 48h			CC ₅₀ (µM) 48h		La	Lb	Li
FT1	205,4	50,4	121,3	2887,36	3030,35	14,05	57,4	23,3
FT2	85,2	151,9	126,5	2578,44	2374,90	30,26	16,97	20,3

Legenda: Comp.: composto; Fib: fibroblastos; La: *Leishmania amazonensis*; Lb: *Leishmania braziliensis*; Li: *Leishmania infantum*; IC₅₀: concentração inibitória de 50% da cultura de parasitos; CC₅₀: concentração citotóxica em 50% da cultura de células de mamíferos; IS: índice de seletividade.

[0065] Conforme mostrado na Tabela 2, os compostos das fórmulas FT1 e FT2 apresentaram baixa citotoxicidade para células de mamíferos, sendo FT1 com CC₅₀ de 907,50 µg/mL (2887,36 µM), para células de macrófagos da linhagem J774 e 952,40 µg/mL (3030,35 µM) em fibroblastos. Já o derivado FT2 apresentou valores semelhantes com CC₅₀ de 846,550 µg/mL (2578,44 µM) e 779,73 µg/mL (2374,90 µM) para células de macrófagos da linhagem J774 e fibroblastos, respectivamente.

[0066] Os compostos das fórmulas FT1 e FT2 foram ainda seletivos contra o parasita com índice de seletividade de 14,05; 57,4 e 23,3 para as espécies de *L. amazonensis*, *L. braziliensis* e *L. infantum*, respectivamente). Estes valores são substancialmente superiores aos referidos na literatura para os fármacos de referência, as quais são altamente tóxicas para a célula do hospedeiro e pouco seletivas para o parasita. Esta constatação constitui uma vantagem dos compostos das fórmulas FT1 e FT2 em relação aos compostos já disponíveis para o tratamento das leishmanioses.

Alterações ultraestruturais causadas pelos compostos das fórmulas FT1 e FT2 sobre promastigotas de *L. amazonensis* e *L. infantum*

[0067] A partir da determinação da concentração inibitória dos compostos das fórmulas FT1 e FT2 nas formas promastigotas, investigou-se o efeito dos sobre a ultraestrutura nas células de *Leishmania*. A análise através da microscopia eletrônica de varredura (MEV) revelou que o tratamento com os compostos das fórmulas FT1 e FT2 induziu alterações expressivas, de forma dose dependente, na morfologia das promastigotas das espécies *L. amazonensis* e *L. infantum*, conforme mostrado nas Figuras 1 a 3, como

19 / 26

diminuição do corpo celular, enrugamento de membrana plasmática, encurtamento do flagelo, aumento no número de vesículas exocíticas e a presença de debris celulares, provavelmente resultado da ruptura dos parasitas. Holanda et al. (2020) relataram danos bastante semelhantes em *L. braziliensis* e *L. amazonensis*, agentes etiológicos da leishmaniose cutânea no Brasil, tratadas com o derivado de ftalimida e triazol o 4-fenil-1-[2-(ftalimido-2-il)etil]-1H-1,2,3-triazol. Estas alterações são compatíveis com a perda de viabilidade e morte celular (ELMORE, 2007; GALLUZZI et al., 2007).

[0068] Portanto, compostos das fórmulas FT1 e FT2 mostraram-se efetivos e seletivos contra as formas promastigotas do parasita, especialmente das espécies *L. amazonensis*, *L. braziliensis* e *L. infantum*, tendo induzido alterações ultraestruturais no parasita compatíveis com a perda da viabilidade celular em concentrações não citotóxicas para macrófagos e fibroblastos.

EXEMPLO 3: AVALIAÇÃO DOS PARÂMETROS FARMACOCINÉTICOS E TOXICOLÓGICOS *IN SILICO*

[0069] Após a determinação do efeito leishmanicida e identificação das alterações ultraestruturais em *L. amazonensis* e *L. infantum*, os compostos das fórmulas FT1 e FT2 foram submetidos a análise *in silico* dos parâmetros farmacocinéticos e farmacológicos, para avaliação do potencial farmacoterapêutico e vias de administração.

Materiais e Métodos

Análise dos parâmetros farmacocinéticos e toxicológicos *in silico*

[0070] A predição da absorção, distribuição, metabolismo, excreção e toxicidade (parâmetros ADMET) dos compostos das fórmulas FT1 e FT2 foi realizada a partir da análise na plataforma de linha SwissADME, Target Prediction (Swiss Institute of Bioinformatic, Lausanne, Suíça), Servidor PROTOX -II (Universidade de Charité, Berlim, Alemanha). Para os parâmetros físico-químicos, potencial de biodisponibilidade oral, semelhanças com outros componentes e os demais utilizou-se também o software OSIRIS

20 / 26

Data Explorer e DataWarrior versão 4.7.

[0050] O perfil farmacocinético e toxicológico de novos medicamentos é uma variável importante e que deve ser investigada durante o processo de produção de novas moléculas bioativas (DAINA et al, 2016). Para um fármaco ser administrado por via oral é necessário que a molécula possa apresentar propriedades físico-químicas adequadas para serem absorvidas no trato gástrico intestinal, conforme pode ser apreciado na **Tabela 3** (LIPINSKI, 2001).

Tabela 3. Parâmetros ideais para formulação de fármacos com potencial de tratamento por via oral (DAINA et al, 2016)

Parâmetro	Referência
MM	150 – 500 g/mol
Polaridade	TPSA entre 20 e 130
n-RotB	Não mais do que 9 ligações rotativas
HBA	≤10
HBD	≤5
C-LogP	≤5

Legenda: MM: massa molecular; n-RotB: número de ligações rotativas; HBA: número de aceptores de ligação hidrogênio; HBD: número de doadores de ligação hidrogênio; C-LogP: Log do Coeficiente de Partição octanol-água Calculado.

Resultados

[0051] A análise preditiva *in silico* das propriedades farmacocinéticas dos compostos das fórmulas FT1 e FT2 mostraram que estas moléculas apresentaram perfil farmacocinético e toxicológico desejáveis para o desenvolvimento de formulações orais e/ou tópicas. Entre elas, o fato de ambas as moléculas não violarem nenhuma das Regras de Lipinsky, não funcionarem como potenciais inibidores do complexo enzimático CYP450, responsável pelo metabolismo de fármacos, e não ultrapassarem a barreira hematoencefálica (**Tabela 4**).

Tabela 4. Avaliação dos Parâmetros Farmacocinéticos e Toxicológicos *in silico*

Propriedades	FT1	FT2
Propriedades Físico-químicas		
HBA	6	6
HBD	0	0
C-logP	1,13	1,49

21 / 26

Propriedades	FT1	FT2
MM (g/mol)	314,3	328,3
n-ROTB	6	7
Farmacocinética		
BBB	Não	Não
HIA	Alto	Alto
P-GPs	Não	Não
Metabolismo		
CYP450 2C9 inibidor	Não	Não
CYP450 2D6 inibidor	Não	Não
CYP450 2C19 inibidor	Não	Não
CYP450 3A4 inibidor	Não	Não
CYP450 1A2 inibidor	Não	Sim
Toxicidade		
MUT	Não	Não
TUM	Não	Não
IRR	Não	Não
DL ₅₀ (mg/Kg)	2500	4000
Percentual de Interação com Alvos Celulares		
KIN	53%	13%
PRO	6%	0%
GPRE	20%	26%
OUTROS	21%	61%

Legenda: HBA: número de aceptores de ligação hidrogênio; HBD: número de doadores de ligação hidrogênio; C-LogP: Log do Coeficiente de Partição octanol-água Calculado; MM: massa molecular; n-ROTB: número de ligações rotativas; BBB: Barreira hematoencefálica; HIA: absorção gastrointestinal; P-GPs: glicoproteínas P; MUT: mutagênico; TUM: tumorigênico; IRR: irritabilidade; DL₅₀: dose necessária causar a morte de 50% de uma população teste; KIN: kinase; PRO: protease, GPre: receptor acoplado à proteína G (família A)

REFERÊNCIAS BIBLIOGRÁFICAS

- [0052] AKGÜN, H. et al. Synthesis and antimycobacterial activity of some phthalimide derivatives. **Bioorganic & medicinal chemistry**, v. 20, n. 13, p. 4149-4154, 2012. ISSN 0968-0896.
- [0053] BACH, D.-H. et al. Synthesis and biological activity of new phthalimides as potential anti-inflammatory agents. **Bioorganic & medicinal chemistry**, v. 25, n. 13, p. 3396-3405, 2017.
- [0054] BATES, P. A. Complete developmental cycle of *Leishmania mexicana* in axenic culture. **Parasitology**, v. 108, n. 01, p. 1-9, 1994.
- [0055] BERGE, S.M., BIGHLEY, L.D., AND MONKHOUSE, D.C. Pharmaceutical salts. **J. Pharm. Sci.** v. 66, p. 1-19, 1977.
- [0056] BOZOROV, K.; ZHAO, J.; AISA, H. A. 1,2,3-Triazole-containing hybrids as leads in medicinal chemistry: A recent overview.

22 / 26

Bioorganic & Medicinal Chemistry, v. 27, n. 16, p. 3511-3531, 2019.

[0057] BRASIL. MINISTÉRIO DA SAÚDE. SECRETARIA DE VIGILÂNCIA EM SAÚDE. Departamento de Vigilância das Doenças Transmissíveis. Manual de vigilância da leishmaniose tegumentar. Brasília: Ministério da Saúde, 2017. 189 p.

[0058] CHAPPUIS, F. et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? **Nature reviews microbiology**, v. 5, n. 11, p. 873-882, 2007.

[0059] CROFT, S. L.; SUNDAR, S.; FAIRLAMB, A. Drug Resistance in Leishmaniasis. **C. Mic. Reviews**. v. 19, n. 1, p. 111-126, 2006.

[0060] DAINA, A.; MICHIELIN, O.; ZOETE, V. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. **Scientific reports**, v. 7, p. 42717-42717, 2017.

[0061] DA SILVA, M. T. et al. Design and Synthesis of Triazole-Phthalimide Hybrids with Antiinflammatory Activity. **Chemical and Pharmaceutical Bulletin**, v. 67, n. 2, p. 96-105, 2019.

[0062] DESJEUX, P. Leishmaniasis: current situation and new perspectives. **Comparative immunology, microbiology and infectious diseases**, v. 27, n. 5, p. 305-318, 2004.

[0063] EFTHYMIU, T.; GONG, W.; DESAULNIERS, J.-P. Chemical Architecture and Applications of Nucleic Acid Derivatives Containing 1,2,3-Triazole Functionalities Synthesized via Click Chemistry. **Molecules**, v. 17, n. 11, p. 12665-12703, 2012.

[0064] EL-AZAB, A. S. et al. Inhibition of carbonic anhydrase isoforms I, II, IV, VII and XII with carboxylates and sulfonamides incorporating phthalimide/phthalic anhydride scaffolds. **Bioorganic & medicinal chemistry**, v. 24, n. 1, p. 20-25, 2016.

[0065] ELMORE, S. Apoptosis: a review of programmed cell death.

Toxicologic pathology, v. 35, n. 4, p. 495-516, 2007.

[0066] GALLUZZI, L. et al. **Cell death modalities: classification and pathophysiological implications**: Nature Publishing Group 2007.

[0067] GENNARO, A. R. **Remington, a ciência e a prática da farmácia**. Guanabara Koogan, 2004.

[0068] GOMES, P. A. T. D. M. et al. Phthalimido-thiazoles as building blocks and their effects on the growth and morphology of *Trypanosoma cruzi*. **European Journal of Medicinal Chemistry**, v. 111, p. 46-57, 2016/03/23/ 2016.

[0069] GUEIRARD, P. et al. Trafficking of *Leishmania donovani* promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. **Cellular microbiology**, v. 10, n. 1, p. 100-111, 2008.

[0070] HAIDER, S.; ALAM, M.S.; HAMID, H. 1, 2, 3-Triazoles: scaffold with medicinal significance. **Inflammation and Cell Signaling**, v. 1, n. 2, 1-12, 2014.

[0071] IOSET, J. R.; BRUN, R.; WENZLER, T.; KAISER, M.; YARDLEY, V. Drug Screening for Kinetoplastids Diseases: a Training Manual for Screening in Neglected Diseases. **Drugs for Neglected Diseases - The Pan-Asian Screening Network**, v. 1, n. 1, p. 74, 2009.

[0072] KUMAR, B; SINGH, V.; SHANKAR, R.; KUMAR, K.; RAWAL, R.K. Synthetic and Medicinal Prospective of Structurally Modified Curcumins. **Curr Top Med Chem**. v. 17, n. 2, p. 148-161, 2017.

[0073] LIPINSKI, C. A; LOMBARDO, F.; DOMINY, B.W.; FEENEY, P.J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. **Adv. Drug Deliv. Rev**, v. 46, n. 1-3, p. 3, 2001.

[0074] MARZOCHI, M. C. de A. Curso-Doenças infecto-parasitárias: leishmanioses no Brasil: as leishmanioses tegumentares. **J. bras. Med**, v. 63,

n. 5/6, p. 82-104, 1992.

[0075] MARZOCHI, M. C. A.; MARZOCHI, K. B. F. Tegumentary and visceral leishmanioses in Brazil – Emerging anthroponosis and possibilities for their control. **Cad. Sau. Publica.**, v. 10, n. 2, p. 359-375. 1994.

[0076] MARZOCHI, M. C. A.; MARZOCHI, K. B. F.; FACUNDES, A.; CONCEIÇÃO-SILVA, F. A questão do controle das Leishmanioses no Brasil. In: CONCEIÇÃO-SILVA, F.; ALVES, C. R (Org.). **Leishmanioses do Continente Americano**. Rio de Janeiro: Fiocruz, 2014. v. 1, p. 431-463.

[0077] MEINEL, R.S.; ALMEIDA, A.D.C.; STROPPIA, P.H.F.; GLANZMANN, N.; COIMBRA, E.S.; DA SILVA, A.D. Novel functionalized 1,2,3-triazole derivatives promote antileishmanial activity, increase in total and mitochondrial-ROS and depolarization of mitochondrial membrane potential of *Leishmania amazonensis*. **Chemico-Biological Interactions**, v. 315, p. 108850-108859, 2019.

[0078] MICHALICK, M. S. M.; RIBEIRO, R. R. Gênero *Leishmania*. In: NEVES, D.P. **Parasitologia Humana**. 12 ed. São Paulo: Editora Atheneu, 2011. Cap 7, p 41-44.

[0079] MONGE-MAILLO, B.; LÓPEZ-VÉLEZ, R. Therapeutic options for old world cutaneous leishmaniasis and new world cutaneous and mucocutaneous. **Drugs**, v. 73, p. 1889–1920, 2013.

[0080] MOSMANN, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. **Journal of Immunological Methods**, v. 65, n. 1, p. 55-63, 1983.

[0081] PAN, L. et al. Synthesis of N-substituted phthalimides and their antifungal activity against *Alternaria solani* and *Botrytis cinerea*. **Microbial pathogenesis**, v. 95, p. 186192, 2016. ISSN 0882-4010.

[0082] PETERSEN, A. L. de O. A. et al. 17-AAG kills intracellular *Leishmania amazonensis* while reducing inflammatory responses in infected

macrophages. **PLoS One**, v. 7, n. 11, p. e49496, 2012.

[0083] PUDIPEDDI, M.; SERAJUDDIN, A.T.M.; GRANT, D.J.W.; STAHL, P.H. Solubility and Dissolution of Weak Acids, Bases and Salts. In: STAHL, P.H.; WERMUTH, C.G. **Handbook of Pharmaceutical Salts: Properties, Selection and Use**. Zürich: Wiley-VCH, 2002. Cap.2, p.19-40.

[0084] REY, L. **Parasitologia**. Guanabara Koogan, 4. ed. Rio de Janeiro, 2008.

[0085] RÍOS, Y. K. et al. Actividad citotóxica y leishmanicida in vitro del aceite esencial de manzanilla (*Matricaria chamomilla*). **Revista Colombiana de Ciencias QuímicoFarmacéuticas**, v. 37, n. 2, 2008.

[0086] SILVA, J. F. M. *Avaliação do Potencial Leishmanicida de Derivados Fialimido-1,4-Naftoquinonas e Fialimido-1,2,3-Triazolil-1,4-Naftoquinonas*. 2018. 73 f. Dissertação (Mestrado em Ciências da Saúde) – Instituto de Ciências Biológicas e da Saúde, Universidade Federal de Alagoas, Maceió. 2018.

[0087] SILVA, M. T.; OLIVEIRA, R. N.; VALENÇA, W. O.; BARBOSA, F. C. G.; SILVA, M. G.; CAMARA, C. A. Synthesis of N-substituted phthalimidoalkyl 1H-1,2,3-triazoles: a molecular diversity combining click chemistry and ultrasound irradiation. **Journal of the Brazilian Chemical Society**, v. 23 n. 10, p. 1839-1843, 2012.

[0088] SILVEIRA, F. T.; LAINSON, R.; CORBETT, C. E. P. Clinical and Immunopathological Spectrum of American Cutaneous Leishmaniasis with Special Reference to the Disease in Amazonian Brazil - A Review. **Mem. Inst. Oswaldo Cruz**, Rio de Janeiro, v. 99, n. 3, p. 239-251, 2004.

[0089] STUART, K. et al. Kinetoplastids: related protozoan pathogens, different diseases. **The Journal of clinical investigation**, v. 118, n. 4, p. 1301-1310, 2008.

[0090] TIUMAN, T. S. et al. Recent advances in leishmaniasis

treatment. **International Journal of Infectious Diseases**, v. 15, n. 8, p. e525-e532, 2011.

[0091] WANG, W. et al. Protective effect of PEGylation against poly (amidoamine) dendrimer-induced hemolysis of human red blood cells. **J. Biomed. Mater. Res.**, v. 93, n. 1, p. 59-64, 2010.

[0092] WHO (WORLD HEALTH ORGANIZATION). Control of the leishmaniasis. Technical Report Series. WHO, Geneva, n. 793, p. 158, 1990.

[0093] WHO/OMS – World Health Organization/Programmes and projects/Leishmaniasis 07/04/2010

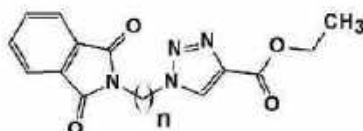
[0094] WHO, Expert Committee on the Control of the Leishmaniasis. II. World Health Organization. III. Series. www.who.int/leishmaniasis/en; Pesquisado em 07/04/11. 2011.

[0095] YANG, Y. J. et al. Synthesis and cytotoxic activity of heterocycle-substituted phthalimide derivatives. **Chinese Chemical Letters**, v. 21, n. 8, p. 902-904, 2010.

1 / 2

REIVINDICAÇÕES

1. Uso do composto de fórmula FT1 ou FT2

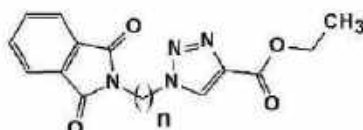


FT1 = n: 2

FT2 = n: 3

ou um sal farmacêuticamente aceitável do mesmo,
caracterizado pelo fato de que é para preparar um
 medicamento para tratamento de leishmaniose.

2. Composto de fórmula FT1 ou FT2

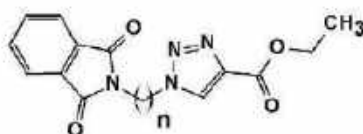


FT1 = n: 2

FT2 = n: 3

ou um sal farmacêuticamente aceitável do mesmo,
caracterizado pelo fato de que é para uso no tratamento de
 leishmaniose.

3. Composição farmacêutica caracterizada pelo fato de que
 compreende o composto de fórmula FT1 ou FT2



FT1 = n: 2

FT2 = n: 3

ou um sal farmacêuticamente aceitável do mesmo e um
 excipiente farmacêuticamente aceitável.

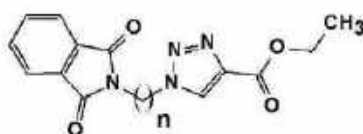
2 / 2

4. Composição farmacêutica, de acordo com a reivindicação 3, caracterizada pelo fato de que compreende adicionalmente um ou mais ingredientes ativos com atividade leishmanicida.

5. Composição farmacêutica, de acordo com a reivindicação 4, caracterizada pelo fato de que o um ou mais ingredientes ativos é selecionado do grupo que consiste em: compostos antimoniais pentavalentes, pentamidina e anfotericina B.

6. Uso de uma composição farmacêutica, como definida em qualquer uma das reivindicações 3 a 5, caracterizado pelo fato de que é preparar um medicamento para tratamento de leishmaniose.

7. Método de tratamento de leishmaniose caracterizado pelo fato de que compreende administrar uma quantidade terapeuticamente eficaz do composto de fórmula FT1 ou FT2



FT1 = n: 2

FT2 = n: 3

ou de um sal farmacêuticamente aceitável do mesmo, a um indivíduo com leishmaniose.

1/3

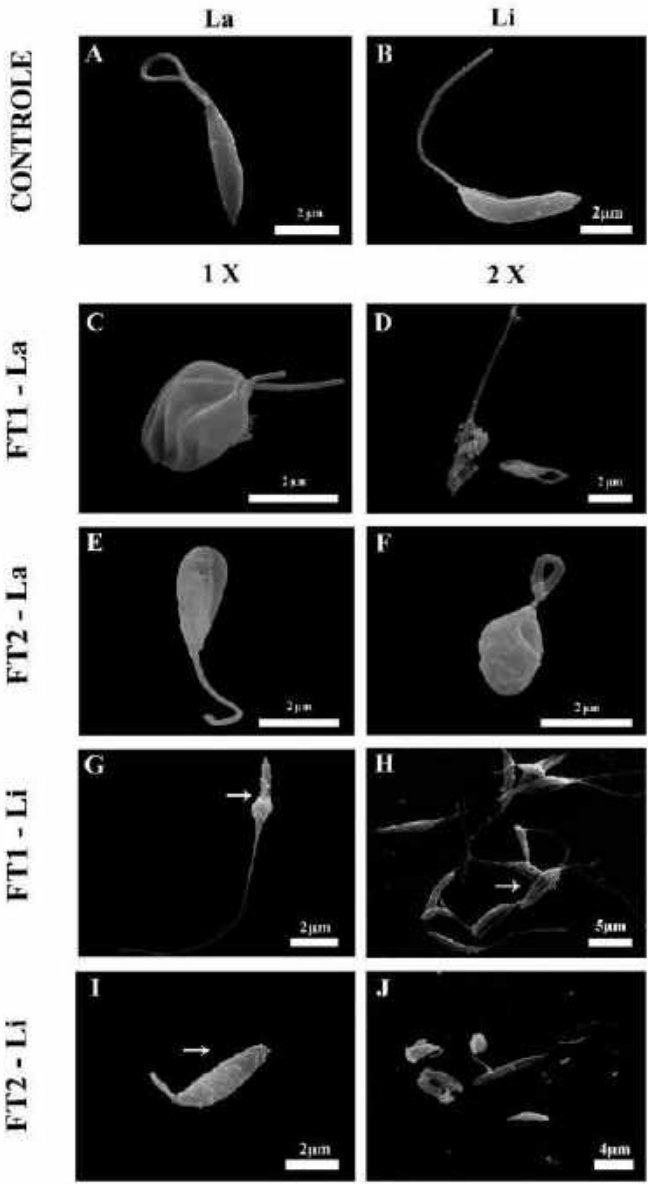
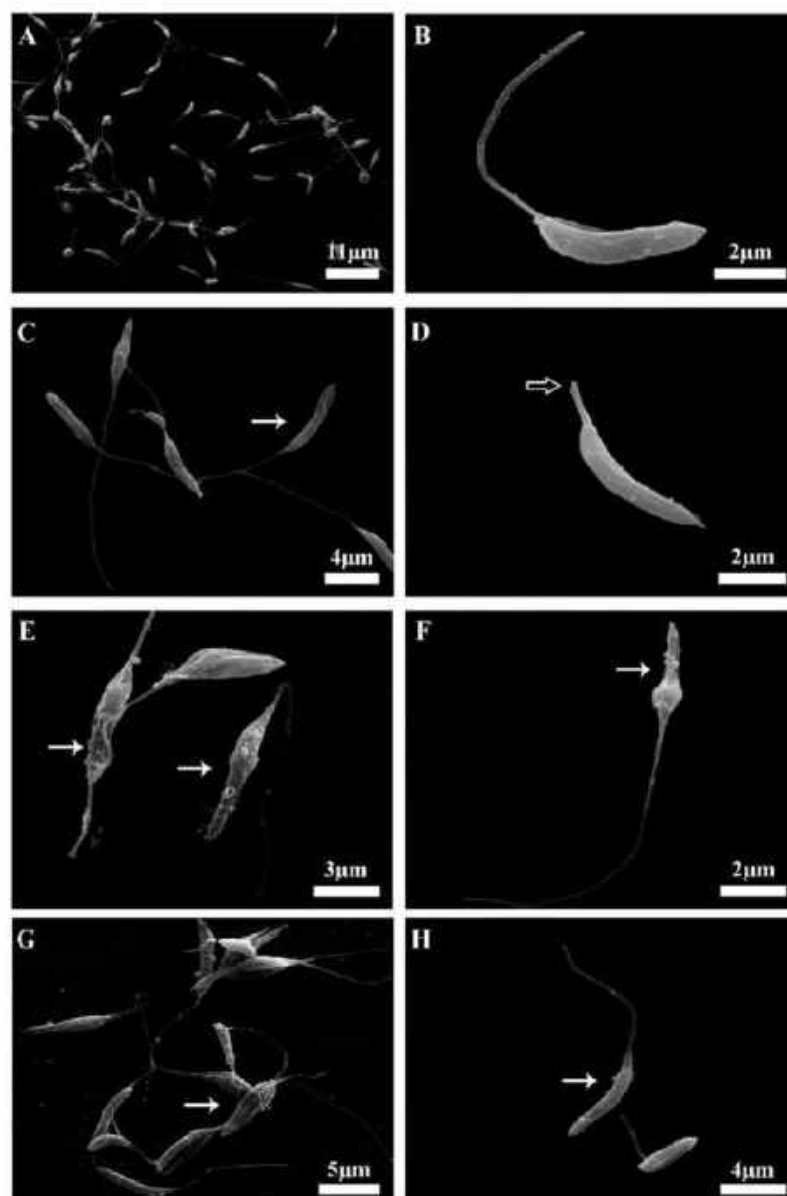
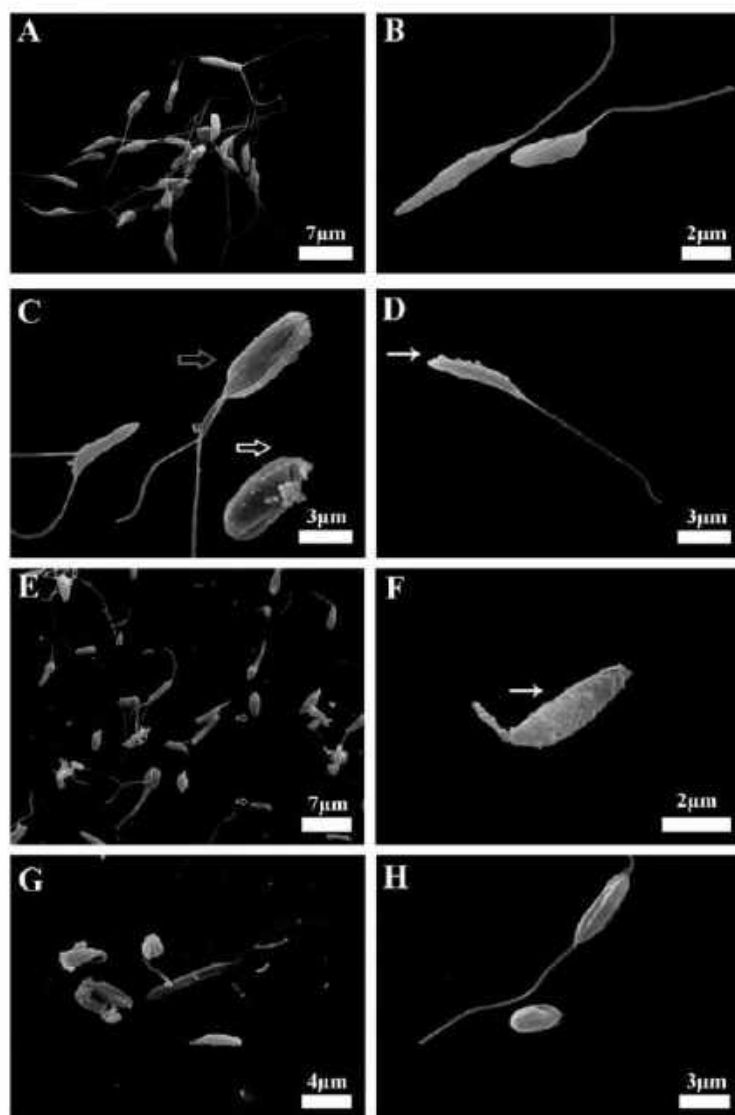


Fig. 1

2/3

**Fig. 2**

3/3

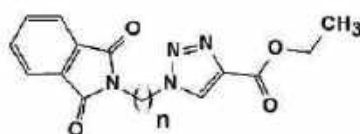
**Fig. 3**

1 / 1

RESUMO

DERIVADOS FTALIMÍDICOS-TRIAZÓLICOS ÚTEIS NO TRATAMENTO DE LEISHMANIOSE, COMPOSIÇÃO FARMACÊUTICA, USO DA COMPOSIÇÃO FARMACÊUTICA E MÉTODO DE TRATAMENTO DE LEISHMANIOSE

A invenção descreve a aplicação terapêutica do composto da fórmula FT1 ou FT2



FT1 = n: 2

FT2 = n: 3

ou um sal farmaceuticamente aceitável do mesmo, no tratamento de leishmaniose. Composições farmacêuticas compreendendo os referidos compostos ou um sal farmaceuticamente aceitável do mesmo, e uso destas são também contempladas no presente pedido.

5.4 ARTIGO 3

O artigo “Therapeutic potential of phthalimide derivatives: A Review” foi publicado no periódico *American Journal of Biomedical Science and Research*, volume 3, número 4, link para acesso: 10.34297/AJBSR.2019.03.000699



American Journal of
Biomedical Science & Research

www.biomedgrid.com

ISSN: 2642-1747

Review Article

Copyright© G.SilvaJunior Jose

Therapeutic Potential of Phthalimide Derivatives: A Review

José Guedes da Silva Júnior^{1*}, Vanderlan Nogueira Holanda¹, Daywison Silva Rodrigues Gambôa¹, Talyta Valéria Siqueira do Monte², Hallysson Douglas Andrade de Araújo¹, José Adelson Alves do Nascimento Júnior¹, Victor Felipe da Silva Araújo¹, Maria Auxiliadora Macedo Callôu¹, Shalom Pôrto de Oliveira Assis³, Vera Lúcia Menezes Lima¹

¹Laboratório de Lipídeos e Biomoléculas e suas Aplicações em Doenças Prevalentes e Negligenciadas, Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, Brasil.

²Hospital Olívio de Freitas, Recife, Pernambuco, Brasil.

³Universidade Católica de Pernambuco, Brasil.

*Corresponding author: G.SilvaJunior Jose, Laboratório de Lipídeos e Biomoléculas e suas Aplicações em Doenças Prevalentes e Negligenciadas, Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, Brasil.

To Cite This Article: G.SilvaJunior Jose, Therapeutic Potential of Phthalimide Derivatives: A Review, Am J Biomed Sci & Res. 2019 - 3(4): AJBSR MS.10.000699. DOI: 10.34297/AJBSR.2019.03.000699

Received: June 18, 2019 | Published: June 27, 2019

Abstract

Phthalimides belong to the group of cyclic imides and have as common chemical characteristic two two carbonyls bound to the same nitrogen. Such compounds are obtained by various organic synthesis processes, generally using phthalic anhydride as the precursor. Because they present various biological activities such as antineoplastic, anti-inflammatory, antitumor, anti-convulsant, antimicrobial, among other actions, these molecules have been object of study in several research centers worldwide. This review article is intended to provide highly relevant information on the biological activities of these compounds and their mechanisms of systemic action that have already been elucidated. The articles used in these studies were obtained through the Internet (from Elsevier, RSC and Science direct) in the period of July and August of 2018. Several compounds were identified acting in various pharmacological actions. The phthalimides and their derivatives studied in this study presented individual molecules, but also conjugated with other pharmacophoric groups, in order to improve their pharmacokinetic and pharmacodynamic aspects and thus generate better therapeutic responses. In spite of the studies with phthalimides, it is still necessary to investigate, mainly the mechanisms of action of these molecules, explaining all the interactions that they can make, so that new and more accurate studies can be carried out.

Introduction



Figure 1: Model of the phthalimide molecule with the two carbonyls attached to the secondary amine. Molecule obtained in ChemSketch program 12.0, 2018

Pharmaceutical innovation has promoted great therapeutic advances against the most diverse diseases, be it infectious, metabolic or oncological [1]. These new therapeutic molecules have allowed a significant improvement in the quality of health worldwide. In the knowledge economy there is no productivity and no new pharmacological agents without a strong scientific and technological base, so encouraging the search for new pharmacophoric groups is fundamental for the progress of health systems [2]. Within the class



This work is licensed under Creative Commons Attribution 4.0 License [AJBSRMS10.000699]

278

of the cyclic imides where they are inserted, glutarimides, maleimides, succinimides and the naphthalimides, the phthalimides, a class of compounds with several biological actions already mentioned, stand out [3]. Like the other cyclic imides, phthalimides are characterized by a pair of carbonyls that are bound to the same nitrogen atom (Figure 1) [4]. These compounds have a number of similar characteristics such as hydrophobicity, which assures greater bioavailability and thus greater membrane diffusion capacity [5].

Phthalimides heterocyclic derivatives present several biological activities already described in the literature and this has attracted the attention of the scientific community [6]. Many phthalimide derivatives have been promising for the following biological activities: hypolipidemic [7-9], analgesic [10], anticonvulsant, anti-inflammatory [10,11], antiviral [12], antitubercular [9-13], and antimicrobials (Figure 2) [13-14].

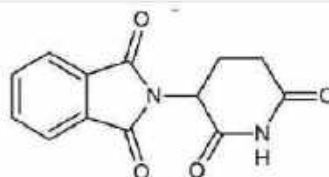


Figure 2: Chemical structure of Thalidomide, two amide rings and a single chiral center that form teratogenic and (R) teratogenic isomers with sedative effects. Molecule obtained in Chem. Sketch program 12.0, 2018.

Hypolipidemic activity

Obesity is defined as the accumulation of fat in the organism in association with health risks due to its relationship with metabolic complications. This condition progresses when there is an imbalance between the calories consumed and the calories consumed, contributing also to this process: endocrinological disorders and sedentarism, for example [15]. The major aggravating factor of diseases is the greater risk they cause for non-transmissible degenerative chronic diseases such as cardiovascular diseases, type 2 diabetes mellitus, musculoskeletal injuries such as osteoarthritis and some types of neoplasias [16]. Due to the serious risks that obesity can have on its patients, the search for therapies that can reduce high parameters such as hypercholesterolemia, hypotriglyceridemia and hyperglycemia is fundamental to avoid the installation of secondary complications that further aggravate the state of health of individuals. In this pharmacological aspect phthalimide derivatives have been presented as promising molecules in reducing high levels of cholesterol, triglycerides and serum glucose in animal models. In addition, some of these drugs have pleiotropic action, acting in a way to inhibit proinflammatory factors, which are released in obese individuals by adipose tissue [17].

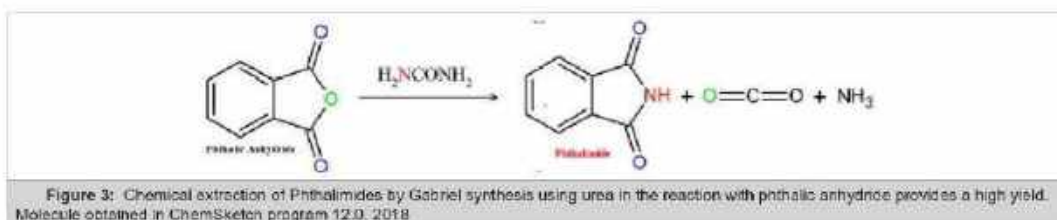
The hypolipidemic action of phthalimide derivatives is one of the first activities described. Compounds containing 1-N-phthalimidobutane-3-one demonstrated significant hypocholesterolemic action; in another study, it was observed that, after 16 days of treatment [20 mg / kg / day] with 4,8-di-O-acetyl-3-dideoxy- α -D-erythro-hex-2-enopyranoside, there was a reduction of up to 25% in plasma cholesterol and triglycerides. After treatment of 20 mg / kg / day mice with the phthalimideN- [4-chlorophenyl] phthalimide derivative, a 63-47% decrease in cholesterol and triglyceride levels, respectively, was observed. These studies demonstrate that compounds containing the phthalimide group are promising with hypolipidemic agents [7,18,19]. A new phthalimide derivative, LASSBio-1425, showed, among other activities, the ability to reduce serum levels of total cholesterol [$248.9 \pm 30.6 \times 519.2 \pm 17.4$ mg / dL], triglycerides [$7.3 \pm 7.8 \times 192.5 \pm 46.0$ mg / dL], LDL [135.7

$\pm 31.2 \times 336.6 \pm 18.5$ mg / dL] and increased HDL levels [$96.7 \pm 17.0 \times 25.4 \pm 3.8$ mg / dL] in wistar rats fed a hypercholesterolemic diet. Other new halide cyclic imides with N-phthalimide fraction presented significant hypolipidemic action in diabetic mice, with rates close to normal values, reinforcing the perspective of using phthalimide derivatives as hypolipidemic agents [9,20].

Analgesic activity

Pain can be defined as a disagreeable unpleasant sensory and emotional experience that is related to a tissue or potential damage to the individual. The sensation of pain is responsive to several damages that the organism can be affected by virtue of physical traumas, inflammatory processes or of infectious or metabolic diseases of chronic character. The pain signal for the cerebral cortex follows the steps of transduction, conduction, transmission, perception and modulation of this signal. These mechanisms of nociception are composed of neuronal events that drive the noxious stimuli through nerve impulses to the central nervous system so that it perceives and generates a response [21]. The pharmacological search for agents that block the sensation of pain is strongly echoed in the scientific studies of medicinal chemistry, mainly due to some adverse effects that therapeutic agents used in clinical practice have, such as: dependence, hepatotoxicity, nephrotoxicity and negative effects on the gastrointestinal tract. As a result, new molecules with more potent analgesic action and with lower collateral effects are sought. Several studies have shown that analogues of phthalimide have an important antinociceptive effect [22].

Phthalimide derivatives also present analgesic activities with well established mechanisms already described in the literature (Figure 3). Seven new compounds containing the phthalimide group linked to methyl 1,2,4-oxadiazol-5-yl presented potent analgesic effect [ED₅₀ = 2.2 mg / kg / ip] through the acetic acid-induced abdominal writhing evaluation in mice. Recently, a group composed of 30 cyclic imides derived from phthalimide, was designed as selective inhibitors of COX-2 in order to obtain anti-inflammatory and analgesic activities. Among the derivatives, a potent inhibitor [IC₅₀ = 0.18 μ M] and highly selective [SI = 668] COX-2 [10,23].



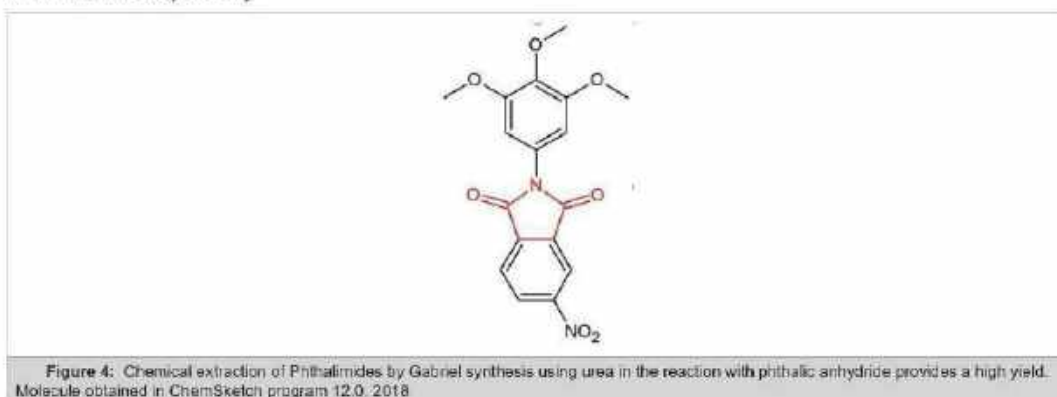
Anticonvulsant activity

Some stress situations may lead to a state of electrophysiological disturbance in the central nervous system, resulting in a lack of synchrony of the neuronal activity, generating the state of convulsion. Situations such as hypoglycemia, physical trauma, meningitis and high febrile state can trigger a convulsive state. These convulsions can affect specific parts of the body, and can be of two main types: clonic when they cause involuntary movements on both sides of the body and tonic when there is sudden contraction of the skeletal muscles [24]. Therapy of seizures is varied and depends on the type and intensity of seizures that the patient presents. However, anticonvulsant drugs still present adverse reactions, such as Phenytoin that causes ataxia, vertigo, megaloblastic anemia, and even fetal malformations. Like Phenytoin, Carbamazepine also generates ataxia, in addition to fluid retention and leucopenia, which leads to complications secondary to the individual, which makes it possible to find compounds with an efficient action in blocking the disordered neuronal discharges and that present smaller adverse effects and toxic to the body [25]. Anticonvulsant activity has also been reported after experiments with synthetic products containing phthalimide as the central component. Among the compounds

described above, 2-phthalimide which showed protection against electrically induced seizures at doses of 30 mg / kg and 100 mg / kg and was shown to be more potent than phenytoin, a reference drug in the treatment of seizures [26].

Since 2000 with the hybridization of Thalidomide with Ameltoide, phthalimides have been studied as promising anticonvulsant agents, mainly because they have low side effects. A series of fluorophthalimides were tested to evaluate their effects on tonic and clonic convulsions in rats and the results showed that among the phthalimide derivatives with the aromatic double-ring compound, therefore more lipophilic, presented more satisfactory results than the control Phenytoin against clonic convulsions, not demonstrating the same effect on tonic manifestations. Seizure reactions were induced using pentylenetetrazole [27]. Another study using two series of phthalimides, one with phenyl ring and one with an N-alkenyl, also inducing convulsion in rats with pentylenetetrazole observed that the alkenyl derivatives were more pharmacologically active against convulsion. This study also evaluated *in silico* the lipophilicity of these compounds using computational models and observed that there was no relation with higher lipophilicity of the molecules tested [28].

Anti-inflammatory activity



Another biological activity of phthalimides described in the literature is the anti-inflammatory action. A series of derivatives was synthesized to modulate the overproduction of TNF- α , the primary mediator of the inflammatory response. It was observed that the phthalimide derivatives presented significant results when compared to TNF- α modulation at concentrations of 10 and 100 μ M,

surpassing thalidomide activity. In addition, the same derivatives were able to significantly inhibit [36%] paw edema in mice treated with 10 mg / kg and decreased [45%] carrageenan-induced hyperalgesia. In another study, where the 2-phthalimidethanol and 2-phthalimide derivatives were used, both inhibited paw edema when administered 1 hour before the application of carrageenan

[11,29]. Another study using cyclic imides designed to have selective anti-inflammatory action against cyclooxygenase 2 [COX-2] was performed with 30 imide compounds. Four of these compounds showed high anti-inflammatory action and ability to inhibit COX-2 with high selectivity index against this enzyme. The phthalimides tested showed a high structural reactivity with COX-2, similar to the COX-2 inhibitor Celecoxib. In this study the molecular docking analysis demonstrated that one of the phthalimide derivatives has a binding mode with great specificity with COX-2 (Figure 4) [10].

Research using triazole-conjugated phthalimides demonstrated a high anti-inflammatory action of these compounds which were obtained through 1,3-dipolar cycloaddition cycle from N-[azido-alkyl] phthalimides and terminal alkynes. After the synthesis, purification and characterization of the compounds the anti-inflammatory potential of these cyclic imides in Swiss albino mice was evaluated by inducing inflammation with carrageenan through the plantar tissue of the right hind paw. All compounds showed anti-inflammatory activity, especially the 4-[4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside]-O-methyl-1-[3-phthalimidopropyl]-1,2,3-triazole [3b] and 4-[N-Phthalimidomethyl]-1-[4-phthalimidobutyl]-1,2,3-triazole [5c] which showed 69% and 56% of anti-inflammatory activity [30].

One of the triggering mechanisms of the inflammatory process is the overproduction of nitric oxide [NO], which is also linked to the pathophysiology of other comorbidities involving inflammation. Drugs that may inhibit the overproduction of nitric oxide are promising molecules that can act against several diseases of a more diverse nature. In light of this, a study using a series of phthalimide analogues tested these pharmacophoric groups against a lipopolysaccharide [LPS]-induced inflammatory process. In the tests cultures of RAW264.7 murine macrophages were used. In addition, an analysis of the structural relationship with biological activity showed that the free hydroxyls at the C-4 and C-6 carbons along with the alkyl chain volume correlated positively with the anti-inflammatory potential. Phthalimide IIh showed an IC₅₀ of 8.7 μ g / mL. The researchers observed that the effects on NO reduction were not related to a cytotoxic activity of phthalimides, so the levels of nitric oxide synthase in the inducible isoform [iNOS] and mRNA expression were investigated [31]. For this investigation RAW264.7 cells were incubated for 12 hours with LPS and treated with compound IIh and others without phthalimide and subsequently Total RNA was isolated for Polymerase Chain Reaction [PCR] and the proteins analyzed by Western blot. The results demonstrated that cells that were not treated with the phthalimide derivative IIh had a marked elevation of iNOS induced by LPS, unlike those treated with IIh which was capable of significantly suppressing the iNOS concentration and in a concentration dependent manner [31].

In addition, compound IIh promoted a reduction of proinflammatory cytokines such as Tumor Necrosis Factor-Alpha [TNF- α], Interleukin 1 β [IL-1 β] in the treated cells. The authors observed that the anti-inflammatory activity of compound IIh was linked to overexpression of the TLR-4 Toll-like receptor, which is activated by LPS and elicits a cascade of signals leading to the synthesis of pro-inflammatory cytokines and mediators of inflammatory pro-

cess such as nitric oxide. This TLR-4 signaling is divided into two ways: MyD88-dependent and MyD88-independent. The dependent pathway leads to the activation of the kappa nuclear factor protein complex B [NF- κ B] which results in a greater expression of proinflammatory cytokines. The independent pathway leads to the expression of interferon- β . Acting on these signaling cascades, phthalimide IIh reduced the production of nitric oxide, TNF- α , IL-1 β and iNOS, which credited it as a strong candidate for anti-inflammatory drug [31].

Antimicrobial activity

Despite the diverse metabolic activities of phthalimide derivatives, the antimicrobial assays deserve to be highlighted by the breadth of action of these compounds and their excellence in the fight against pathogenic microorganisms. Many infections by viruses, bacteria, protozoa, fungi and so on, drug cocktails, including potent drugs that bring the user several side effects, in addition to requiring long-term and multi-drug therapies [32]. The antiviral potential of new compounds, whether of synthetic or natural origin, has been of great interest throughout the development of science. The N-1-adamantyl-4-aminophthalimide derivative was prominent when it presented antiviral activity, with EC₅₀ = 4.78 μ g / mL for the HIV virus. In the same study, the importance of the amino group on the phthalyl moiety in the biological activity investigated was emphasized, since other compounds with significant results also present the same chemical characteristic in common. A series of flubuzone analogues, prepared from potassium phthalimide, showed significant inhibitory results against herpes simplex virus 1 and 2. The compounds with phthalimide moieties presented as potential antiviral agents, with IC₅₀ = 2.85 μ g / mL for herpes simplex viruses 1 [33,34].

Other pyrimidine ring-containing phthalimide derivatives were also synthesized and tested against *M. tuberculosis* and showed significant values of Minimum Inhibitory Concentration [MIC] = 3.9 μ g / mL, cytotoxicity [CC₅₀] = 312.5, and selectivity index to macrophages [I₇₇₄] of 80. Fluorinated phthalimide derivatives were also tested for efficacy in combating the tuberculous agent *M. tuberculosis* H37Ra [ATCC 25177] and it was found that the fluorinated phthalimide hybrid molecules had similar anti-mycobacterial activity sulfonamide drugs. In another study, in which 12 phthalimide derivatives with fluorinated radicals were synthesized, the compounds showed potent antimicrobial activities, with a mean MIC of 1.8 μ g / mL [13,35,36]. The analogs obtained from the synthesis of N-[triethoxysilylpropyl] phthalimide by the transimidation reaction between phthalic anhydride and 3-aminopropyl triethoxysilane were tested for possible antimicrobial effect on strains of *Staphylococcus aureus* [ATCC 25923], *Acinetobacter baumannii* [ATCC 19606], *Pseudomonas aeruginosa* [ATCC 27853] and *Escherichia coli* [ATCC 25922]. The drugs tested were considered to be effective antimicrobials against all tested microorganisms, and the compounds with the phthalimide structure showed a significantly higher result, and this effect can be attributed to the presence of phthalimides in their chemical composition [14].

The compounds N-vinylphthalimide and 8-[4-[phthalimid-2-yl] butyloxy] quinoline, phthalimide derivatives obtained by

modified condensation reactions, are described in the literature as good fungicidal agents. Synthetic analogues were tested against phytopathogens, *Alternaria solani* and *Botrytis cinerea*, with IC₅₀ values of 7.92 µg / mL and 10.85 µg / mL [37]. Other compounds containing the phthalimide group also showed excellent activity against the etiologic agent of malaria, *Plasmodium falciparum*. In general, in the study, the phthalimide group was introduced into [α]-dehydroabietylamine and from further reactions, twelve derivatives were obtained. Among the compounds obtained, 12-acetyl-N, N-phthaloylhydroabietylamine showed excellent activity against the protozoan with EC₅₀ [antiplasmodic activity] = 0.086 µM and SI [selectivity index] => 290, confirming that compounds with phthalimide grouping are promising in the treatment of intracellular protozoal infections [38].

Antitumor activity

Research with phthalimides with antitumor action has grown significantly, mainly because some types of cancer present resistance to medications already used, which reinforces the need to seek new antimitotic molecules. Blockage of tumor growth may oc-

cur in one or more of the six phenotypes of the cancer cell that confer indiscriminate proliferative capacity, the disruption of the activity of cell signaling pathways for proliferation is one of the main therapeutic pathways used to curb tumor growth. Many phthalimides have been proposed as promising chemotherapeutic agents to block cell growth signaling pathways [39]. Like P13K / Akt / mTOR, a signaling pathway linked to cell growth, differentiation and differentiation, and when altered, are operative for initiation and tumor development. A study using glycoconjugated phthalimides demonstrated that these compounds showed antitumor activity by being able to inhibit the enzymes of the P13K pathway by means of proteolysis. In addition, the compounds regulated the phosphorylation of the Akt, S6K and GSK-3β pathway in cells of the HepG2 hepatic lineage. One of the study's phthalimide molecules was even more promising by inhibiting cell proliferation and inducing an autophagy process in a process that includes the formation of autophagosomes, fusion with lysosomes, and degradation of cell structures. This process was evidenced by the conversion of autophagy marker protein LC3BI and LC3BII detected by western blot (Figure 5) [40].

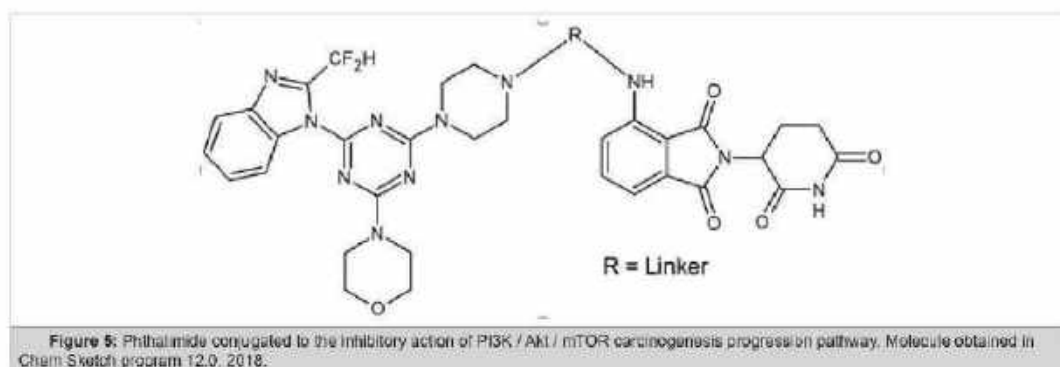


Figure 5: Phthalimide conjugated to the inhibitory action of P13K / AKT / mTOR carcinogenesis progression pathway. Molecule obtained in Chem Sketch program 12.0, 2018.

A number of chemical derivatives of thalidomide and phthalimide esters were synthesized and purified for evaluation of their antitumor potential against HepG2 cell lines of liver cancer and MCF-7 mammary neoplasia. One of the phthalimide esters demonstrated high inhibition of MCF-7 lineage growth, which was not observed by the other molecules. None of the compounds were effective in inhibiting the hepatic lineage. However, three phthalimide derivatives elicited an immunosuppressive effect and six an immunostimulatory effect. Of the compounds tested two Thalidomide derivatives and a phthalimide ester showed a high potential to inhibit vascular endothelial growth factor [VEGF] that in this study was used as a recombinant form [VEGFR] and analyzed by sandwich ELISA [41]. Phthalimide derivatives have also been proposed as anticancer therapeutic agents in combination with chemotherapeutics already used in antimitotic therapies. A study with Oprozomib [OPZ] a protease inhibitor and Pomalidomide [Pom] a phthalimide derivative of thalidomide has shown that phthalimide in a study of antiangiogenic action on choroidal membrane against the development of multiple myeloma.

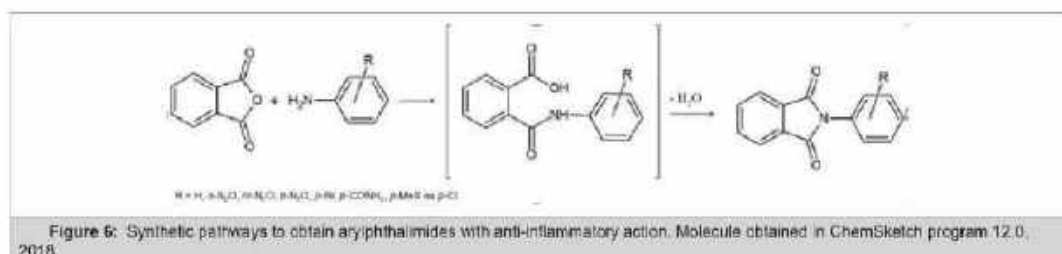
This study showed that Oprozomib was able to significantly reduce neoangiogenesis and expression of genes related to endothelial protein synthesis. The study then found that when Oprozomib was combined with dexamethasone [Dex] or with Pomalidomide the effects were even more significant when compared with monotherapy. There was even more therapeutic significance when the three molecules [OPZ + Pom + Dex] were used, demonstrating that the pharmacological combinations may contribute to a better therapeutic efficacy against cancer. In addition, this study using animal model allows to investigate other forms of phthalimide derivatives, such as the tumor microenvironment, the transition of the mesenchymal epithelium and mainly the angiogenesis process, which are not analyzed in cell culture analyzes [42].

Toxicity

Studies using phthalimides and their derivatives have shown low toxic effects at the acute level of these molecules. In a study with arylphthalimides and its hypolipidemic and anti-inflammatory activity, a therapeutic safety test was performed to determine its

acute toxicity [LD50]. The test demonstrated that the therapeutic dose for the biological activities tested [250 mg / kg] was safe, since even at the highest tested dose of 2 g / kg there was no lethality

and neither did the animals show signs of adverse reactions such as behavioral changes such as twitching, breathing breathlessness, palpitations, and decreased respiratory rate (Figure 6) [43].



Conclusion

Despite the many advances in modern medicine, many diseases of a diversified nature still cause various health complications and deaths. Pharmacological treatments are one of the main alternative to seek resolution or placate symptoms of diseases. In this scenario, the compounds originated from organic synthesis are highlighted because they are quickly obtained molecules and do not face problems of sustainability and seasonality as the compounds extracted from bioprospecting. Among the various classes of organic synthesis molecules, phthalimides are distinguished by their extremely pharmacological effects and often pleiotropic, acting on more than one front in the same organism. The actions of these compounds are from antimicrobial agents, mainly against fungi, bacteria and parasites, to effects on the metabolism of macromolecules and regulation of the processes of pain and inflammation. This great variability of pharmacological actions along with the constant possibility of improvement and conjugation of the phthalimide molecules so that they become increasingly active against the various insults and less toxic, places them in a prominent place in the scenario of medicinal chemistry, so that more and more this important class of cyclic imides is elucidated, until it materializes in effective therapeutic tools against diverse comorbidities.

References

- Verweij J, Hendriks HR, Zwietersma H (2019) Innovation in Oncology Clinical Trial design. *Cancer Treat Rev* 74: 15-20.
- Daniel GW, Cazé A, Romine MH, Audibert C, Leff JS, et al. (2015) Improving Pharmaceutical Innovation by Building a more comprehensive Database on Drug Development and use. *Health Aff (Millwood)* 34(2): 319-327.
- Yuan YC, Bruneau C, Dorcet V, Rolsnel T, Grumage-Doris R, et al. (2019) Ru-Catalyzed Selective C-H Bond Hydroxylation of Cyclic Imides. *J Org Chem* 84(4): 1898-1907.
- Lian B, Sun C, Xu K, Zeng C (2019) Electrochemical Dehydrogenative Iridation of N-Methyl-Substituted Benzylamines with Phthalimides for the Direct Synthesis of Phthalimide-Protected gem-Diamines. *Org Lett* 21(1): 156-159.
- Georgiev A, Stoitcheva A, Dimov D, Yordanov D, Zhivkov I, et al. (2019) Synthesis and photochromic properties of some N-phthalimide azoazomethine dyes: A DFT quantum mechanical calculations on imine-enamine tautomerism and trans-cis photoisomerization. *Spectrochim Acta A Mol Biomol Spectrosc* 210: 230-244.
- Winter GE, Buckley DL, Paulk J, Roberts JM, Souza A, et al. (2015) Selective Target Protein Degradation via Phthalimide Conjugation. *Science* 348(6241): 1376-1381.
- Chapman Jr JM, Coccolas GH, Hall IH. (1983) Hypolipidemic activity of phthalimide derivatives. IV. Further chemical modification and investigation of the hypolipidemic activity of N-substituted imides. *J Pharm Sci* 72(11): 1344-1347.
- Murthy A, Wyrick S, Hall, IH (1985) Hypolipidemic activity of indan-1, 3-dione derivatives in rodents. *J Med Chem* 28 (11): 1591-1596.
- Abdel-Aziz AAM, El-Azab AS, Attia SM, Al-Obaidi Mohamed AM, Al-Omari MA, et al. (2011) Synthesis and biological evaluation of some novel cyclic-imides as hypoglycaemic, anti-hyperlipidemic agents. *Eur J Med Chem* 46 (8): 4324-4329.
- Alenazi AM, El-Azab AS, Al-Sowaidan IA, ElTahir KEH, Asiri VA, et al. (2015) Structure-based design of phthalimide derivatives as potential cyclooxygenase-2 (COX-2) inhibitors: anti-inflammatory and analgesic activities. *Eur J Med Chem* 6(92):115-123.
- Casal JJ, Bollini M, Lombardo MK, Bruno AM (2016) Thalidomide analogues: tumor necrosis factor- α inhibitors and their evaluation as anti-inflammatory agents. *Eur J Pharm Sci* 83:114-119.
- Saari WS, Wai JS, Fisher TE, Craig MT, Hoffman JM, et al. (1992) *Journal of Medicinal Chemistry*. 35(21): 3792-3802.
- Akgun H, Karamelkoculu I, Berk B, Kurnaz I, Saribayrak G, et al. (2012) Synthesis and antimicrobial activity of some phthalimide derivatives. *Bioorg Med Chem* 20: 4149-4154.
- Singh G, Sarda A, Girdhar S, Rani S, Sahoo S, et al. (2015) Synthesis, characterization, electronic absorption and antimicrobial studies of N-(sulfamoylpropyl) phthalimide derived from phthalic anhydride. *Inorganica Chimica Acta* 427: 232-239.
- Sharon (2017) MF Obesity: Risk factors, complications, and strategies for sustainable long-term weight management. *J Am Assoc Nurse Pract* 29(S1): S3-S14.
- Martin-Rodríguez E, Guillen-Gracia F, Martí A, Irugos-Larumbe A (2015) Comorbidity associated with obesity in a large population: The APNA study. *Obes Res Clin Pract* 9(5): 435-447.
- Mc Donnell DP, Park S, Goulet MT, Jasper J, Wardell SE, et al. (2014) Obesity, Cholesterol Metabolism and Breast Cancer Pathogenesis. *Cancer Res* 74(18): 4976-4982.
- Srivastava RM, Oliveira FJ, da Silva LP, de Freitas Filho JR, Oliveira SP, et al. (2003) Synthesis and hypolipidemic activity of N-phthalimidomethyl tetra-O-acetyl- α -D-mannopyranosides. *Carbohydr Res* 332(3): 335-340.
- Sena YLM, Srivastava MR, Silva SO, Lima VLM (2003) Synthesis and hypolipidemic activity of N-substituted phthalimides. Part V. *Pharmazie* 58(12): 1283-1288.

20. Fomlan MM, da Mota NA, Maia R, Fraga CA, Borreiro EJ, et al. (2016) LASSBio-1425, an analog of ibuprofen, decreases triglyceride and increases HDL cholesterol levels by inhibition of TNF- α production. *Int J Cardiol* 202: 497-499.
21. Vanderah TW (2007) Pathophysiology of Pain. *The Medical Clinics of North America* 91(1): 1-12.
22. Silva GF, Anjos MF, Wunsch RL, Ferreira LFGR, Stiz DS, et al. (2017) Antihypersensitivity effects of the phthalimide derivative N-(4-methylphenyl)-4-methylphthalimide in different pain models in mice. *Biomedicine & Pharmacotherapy* 96: 503-512.
23. Antunes R, Batista H, Srivastava RM, Thomas G, Araujo CC, et al. (1998) New phthalimide derivatives with potent analgesic activity. II. *Bioorg Med Chem Lett* 8(21): 3071-3076.
24. Asadi-Pooya AA (2017) Psychogenic nonepileptic seizures: a concise review. *Neurol Sci* 38(6): 935-940.
25. Gierbolini JR, Garretano M, Benbadis SR (2016) Carbamazepine-related antiepileptic drugs for the treatment of epilepsy - a comparative review. *Expert Opin on Pharmacotherapy* 17(7): 885-898.
26. Kamiński K, Obniska J, Wiklik B, Atamanjuk D (2011) Synthesis and anticonvulsant properties of new acetamide derivatives of phthalimide, and its saturated cyclohexane and norbornene analogs. *Eur J Med Chem* 46(9): 4634-4641.
27. Iman M, Fakhari S, Jahanspanah M, Nadiri N, Drouot A, et al. (2018) Design and Synthesis of 4-fluorophthalimides as potential anticonvulsant agents. *Iranian Journal of Pharmaceutical Research* 17(3): 896-905.
28. Wincz M, Kice-Kononowicz K (2006) Synthesis and Anticonvulsant Evaluation of Some N-Substituted Phthalimides. *Acta Pol Pharm* 55(3): 249-257.
29. Coelho LCD, Cardoso MVD, Moreira DRM, Gomes PATM, Cavalcanti SMT, et al. (2014) Novel phthalimide derivatives with TNF- α and IL-1 β expression inhibitory and apoptotic inducing properties. *Med Chem Comm* 5(6): 758-765.
30. Assis SPO, Silva MT, Oliveira RN, Lima VL (2012) Synthesis and Anti-Inflammatory Activity of New Alkyl-Substituted Phthalimide 1H-1,2,3-Triazole Derivatives. *Scientific World Journal* 5: 1-7.
31. Bach DH, Liu JY, Kim WK, Hong JY, Park SH, et al. (2017) Synthesis and biological activity of new phthalimides as potential anti-inflammatory agents. *Bioorg Med Chem* 25(13): 3396-3405.
32. Bentrup ZKH, Russell DG (2001) Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol* 9 (12): 597-605.
33. Van Derpoorten K, Balzarini J, De Clercq E, Poupard JH (1997) Anti-HIV activity of N-1-adamantyl-4-aminophthalimide. *Biomed Pharmacother* 51(10): 464-468.
34. Yang YJ, Zhao JH, Pan XD, Zhang PC. (2010) *Chemical and Pharmaceutical Bulletin*. 50(2): 200-211.
35. Santos JL, Yamasaki PR, Chin CM, Takashi CH, Pavan ER, et al. (2009) Synthesis and in vitro anti Mycobacterium tuberculosis activity of a series of phthalimide derivatives. *Bioorg Med Chem* 17 (11): 3795-3799.
36. Elumalai K, Ali MA, Elumalai M, Eluri K, Srinivasan S, et al. (2013) characterization and biological evaluation of acetazolamide, cycloserine and meniazid condensed some novel phthalimide derivatives. *International Journal of Chemical and Analytical Science* 4 (2): 57-61.
37. Pan L, Li X, Gong C, Jin H, Qiu B (2016) Synthesis of N-substituted phthalimides and their antifungal activity against *Alternaria solani* and *Bocrytis cinerea*. *Microb Pathog* 95: 186-192.
38. González MA, Clark J, Connolly M, Rivas F (2014) Antimalarial activity of abietane ferriquinol analogues possessing a phthalimide group. *Bioorg Med Chem Lett* 24(22): 5234-5237.
39. Belluti S, Orteca G, Someghini V, Rigillo G, Parenti E, et al. (2019) Potent Anti-Cancer Properties of Phthalimide-Based Curcumin Derivatives on Prostate Tumor Cells. *Int J Mol Sci* 20 (1): 28.
40. Li W, Gao C, Zhao L, Yuan Z, Chen Y, et al. (2018) Phthalimide conjugations for the degradation of oncogenic PI3K. *European Journal of Medicinal Chemistry*. 2018; 10 (151): 237-247.
41. Zahran MA, Abdin YG, Osman AM, Gamal-Eldeen AM, Talaat RM, et al. (2014) Synthesis and Evaluation of Thalidomide and Phthalimide Esters as Antitumor Agents. *Arch Pharm (Weinheim)* 347(9): 642-649.
42. Sanchez E, Li M, Wang CS, Tang G, Gillespie A, et al. (2017) Anti-angiogenic and anti-multiple myeloma effects of oprozomib (OPZ) alone and in combination with pomalidomide (Pom) and/or dexamethasone (Dex). *Leuk Res* 57: 45-54.
43. Assis SPO, Araújo TG, Sena VLM, Catanho MTJA, Ramos MN, et al. (2014) Synthesis, hypolipidemic, and anti-inflammatory activities of acylphthalimides. *Medicinal Chemistry Research* 23(2): 708-716.

5.5 ARTIGO 4

O artigo “Identification of 1,2,3-triazole-phthalimide derivatives as potential drugs against COVID-19: a virtual screening, docking and molecular dynamic study” foi publicado no periódico *Journal of Biomolecular Structure and Dynamics*, volume 40, número 12, link para acesso: <https://doi.org/10.1080/07391102.2020.1871073>



Journal of Biomolecular Structure and Dynamics



ISSN: (Print) (Online) journal homepage: <https://www.tandfonline.com/loi/tbsd20>

Identification of 1,2,3-triazole-phthalimide derivatives as potential drugs against COVID-19: a virtual screening, docking and molecular dynamic study

Vanderlan Nogueira Holanda , Elton Marlon de Araújo Lima , Welson Vicente da Silva , Rafael Trindade Maia , Rafael de Lima Medeiros , Arabinda Ghosh , Vera Lúcia de Menezes Lima & Regina Celia Bressan Queiroz de Figueiredo

To cite this article: Vanderlan Nogueira Holanda , Elton Marlon de Araújo Lima , Welson Vicente da Silva , Rafael Trindade Maia , Rafael de Lima Medeiros , Arabinda Ghosh , Vera Lúcia de Menezes Lima & Regina Celia Bressan Queiroz de Figueiredo (2021): Identification of 1,2,3-triazole-phthalimide derivatives as potential drugs against COVID-19: a virtual screening, docking and molecular dynamic study, *Journal of Biomolecular Structure and Dynamics*, DOI: 10.1080/07391102.2020.1871073

To link to this article: <https://doi.org/10.1080/07391102.2020.1871073>



View supplementary material



Published online: 18 Jan 2021.



Submit your article to this journal



Article views: 690



View related articles



View Crossmark data

Identification of 1,2,3-triazole-phthalimide derivatives as potential drugs against COVID-19: a virtual screening, docking and molecular dynamic study

Vanderlan Nogueira Holanda^{a,b}, Elton Marlon de Araújo Lima^{b,c}, Welson Vicente da Silva^d, Rafael Trindade Maia^d, Rafael de Lima Medeiros^e, Arabinha Ghosh^f, Vera Lúcia de Menezes Lima^{a,g} and Regina Celia Bressan Queiroz de Figueiredo^{b,h}^aLaboratório de Lipídios e Aplicação de Biomoléculas em Doenças Prevalentes e Negligenciadas, Departamento de Bioquímica, Centro de Biotecnologia, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil; ^bLaboratório de Biologia Celular de Patógenos, Instituto Aggeu Magalhães, Departamento de Microbiologia, Instituto Aggeu Magalhães – IAM/FIOCRUZ-PE, Recife, Pernambuco, Brazil; ^cLaboratório de Polímeros Não-Convencionais, Departamento de Física, Centro de Ciências Exatas e da Natureza, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil; ^dCentro de Desenvolvimento Sustentável do Semiárido, Universidade Federal de Campina Grande, Sumé, Paraíba, Brazil; ^eInstituto de Física, Universidade Federal de Alagoas, Maceió, Alagoas, Brazil; ^fMicrobiology Division, Department of Botany, Gauhati University, Guwahati, Assam, India

Communicated by Ramaswamy H. Sarma

ABSTRACT

In this work we aimed to perform an *in silico* predictive screening, docking and molecular dynamic study to identify 1,2,3-triazole-phthalimide derivatives as drug candidates against SARS-CoV-2. The *in silico* prediction of pharmacokinetic and toxicological properties of hundred one 1,2,3-triazole-phthalimide derivatives, obtained from SciFinder® library, were investigated. Compounds that did not show good gastrointestinal absorption, violated the Lipinski's rules, proved to be positive for the AMES test, and showed to be hepatotoxic or immunotoxic in our ADMET analysis, were filtered out of our study. The hit compounds were further subjected to molecular docking on SARS-CoV-2 target proteins. The ADMET analysis revealed that 43 derivatives violated the Lipinski's rules and 51 other compounds showed to be positive for the toxicity test. Seven 1,2,3-triazole-phthalimide derivatives (**A7**, **A8**, **B05**, **E35**, **E38**, **E39**, and **E40**) were selected for molecular docking and MFCC—*ab initio* analysis. The results of molecular docking pointed the derivative **E40** as a promising compound interacting with multiple target proteins of SARS-CoV-2. The complex **E40-M^{pro}** was found to have minimum binding energy of -10.26 kcal/mol and a general energy balance, calculated by the quantum mechanical analysis, of -8.63 eV. MD simulation and MMGBSA calculations confirmed that the derivatives **E38** and **E40** have high binding energies of -63.47 ± 3 and -63.31 ± 7 kcal/mol against SARS-CoV-2 main protease. In addition, the derivative **E40** exhibited excellent interaction values and inhibitory potential against SARS-CoV-2 main protease and viral nucleocapsid proteins, suggesting this derivative as a potent antiviral for the treatment and/or prophylaxis of COVID-19.

Abbreviations: 3CLPro: 3-C Like Protease; ACE2: Angiotensin Converting Enzyme 2; ADME: Absorption, Distribution, Metabolism and Excretion; ALA: Alanine; ARDS: Acute Respiratory Distress Syndrome; ARG: Arginine; BBB: Blood-Brain Barrier; CLogP: Octanol-water partition coefficient; Comp: Compounds; COVID-19: Coronavirus Disease 2019; CYS: Cysteine; DNA: Deoxyribonucleic Acid; E: Total Energy; Elec: Electrostatic; GIA: Gastrointestinal Absorption; GLN: Glutamine; GLU: Glutamic Acid; GLY: Glycine; HB: Hydrogen-bond; HBA: Hydrogen-Bond Acceptors; HBD: Hydrogen-Bond Donors; HEP: Hepatotoxicity; hERG: Human Ether-a-go-go-related Gene; HIS: Histidine; HIV: Human Immunodeficiency Virus; ILE: Isoleucine; IT: Immunotoxicity; K_i: Inhibition Constant; LYS: Lysine; MET: Methionine; MFCC: Molecular Fractionation with Conjugate Caps; M^{pro}: Main Protein; MTD: Maximum Tolerated Dose; MW: Molecular Weight; NCP: Nucleocapsid Protein; NR: Number Rotatable Bonds; NSP: Non-structural Proteins; ORAT: Acute Oral Toxicity in Rats; ORCT: Oral Rat Chronic Toxicity; PDB: Protein Data Bank; PGs: P-glycoprotein Substrate; PHE: Phenylalanine; PSA: Polar Surface Area; RBD: Receptor Binding Domain; RNA: Ribonucleic Acid; ROS: Lipinski's Rule of Five; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SS: Skin Sensitization; TC: Total Clearance; TRP: Tryptophan; TYR: Tyrosine; VAL: Valine; VDW: van der Waals; MD: Molecular Dynamic; MMGBSA: Molecular mechanics and generalized Born and surface area

ARTICLE HISTORY

Received 8 September 2020
Accepted 28 December 2020

KEYWORDS

Antiviral; SARS-CoV-2;
phthalimide; 1,2,3-triazole;
MD simulation

CONTACT Vanderlan Nogueira Holanda  vanderlan.holanda@ufpe.br  Laboratório de Lipídios e Aplicação de Biomoléculas em Doenças Prevalentes e Negligenciadas, Departamento de Bioquímica, Centro de Biotecnologia, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil;  Laboratório de Biologia Celular de Patógenos, Instituto Aggeu Magalhães, Departamento de Microbiologia, Instituto Aggeu Magalhães – IAM/FIOCRUZ-PE, Avenida Professor Moraes Rego s/n 50670-420, Recife, Pernambuco, Brazil.

[†]Both authors contributed equally to this work. Supplemental data for this article can be accessed online at <https://doi.org/10.1080/07391102.2020.1871073>.

© 2021 Informa UK Limited, trading as Taylor & Francis Group

1. Introduction

Nowadays respiratory diseases caused by virus are among the main causes of morbidity and mortality due to their easy spreading and the high pathogenicity of viral agents (Pinana et al., 2020). At the end of 2019 a new Coronavirus, called SARS-CoV-2, appeared in Wuhan, China and became one of the most aggressive pandemics in history (Meo et al., 2020), exceeding 3 million infected individuals and causing thousands of deaths worldwide by the beginning of May 2020. The clinical manifestations of Coronavirus disease 2019 (COVID-19) are characterized by fever, fatigue, cough, which can rapidly progress into Acute Respiratory Distress Syndrome (ARDS) and death (Huang et al., 2020). Although vaccinal candidates against COVID-19 are already under development stage, a safe and effective vaccine for large-scale vaccination is far from becoming a reality. Hydroxychloroquine or chloroquine alone or in combination with a second-generation of macrolide, have been preconized for the treatment of COVID-19 despite no conclusive evidence of their benefits (Mehra et al., 2020). In this regard, the search for a safe and effective treatment against the severe form of COVID-19 is still necessary (Zhang et al., 2020).

Among the arsenal of bioactive compounds, the 1,2,3-triazole group has been attracting interest of scientific community due to their wide-ranging and versatile medicinal applications such as antivirals (Sun et al., 2020), antibacterial (Gonzaga et al., 2013), antifungal (Thanh et al., 2019) and antiparasitic (Leite et al., 2018) agents. Furthermore, 1,2,3-triazoles can be easily synthesized by low-cost copper catalysed reactions between alkynes and azides, namely *click chemistry*. Besides being used to synthesize triazoles derivatives, *click chemistry* is also widely applied for the fusion of two or more chemical groups in a single hybrid molecule with improved biological activity (Ouyang et al., 2018). As triazole derivatives, the phthalimide group has been shown to be useful in the formation of hybrid compounds with antiviral activity against HIV (Al-Masoudi et al., 2016), cytomegalovirus and varicella-zoster (Mandić et al., 2020). Importantly, both triazole and phthalimide derivatives also have relevant anti-inflammatory activity (Assis et al., 2019). Therefore, 1,2,3-triazole-phthalimide hybrids may not only be able to prevent viral replication but also treat the severe form of COVID-19, characterized by an intense inflammatory process called 'cytokine storm' (Zhang et al., 2020).

One of the strategies for the discovery of antiviral candidates against SARS-CoV-2 is the association of *in silico* screening-based approaches with the molecular docking of selected molecules against SARS-CoV-2 target proteins, to identify compounds having good drug-likeness properties. Because experimental approaches for drug discovery are highly costly and time-consuming, screening-based methods offer a fast and low-cost way for prospecting new potential drugs (Hall & Ji, 2020). The SARS-CoV-2 viral genome encodes for 29 proteins—many of which could serve as potential targets for anti-viral drugs. Among these proteins, spike protein (S), C-like proteases and nucleocapsid proteins have gained growing interest due to their relevant roles in the host cell recognition, viral replication, processing and formation of nucleocapsid structure. Spike glycoproteins, found on the surface of SARS-CoV-2, are a class I fusion proteins which bind to human Angiotensin Converting

Enzyme 2 (ACE2) receptor mediating both the fusion of the viral envelope with the host-cell membrane and the virus entry into host cells. The chymotrypsin-like cysteine protease, also known as main protease (M^{pro}) or 3C-like protease (3CLPro), is also an interesting target for drugs against COVID-19. This protease cleaves the C-terminal sequence of the polyprotein PP1A and PP1AB into 16 functional non-structural proteins (nsps), which play an important role in viral replication (Chen et al., 2020). Besides proteases and spike proteins, the nucleocapsid protein of SARS-CoV-2 has been investigated as a target protein for drug development. The nucleocapsid protein comprises two structured domains, the N-terminal domain (NTD) and the C-terminal domain (CTD), interspersed by three intrinsically disordered regions (IDRs) known as the N-terminal arm (N-arm), the central linker region (LKR), and the C-terminal tail (C-tail) arm (Zhou et al., 2008, 2020). The main function of these proteins is to form complexes with ribonucleoproteins. However, they also regulate the replication, transcription of viral RNA and, in the host cells, inhibit protein translation, leading to disruption of host cell metabolism. Hence, targeting this protein can lead to blockbuster therapeutic agents for COVID-19 treatment (Bhatia, Narang & Rawal, 2020; Zeng et al., 2008). In this study, we have performed docking study of selected 1,2,3-triazole-phthalimide derivatives against COVID-19 targets.

2. Materials and methods

2.1. Selection and preparation of ligands

The access to the chemical structures of 1,2,3-triazole-phthalimide hybrid compounds was performed using the SciFinder® database (<https://scifinder.cas.org>). For this study, only compounds that were obtained by *click chemistry* and have biological activity already reported in the literature were chosen for the analysis. The chemical structure of compounds was built using ACD/ChemSketch 2.1 and optimized using the PM3 semi-empirical method (Stewart, 1989) implemented in Mercury 4.3.1 (Macrae et al., 2008). The protonation states of the ligands were verified by MarvinSketch 20.19.0 software assuming a physiological pH (Butcher et al., 2015). The canonical smiles were obtained for ADMET *in silico* analysis (Karwath & Raedt, 2006).

2.2. In silico analysis of physico-chemical, pharmacokinetic and toxicological profile (ADMET) of ligands

The analysis of the pharmacokinetic and druglike properties of 1,2,3-triazole-phthalimide derivatives was carried out using the Web server SwissADME, <http://www.swissadme.ch/> (Swiss Institute of Bioinformatics). Only the compounds that have good gastrointestinal adsorption and did not violate the following parameters of Lipinski's Rule of Five: (i) $\text{clogP} \leq 5$; (ii) Molecular weight (MW) $\leq 500 \text{ g/mol}$; (iii) Number of hydrogen bond acceptors (HBA) (sum of N and O atoms) ≤ 10 ; (iv) Number of hydrogen bond donors (HBD) (sum of OH and NH groups) ≤ 5 , were selected (Lipinski, 2004). The number of rotatable bonds ($n\text{Rotb}$) ≤ 10 , were also considered for the further toxicity and molecular docking analysis. The prediction of

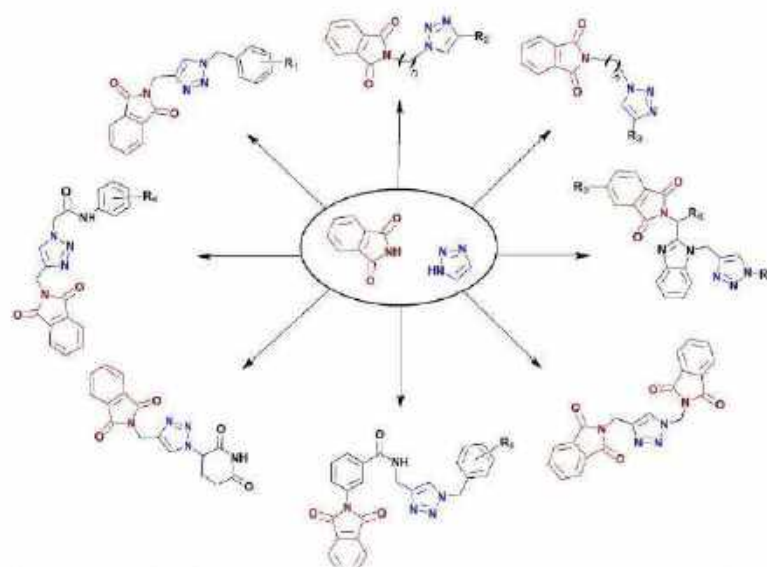


Figure 1. The chemical structures of 1,2,3-triazole-phthalimide bioactive derivatives obtained from Scifinder database: R_1 : 4-methylbenzyl, 2-methylbenzyl, 4-methoxybenzyl, 2-fluorobenzyl, 3-fluorobenzyl, 4-fluorobenzyl, 2-chlorobenzyl, 3-chlorobenzyl, 2,3-dichlorobenzyl, 3,4-dichlorobenzyl, 2-bromobenzyl, 4-bromobenzyl, 2-nitrobenzyl; R_2 : 2-(methylsulfonyl)-1,3-benzothiazole or phenyl; R_3 : carbohydrate or phthalimide; R_4 : 4-methyl, 4-chloro, H, 4-bromo, 3-nitro, 4-methoxy, 2-chloro, 3-chloro, 4-fluoro, 2-methoxy, 3-methoxy, 2-methyl; R_5 : Methyl or H; R_6 : methylbutyl, methyl and 2-methylpropyl; R_7 : 4-fluorophenyl, p-tolyl, H, 1-pentafluorophenyl, 4-(trifluoromethyl)phenyl, 1-benzyl, 1-phenyl, 4-methoxyphenyl, 2,4-difluorophenyl, 2,6-difluorophenyl, 1-perfluorophenyl, 2-fluorophenyl, 4-pyridinyl; R_8 : H, 4-methoxybenzyl, 2-fluorobenzyl, 4-fluorobenzyl, 4-chlorobenzyl, 2,6-dichlorobenzyl, 3-bromobenzyl, 4-bromobenzyl, 2-nitrobenzyl, 3-nitrobenzyl, 4-nitrobenzyl.

toxicological potential of these compounds was performed using the pKCSM on-line platforms (Pires et al., 2015). The AMES toxicity, hepatotoxicity, and immunotoxicity parameters were also used to filter out compounds.

2.3. Molecular docking of 1,2,3-triazole-phthalimide hybrids on SARS-CoV-2 target proteins

The molecular docking of selected compounds on SARS-CoV-2 target proteins was carried out using rDock 2.1 and AutoDock 4.2 software. The main protease (M^{pro}), spike (SP) and viral nucleocapsid (NCP) proteins were chosen according to the stereochemical parameters provided by Ramachandran analysis, using the PROCHECK server (Laskowski et al., 2006). Molecular docking was performed based on the confrontation between optimized ligands structure and biomacromolecules from SARS-CoV-2. The crystal structure of viral proteins was obtained from the Protein Data Bank (PDB) under the codes: spike: 6V5B, in its open (6VYB) and closed states (6VXX) (Walls et al., 2020; Wrapp et al., 2020); nucleocapsid protein, 6VYQ (Yadav et al., 2020); and M^{pro} , 5R80 (Razzaghi-Asl et al., 2020). The validation of target protein-ligand complex structures was performed using the co-crystallized standard ligand of target proteins to ensure the virtual screening process. For this, M^{pro} was redocked with its co-crystallized ligand RGZ: methyl 4-sulfamoylbenzoate. 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 analysed and rendered using Discovery Studio v20.1.0.19295.

2.4. Molecular fractionation with conjugate caps (MFCC—ab initio)

To calculate the interaction energy between receptors and ligands, the complexes obtained by molecular docking were used as an input for the molecular fractionation method with conjugated caps (MFCC). For this purpose, a convergence radius of 15 Å was defined (having its origin in the geometric center of the ligands). All the residues with at least one atom inside the spherical volume were taken into account. All atomic positions were kept fixed, with the exception of hydrogen atoms, which were optimized using consistent valence force field (CVFF). Subsequently, simulations within the Density Functional Theory formalism using the Local Density Approximation (DFT/LDA) were carried out using the DMOL3 code.

To obtain the interaction energy between each eligible amino acid and the ligands, a fractionation was applied, respecting Eq. (1):

$$E(L-R^i) = E(L-C^{(i-1)}R^iC^{(i+1)}) - E(C^{(i-1)}R^iC^{(i+1)}) \\ - E(L-C^{(i-1)}C^{(i+1)}) + E(C^{(i-1)}C^{(i+1)})$$

where $E(L-R^i)$ represents the interaction energy between the ligand L and the amino acid R^i , where cap $C^{(i-1)}$ and $C^{(i+1)}$ is arranged including the predecessor amino acid and posteriorly

Table 1. Analysis of physicochemical and pharmacokinetic profiles of selected phthalimide-1,2,3-triazole derivatives.

	Physicochemical properties					Absorption		Metabolism					Excretion TC
	HBA	HBD	ClogP	MW	NRB	BBB	PGP	1A2	2C19	2C9	2D6	3A4	
A01	4	0	2.47	332.36	4	Yes	No	Yes	Yes	Yes	No	No	-0.07
A02	4	0	2.43	332.36	4	Yes	No	Yes	Yes	Yes	No	No	-0.06
A03	5	0	2.16	349.46	5	No	No	Yes	Yes	Yes	No	Yes	-0.04
A04	5	0	2.62	336.32	4	Yes	No	Yes	Yes	Yes	No	No	0.15
A05	5	0	2.42	336.32	4	Yes	No	Yes	Yes	Yes	No	No	0.12
A06	5	0	2.44	336.32	4	Yes	No	Yes	Yes	Yes	No	No	0.10
A07	4	0	2.63	352.77	4	Yes	No	Yes	Yes	Yes	No	No	0.34
A08	4	0	2.64	352.77	4	Yes	No	Yes	Yes	Yes	No	No	0.28
A09	4	0	3.15	387.22	4	Yes	No	Yes	Yes	Yes	No	No	0.46
A10	4	0	3.17	387.22	4	Yes	No	Yes	Yes	Yes	No	No	0.40
A11	4	0	2.71	397.23	4	Yes	No	Yes	Yes	Yes	No	No	0.32
A12	4	0	2.75	397.23	4	Yes	No	Yes	Yes	Yes	No	No	0.25
A13	6	0	1.89	363.33	5	No	No	No	Yes	No	No	No	-0.07
B01	5	0	3.03	407.47	5	No	No	Yes	Yes	Yes	No	Yes	0.41
B02	5	0	3.19	421.50	6	No	No	Yes	Yes	Yes	No	Yes	0.49
B05	4	0	2.04	304.30	3	Yes	No	Yes	Yes	Yes	No	No	0.11
B06	4	0	2.23	318.33	4	Yes	No	Yes	Yes	Yes	No	No	0.11
B07	4	0	2.54	332.36	5	Yes	No	Yes	Yes	Yes	No	No	0.19
B08	4	0	2.87	348.38	6	Yes	No	Yes	Yes	Yes	No	Yes	0.15
C01	10	0	1.03	484.46	11	No	No	No	No	No	No	Yes	1.37
C04	6	0	1.46	401.37	5	No	Yes	No	Yes	Yes	No	Yes	-0.23
C05	6	0	1.78	415.40	6	No	Yes	No	Yes	Yes	No	Yes	-0.14
C06	6	0	2.09	429.43	7	No	Yes	No	Yes	Yes	No	Yes	-0.13
D01	5	1	1.75	375.38	6	No	Yes	No	No	Yes	No	Yes	-0.07
D02	5	1	1.92	395.80	6	No	No	No	Yes	Yes	No	Yes	0.05
D03	5	1	1.41	361.35	6	No	Yes	No	No	No	No	No	-0.07
D04	5	1	2.06	440.25	6	No	No	No	Yes	Yes	No	Yes	0.03
D05	6	1	1.45	391.38	7	No	Yes	No	No	Yes	No	Yes	-0.05
D07	5	1	2.00	395.80	6	No	No	No	Yes	Yes	No	Yes	0.32
D08	5	1	1.94	395.80	6	No	No	No	Yes	Yes	No	Yes	0.11
D09	6	1	1.72	379.34	6	No	No	No	No	No	No	No	-0.24
D10	6	1	1.46	391.38	7	No	Yes	No	No	Yes	No	Yes	-0.11
D11	6	1	1.42	391.38	7	No	Yes	No	No	Yes	No	Yes	0.02
D12	5	1	1.75	375.38	6	No	Yes	No	No	Yes	No	Yes	-0.06
E03	5	1	3.37	430.50	6	No	No	No	Yes	Yes	Yes	Yes	0.51
E21	6	0	3.31	491.54	7	No	Yes	No	Yes	Yes	No	Yes	0.73
E25	6	0	3.47	466.47	5	No	Yes	No	Yes	Yes	No	No	0.54
E26	5	0	3.53	462.50	5	No	Yes	No	Yes	Yes	No	No	0.72
E27	5	0	3.22	448.48	5	No	Yes	No	Yes	Yes	No	No	0.71
E29	6	0	3.58	466.47	5	No	Yes	No	Yes	Yes	No	No	0.56
E31	5	0	3.29	462.50	5	No	Yes	No	Yes	Yes	No	Yes	0.81
E33	5	1	2.17	374.40	4	No	No	No	Yes	Yes	No	Yes	0.84
E35	5	0	3.90	476.53	5	No	Yes	No	Yes	Yes	No	Yes	0.72
E37	5	1	2.54	388.42	4	No	No	No	Yes	Yes	No	Yes	0.85
E38	5	0	3.62	462.50	5	No	Yes	No	Yes	Yes	No	No	0.72
E39	5	0	3.68	476.53	6	No	Yes	No	Yes	Yes	No	Yes	0.81
E40	6	0	2.89	463.49	5	No	Yes	No	Yes	Yes	No	Yes	0.76
E45	6	0	3.99	494.52	6	No	Yes	No	Yes	Yes	No	No	0.44
F01	6	1	0.33	325.28	2	No	No	No	No	No	No	No	-0.17
F02	6	1	0.24	339.31	3	No	No	No	No	No	No	No	-0.19
G01	5	1	2.87	437.45	7	No	Yes	No	Yes	Yes	No	Yes	0.05
G02	6	1	2.90	467.48	8	No	Yes	No	Yes	Yes	Yes	Yes	0.04
G03	6	1	3.19	455.44	7	No	Yes	No	Yes	Yes	No	Yes	-0.15
G04	6	1	3.21	455.44	7	No	Yes	No	Yes	Yes	No	Yes	-0.20
G05	5	1	3.36	471.90	7	No	Yes	No	Yes	Yes	No	Yes	-0.20
H01	6	0	1.39	387.35	4	No	Yes	No	Yes	No	No	No	-0.25
H02	6	0	1.33	387.35	4	No	Yes	No	Yes	No	No	No	-0.26

HBA: Hydrogen-Bond Acceptors, HBD: Hydrogen-Bond Donors, ClogP: Octanol-water partition coefficient, MW: Molecular Weight, NRB: Number Rotatable Bonds, BBB: Blood Brain Barrier, PGP: P-glycoprotein Substrate, TC: Total Clearance (log mL/min/kg).

respectively of R^i . The term $E(L - C^{(i-1)}R^iC^{(i+1)})$ is the total energy of the system consisting of the ligand and the residue with its caps; the $E(C^{(i-1)}R^iC^{(i+1)})$ is the total energy of the waste and the isolated caps $E(L - C^{(i-1)}R^iC^{(i+1)})$ the total energy of the system formed by the ligand and only the caps; and finally, $E(C^{(i-1)}C^{(i+1)})$ the energy of the system composed by the caps. The total energy of the ligand interaction with the entire receptor is obtained by adding the individual energy of each eligible amino acid, as in Eq. (2).

$$\sum_{i=1}^N E(L - R^i)$$

where N is the number of eligible amino acids.

2.5. Molecular dynamics and binding energy calculations

Molecular dynamics and simulation (MDS) studies were carried out in order to determine the stability and convergence

Table 2. *In silico* toxicological prediction of 1,2,3-triazole-phthalimide derivatives that showed better pharmacokinetic profile.

Comp.	AMES toxicity	MTD	HERG	hERG II	ORAT (LD ₅₀)	ORCT	Hep	IT	SS
A01	No	-0.43	No	Yes	1.984	1.317	Yes	No	No
A02	No	-0.40	No	Yes	1.994	1.305	Yes	No	No
A03	Yes	-0.30	No	Yes	2.112	1.373	Yes	No	No
A04	No	-0.13	No	Yes	1.991	1.823	Yes	No	No
A05	Yes	-0.22	No	Yes	2.062	1.404	Yes	No	No
A06	No	-0.31	No	Yes	2.050	1.384	Yes	No	No
A07	No	-0.39	No	Yes	2.044	1.235	No	No	No
A08	No	-0.40	No	Yes	2.035	1.269	No	No	No
A09	No	-0.40	No	Yes	2.178	1.225	Yes	No	Yes
A10	No	-0.40	No	Yes	2.156	1.257	Yes	No	No
A11	No	-0.39	No	Yes	2.053	1.225	Yes	No	No
A12	No	-0.42	No	Yes	2.040	1.235	Yes	No	No
A13	Yes	-0.38	No	Yes	2.980	1.595	Yes	Yes	No
B01	No	0.38	No	Yes	2.309	0.850	Yes	No	No
B02	No	0.40	No	Yes	2.244	0.924	Yes	No	No
B05	No	0.48	No	Yes	2.541	1.133	No	No	No
B06	No	0.47	No	Yes	2.429	1.298	Yes	No	No
B07	No	0.51	No	Yes	2.460	1.411	Yes	No	No
B08	No	0.55	No	Yes	1.888	1.171	Yes	No	No
C01	No	0.63	No	No	2.439	1.309	Yes	No	No
C04	No	-0.14	No	Yes	2.272	2.020	Yes	No	No
C05	No	-0.13	No	Yes	2.243	2.045	Yes	No	No
C06	No	-0.09	No	Yes	2.221	1.992	Yes	No	No
D01	No	-0.21	No	Yes	2.153	1.674	Yes	No	No
D02	No	-0.18	No	Yes	2.148	1.631	Yes	No	No
D03	No	-0.25	No	Yes	2.109	1.695	Yes	No	No
D04	No	-0.19	No	Yes	2.158	1.624	Yes	No	No
D05	No	-0.14	No	Yes	2.080	1.654	Yes	No	No
D07	No	-0.25	No	Yes	2.157	1.625	Yes	No	No
D08	No	-0.22	No	Yes	2.171	1.693	Yes	No	No
D09	No	-0.12	No	Yes	2.065	1.605	Yes	No	No
D10	No	-0.25	No	Yes	2.093	1.648	Yes	No	No
D11	No	-0.21	No	Yes	2.111	1.691	Yes	No	No
D12	No	-0.28	No	Yes	2.155	1.668	Yes	No	No
E03	No	0.24	No	Yes	2.252	2.644	Yes	No	No
E21	No	0.23	No	Yes	2.480	1.690	Yes	No	No
E25	No	0.12	No	Yes	2.413	0.673	Yes	No	No
E26	Yes	0.08	No	Yes	2.416	0.803	No	No	No
E27	Yes	0.117	No	Yes	2.417	0.721	No	No	No
E29	Yes	0.121	No	Yes	2.416	0.555	Yes	No	No
E31	Yes	-0.028	No	Yes	2.435	0.822	No	No	No
E33	Yes	0.042	No	Yes	2.191	1.663	No	No	No
E35	No	0.200	No	Yes	2.463	1.074	No	No	No
E37	Yes	0.095	No	Yes	2.115	1.668	No	No	No
E38	No	0.217	No	Yes	2.465	0.993	No	No	No
E39	No	0.108	No	Yes	2.459	1.115	No	No	No
E40	No	0.225	No	Yes	2.441	1.590	No	No	No
E45	No	0.276	No	Yes	2.484	0.917	Yes	No	No
F01	No	-0.151	No	No	2.482	1.831	Yes	No	No
F02	No	0.095	No	No	2.364	1.965	Yes	No	No
G01	Yes	0.277	No	Yes	2.647	0.584	Yes	No	No
G02	Yes	0.236	No	Yes	2.853	0.578	Yes	No	No
G03	Yes	0.378	No	Yes	2.776	0.505	Yes	No	No
G04	Yes	0.292	No	Yes	2.816	0.532	Yes	No	No
G05	Yes	0.166	No	Yes	2.710	0.395	Yes	No	No
H01	No	0.015	No	Yes	2.110	1.889	Yes	No	No
H02	No	-0.018	No	Yes	2.216	1.985	Yes	No	No

MTD: Maximum Tolerated Dose—log(mg/kg/day), hERG I/II: Human Ether-a-go-go-related Gene, ORAT: Acute Oral Toxicity in Rats (LD₅₀)—mol/kg, ORCT: oral rat chronic toxicity log(mg/kg/day), Hep: Hepatotoxicity, IT: Immunotoxicity, SS: Skin Sensitization.

of **E38** and **E40** molecules complexed with M^{Pro}. To set up the simulation, initially, the systems were built for complexes of **E38**-M^{Pro} and **E40**-M^{Pro}, respectively, in a system builder. For this purpose, Desmond 2018-4 was used to set up the initial parameters within an explicit SPC water model and placed in the orthorhombic box 4.0 × 4.0 × 4.0 Å. All the protease-ligand complexes were neutralized with NaCl by adding 0.15M Na⁺ ions. ASL module was used to select the

specific residues of ligand and protein molecule. The prepared systems were relaxed using Desmond default protocol of relaxation (Khan et al., 2020). MDs run of 20 ns was set up at constant temperature and constant pressure (NPT) for the final production run. The NPT ensemble was set up using Nosé-Hoover chain coupling scheme (Dayer et al., 2017) at temperature 300 K for final production and throughout the dynamics with relaxation time 1 ps. RESPA integrator was used to calculate the binding interactions for a time step 2 fs (Kaiser, 2005). All the other parameters were associated in the settings as described elsewhere (Chowdhury, 2020). After the final production run, the simulation trajectories of main protease complexed with **E38** and **E40** molecules were analyzed for final outcome of RMSD, RMSF and number of hydrogen bonds formed from the simulation. Binding energies of the complexes were calculated using MM-GBSA (Genheden & Ryde, 2015) for every 1 ns trajectory up to 20 ns and the average binding energies with standard deviations were measured for accurate binding approximation and stability described elsewhere (Genheden & Ryde, 2015).

3. Results and discussion

3.1. *In silico* pharmacokinetic and ADMET assays

In silico screening of compounds against new coronavirus targets is considered a useful strategy for the detection of molecules with great probability of presenting good pharmacokinetic properties and *in vitro* and *in vivo* low toxicity (Yang et al., 2020). Thus, we used *in silico* approaches to predict the pharmacokinetics and the toxicological potential of 101 phthalimide-1,2,3-triazole derivatives, aiming to identify leader compounds against SARS-CoV-2 (Figure 1). These compounds were categorized according to the radicals attached to the 1,2,3-triazole-phthalimide nucleus into groups A-H, as showed in supplementary material (Tables S1–S8): (A) derivatives containing benzyl substituents (Tehrani et al., 2019); (B) derivatives containing phenyl or benzothiazole groups (Da Silva et al., 2019); (C) derivatives containing carbohydrate groups and an additional phthalimide (Assis et al., 2012); (D) compounds with *N*-phenylacetamides substituents (Phatak et al., 2019); (E) presence of benzimidazole group between phthalimide-triazole nucleus and three radicals variations (Singh et al., 2020); (F) thalidomide derivative analogues (Ronnebaum & Luzzio, 2016); (G) derivatives of phthalimide-benzamide-1,2,3-triazole (Sadat-Ebrahimi et al., 2020); and (H) derivatives with an additional phthalimide (López-González et al., 2016).

For hit compounds selection, molecules from the above-mentioned series, that violated the Lipinski's rule of five (RO5) and therefore, those which have poor druglike properties, were filtered out. The lipophilicity and solubility are the key molecular properties for drug absorption. Lipinski's rule of five is a rule of thumb that describes the druggability of a given molecule. Our pharmacokinetic analysis (supplementary material, Tables S9 and S10) revealed that 44 out 101 compounds violated at least one of the Lipinski's rules. Out of these, 11 derivatives were predicted to have low gastrointestinal absorption (GIA). Although the compounds **B03** and **B04**

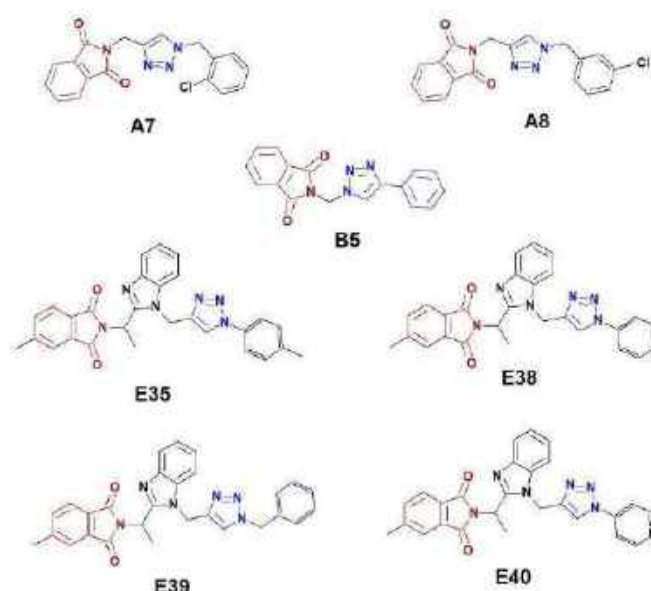


Figure 2. The chemical structures of derivatives A7, A8, B5, E35, E38, E39, and E40 chosen for molecular docking studies.

Table 3. XGBoost predictive profile of binding energies for A07, A08, B05, B35, E38, E39 and E40 triazole-phthalimide derivatives against SARS-CoV-2 targets.

Comp.	Energy (kcal/mol)	Spike protein				
		NPPO SR80	6V5B	6VXX	6VY3	NCP 6VY0
A07	E	-103.8	-94.83	-92	-89.47	-94.5
	VDW	-95.41	-71.12	-79.44	-74.98	-80.5
	HB	-8.4	-23.71	-12.56	-14.5	-14
	Elec	0	0	0	0	0
A08	E	-103.4	-93.35	-91.74	-101.5	-94.5
	VDW	-95.37	-83.81	-84.57	-85.46	-80.5
	HB	-8.1	-9.54	-7.22	-15.1	-14
	Elec	0	0	0	0	0
B05	E	-89.9	-99.46	-107.5	-84.81	-88.16
	VDW	-79.51	-66.62	-76.07	-67.26	-75.4
	HB	-10.4	-32.86	-31.48	-17.55	-12.7
	Elec	0	0	0	0	0
E35	E	-107.5	-98.8	-102.2	-109.1	-102.3
	VDW	-95.5	-78.2	-92.48	-91.94	-92.9
	HB	-12	-26.39	-9.80	-17.24	-9.6
	Elec	0	0	0	0	0
E38	E	-117.2	-107.1	-116.6	-93.2	-105.3
	VDW	-109.1	-91.53	-94.08	-80.36	-97.3
	HB	-8.1	-15.01	-22.61	-12.84	-18
	Elec	0	0	0	0	0
E39	E	-103.3	-99.32	-108.4	-98.55	-108.8
	VDW	-99.3	-83.36	-91.46	-80.55	-91.8
	HB	-4	-15.96	-16.94	-7.7	-17
	Elec	0	0	0	0	0
E40	E	-106.3	-105.1	-94.82	-96.75	-101.9
	VDW	-99.36	-82.52	-91.02	-87.53	-89.4
	HB	-7	-22.65	-3.8	-9.21	-12.5
	Elec	0	0	0	0	0

E: energy; VDW: van der Waals; HB: hydrogen bonds; Elec: electrostatic interactions.

did not violate the RO5, they showed a low GIA profile and were also rejected as hit compounds. Thus, 57 compounds were further submitted to ADMET analysis (Table 1).

The high oral bioavailability, preconized by Lipinski's rules, is one of main desirable characteristics of a druggable compound and an important factor for the optimization of bioactive molecules as therapeutic agents. Oral route is one of the preferred pathways of drug administration due to its unique advantages, including sustained and controllable drug delivery, easy administration and high patient compliance (Ekins et al., 2010; Homayun et al., 2019). Passive intestinal absorption (associated with low MW), reduced molecular flexibility (measured by NRB), low TPSA or total hydrogen bond counts (HBA and HBD) are important predictors of good oral bioavailability (Brito, 2011). A compound having CLogP ranging 0.5 to 3.5 and molecular mass < 500 kDa is considered a druggable candidate (Tetko et al., 2016).

The presence of more than two heterocycles groups in the same molecule can result in compounds with high molecular weight and LogP values. These characteristics can be observed in most of the compounds belonging to the group E (1,2,3-triazole-benzimidazole-phthalimide derivatives) and in compounds G07 and G08 (1,2,3-triazol-benzimidazole-phthalimide), which are therefore excluded from our study. Independently of molecular weight, compounds with high probability of good oral bioavailability have no more than 5 hydrogen bond donors and no more than 10 hydrogen bond acceptors. According to these latter criteria, our predictive analysis showed that the C03 and C02, D05, G09, G10, and G11 have low permeability throughout biological barriers.

The blood-brain barrier (BBB) protects the central nervous system (CNS) preventing certain substances (mostly harmful) from entering the brain tissues. The BBB limits the passage

of most of the external compounds to maintain CNS at a steady state (Durán-Iturbide et al., 2020; Miao et al., 2019). In addition, P-glycoprotein (P-gp) actively transports a wide variety of compounds out of cells. This protein is highly associated with the ADME properties, playing a major role in the multidrug resistance (MDR) phenomenon (Li et al., 2014). Our ADME analysis showed that 15 out of 57 compounds are predicted to cross the BBB, and 27 compounds were identified as a potential substrate of P-gp. Total Clearance (TC) is an important parameter associated with both the half-life and bioavailability of a xenobiotic compound. This parameter has

a direct impact in determining the dose regimen (how often) and dose size (how much) of a given drug. Thus, its prediction helps us to determine the feasibility of clinical dosing and provides a framework for the starting dose in *in vivo* studies (Durán-Iturbide et al., 2020). In this work, the TC values of 1,2,3-triazoles-phthalimide derivatives varied from -0.26 to 0.85 log (mL/min/kg), for H2 and E37 derivatives, respectively. Higher values of TC suggest that the xenobiotic is removed rapidly from the body, whereas a low clearance value indicates slower removal.

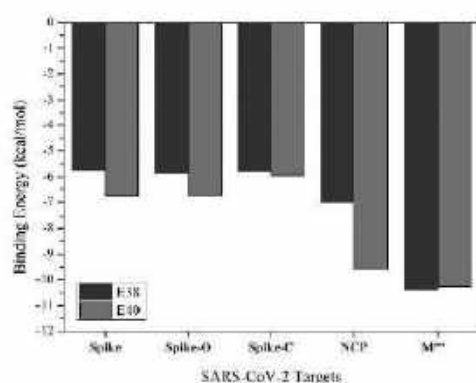


Figure 3. the graphical representation of binding energy of E38 and E40 derivatives on SARS-CoV-2 proteins. Spike (in prefusion conformation), Spike-O: spike protein in its open state, Spike-C: spike protein in its closed state, NCP: nucleocapsid protein, MPP: main protease.

3.2. Prediction of toxicological potential of 1,2,3-triazole-phthalimide compounds

Poor pharmacokinetics and adverse side effects, due to compound toxicity, are pointed as the main causes of late-stage failures in drug development (Li et al., 2019). In this regard, *in silico* predicted toxicological potential of the above-mentioned compounds was investigated. Our results showed that 15 out of 57 compounds were positive for AMES and 46 compounds presented potential to be hepatotoxic. Of these, 10 compounds were both positive for AMES and hepatotoxic. The analysis of 8 selected compounds, that were either negative for AMES test or presented no hepatotoxic potential, revealed a predicted LD₅₀ for acute toxicity in rats ranging from 1,888 to 2,980 mg/kg. The minimum value for oral chronic toxicity (ORCT) was 0.160 and the maximum value was 2,046 mg/kg/day. Besides presenting positive score for AMES test and hepatotoxicity the compound A09 also showed potential to cause adverse effects on the skin (Table 2).

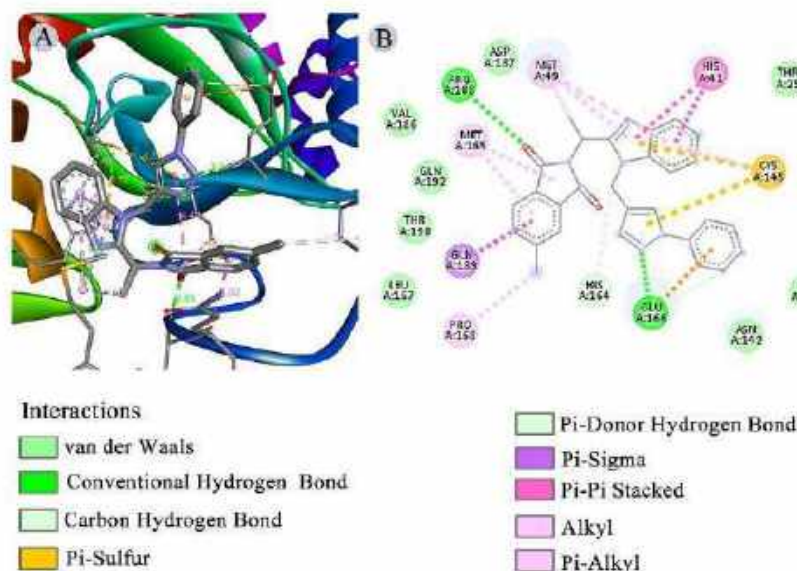


Figure 4. The molecular docking of derivative E40 with MPP. (A) Representative diagram of docking result of E40 with MPP. (B) Binding interaction of E40 with amino acid residues of MPP.

8 V. N. HOLANDA ET AL.

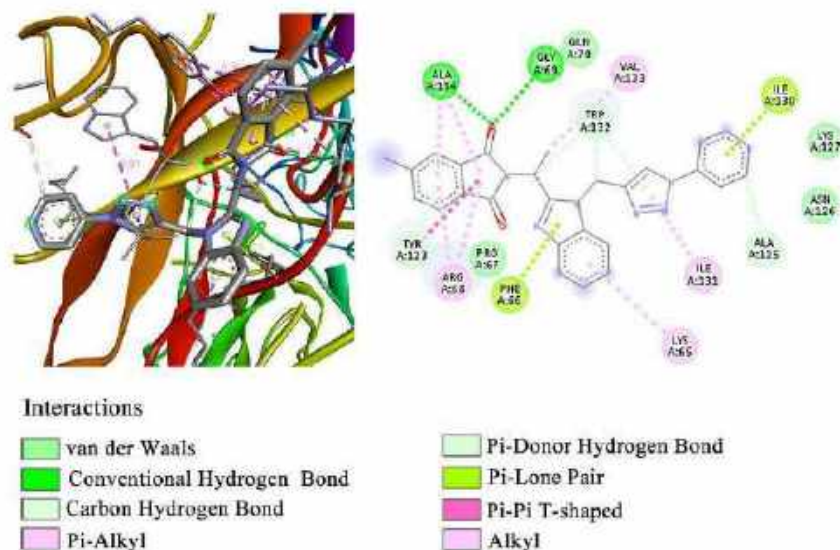


Figure 5. The molecular docking of derivative E40 with NCP. (A) Representative diagram of docking result of E40 with NCP. (B) Binding interaction of E40 with amino acid residues of NCP.

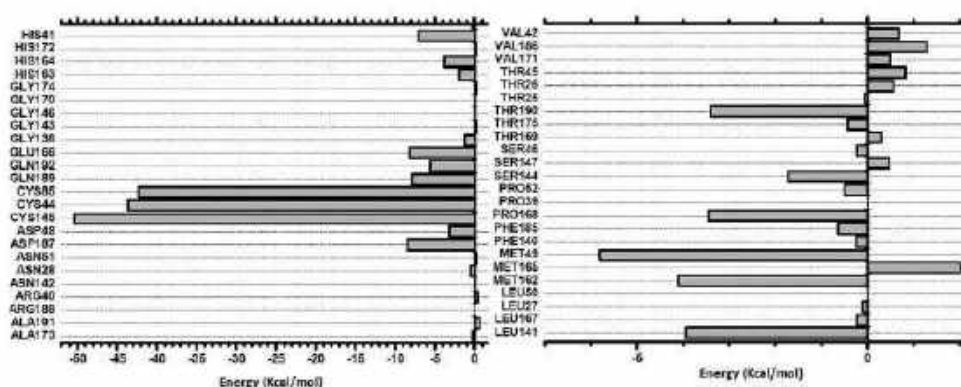


Figure 6. Energy for each nucleocapsid phosphoprotein amino acids in a radius of 15 Å from E40 ligand.

In silico predictive toxicological evaluation is easy to perform, cost-effective, high-throughput alternative to *in vitro* assays and, even replaces time-consuming, expensive *in vivo* experiments (Myatt et al., 2018). Derivatives **E26**, **E27**, **E31**, **E33**, and **E37** showed a positive predictive result for the AMES test, a method that assesses the mutagenic potential of chemical compounds (Kauffmann et al., 2020). The absence of a methyl at the carbon 5 of phthalimide ring and the presence of 1H-benzotriimidazole group may be related to the toxic effect of these compounds. In addition, changes in benzimidazole ligands can modulate their binding to deoxyribonucleic acid and the selectivity of these

compounds towards the host genetic material (Gumus et al., 2009).

Besides the mutagenic potential, another important variable taken in account in our predictive analysis was the evaluation of the hepatotoxic potential of selected phthalimide-1,2,3-triazole derivatives. Although the compounds **A01**, **A02**, **A04**, **A06**, **A09**, **A10**, **A11** and **A12**, that have phthalimide-1,2,3-triazol-phenyl as the main structural group, were negative for AMES test, they showed important hepatotoxic potential. The presence of the methyl-benzyl group linked to 1,2,3-triazole-phthalimide nucleus, as well as the insertion of binders such as fluorine and bromine, may be

related to the deleterious potential of these compounds. Interestingly, the compounds from the same series that did not show a predictive hepatotoxic effect, contained chlorine at the carbon 2 or 3 of phenyl group. However, this same result was not observed when 2,3-dichloro or 3,4-dichloro was present. Like the derivatives from the A series, the molecules **E25**, **E45**, **G05**, and **G07** that also presented predictive hepatotoxic potential, have substituted halogenated groups in their structures. In order to better understand the

Table 4. Interaction energies between RNA binding domain of nucleocapsid phosphoprotein residues and **E40**.

Amino acids	Energy
ASP63	5.00
LEU64	6.10
LYS65	-7.00
PHE66	-4.32
PRO67	0.00
ARG68	-5.89
GLY69	-8.89
GLN70	-4.43
GLY71	-8.12
VAL72	-0.52
ILE84	-0.33
GLY85	-33.74
TYR86	-31.00
TYR87	-24.50
LEU121	-28.65
PRO122	-24.55
TYR123	-21.63
GLY124	-14.20
ALA125	-1.22
ASN126	-1.31
GLY129	-2.01
ILE130	-2.14
ILE131	1.87
TRP132	2.01
VAL133	2.02
ALA134	4.31
THR135	3.22
GLU136	4.87
ALA138	1.51
THR166	6.90
Total energy	-1.87E + 2 kcal/mol -8.03 eV

Table 5. Interaction energies between Spike-O amino acid residues and **E40**.

Amino acids	Energy
LYS1038	2.87
PHE906	-3.51
ASN907	-4.85
GLY908	0.31
ILE909	0.11
GLY910	0.20
VAL911	0.02
THR912	0.17
GLN1036	-2.14
SER1037	-22.17
LYS1038	-31.89
VAL1040	-15.72
ASP1041	-11.59
CYS1043	-9.25
GLY1046	-5.78
TYR1047	-5.23
HIS1048	-4.17
LEU1049	-10.83
THR1066	-4.22
TYR1067	-4.82
VAL1068	-10.11
GLU1092	-7.05
GLY1093	-4.64
GLN1106	4.58
ARG1107	2.89
ASN1108	-5.13
GLY885	-5.01
TRP886	-3.54
THR887	0.17
GLY888	-5.12
ALA890	-3.21
GLN901	-3.71
ALA903	-2.68
TYR904	-1.72
ARG905	-2.47
ASN907	-18.12
GLY908	-2.14
ILE909	5.21
GLY910	5.87
GLU1031	3.17
GLY1035	3.16
GLN1036	-2.51
SER1037	-8.10
LYS1038	17.54
HIS1048	-5.81
Total energy	-1.81E + 02 kcal/mol -7.77 eV

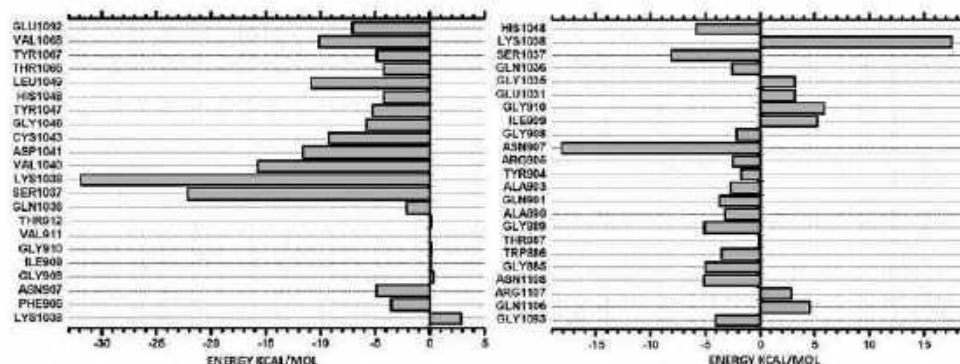


Figure 7. Energy for each Spike-O amino acids in a radius of 15 Å from **E40** ligand.

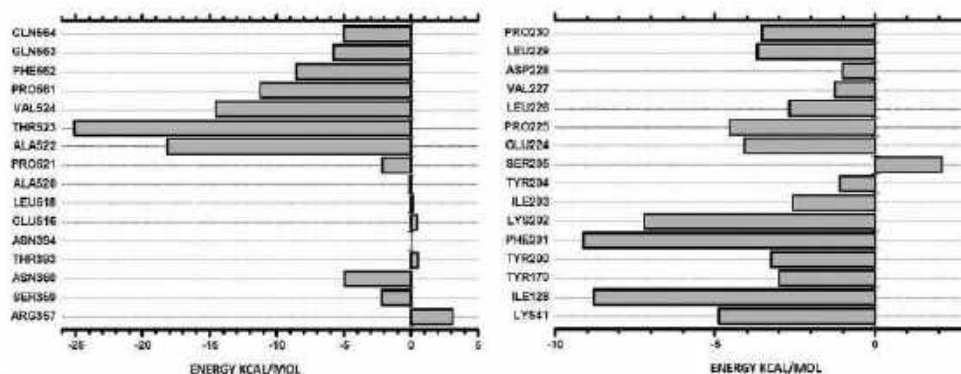


Figure 8. Energy for each Spike-C amino acid in a radius of 15 Å from E40 ligand.

Table 6. Interaction energies between Spike-C amino acid residues and E40.

Amino acid	Energy
ARG357	3.12
SER359	-2.18
ASN360	-4.97
THR393	0.52
ASN394	0.00
GLU516	0.44
LEU518	0.17
ALA520	-0.07
PRO521	-2.13
ALA522	-18.18
THR523	-25.10
VAL524	-14.55
PRO561	-11.20
PHE562	-8.56
GLN563	-5.77
GLN564	-5.01
LYS41	-4.87
ILE128	-8.78
TYR170	-2.99
TYR200	-3.25
PHE201	-9.11
LYS202	-7.21
ILE203	-2.56
TYR204	-1.11
SER205	2.10
GLU224	-4.10
PRO225	-4.55
LEU226	-2.67
VAL227	-1.25
ASP228	-1.02
LEU229	-3.68
PRO230	-3.53
Total energy	-7.52E +02 kcal/mol -6.54 eV

structure-toxicity relationship of the compounds containing halogen atoms, further evaluation of other chemical-structural parameters is required (Kortagere et al., 2008).

The derivatives **B01**, **B02**, **B06**, **B07**, and **B08** also showed positive predictive results for hepatotoxicity. The difference in the number of carbons in the aliphatic chain between phthalimide and triazole, also present in the molecules **C04**, **C05** and **C06**, **H01** and **H02**, could explain, at least in part, the predicted hepatotoxic potential found for compounds belonging to this series. Consistently, the number of carbons present in the aliphatic chain, that joins pharmacophoric

groups in hybrid derivatives of 1,2,3-triazole-phthalimide, has been associated with the occurrence *in vivo* hepatotoxicity (Da Silva et al., 2019). The presence of the benzothiazole group in compounds of the **E** series did not appear to be determinant for the predicted hepatotoxicity of these molecules, since other triazole-phthalimide derivatives containing the same group, like **E26**, **E27**, **E38**, **E31** and **E33** did not present hepatotoxic potential. Heterocyclic molecules containing the benzothiazole group have shown to be more promising drug candidates to treat infectious diseases, reinforcing the pharmacological potential of benzothiazole-containing molecules (Papadopoulou et al., 2013). It has been reported that molecules containing thalidomide group in its structure, as those of series **F**, presented high hepatotoxic potential (Kamiya et al., 2020). In this regard, only the derivatives **A7**, **A8**, **B5**, **E35**, **E38**, **E39**, and **E40** (Figure 2) showed good pharmacokinetic and toxicological profiles, and were therefore chosen for molecular docking.

3.3. Molecular docking of selected 1,2,3-triazole-phthalimide compounds

Computational simulations of ligand-protein docking are an important component of drug discovery, being mainly used for the virtual screening of hit compounds from large databases and to identify and evaluate the effects of chemical changes during leader optimization (Bordogna et al., 2011). In this regard, the accuracy of modeled structures should be taken into account. Our stereochemical analysis of SARS-CoV-2 target proteins, obtained from PDB (Figures S1–S5), showed that the percentage of the sum of amino acid residues in the most favoured and additional allowed regions was $\geq 95\%$. This result confirmed the high quality of our selected target models (Laskowski et al., 2006). In order to validate our molecular docking, the co-crystal standard ligand (RGZ, methyl 4-sulfamoylbenzoate) of M^{pro} complex (SR80) was redocked against this protein and the root-mean-square deviation (RMSD) was calculated for predicting the stability of the protein and protein-ligand complexes. Our results showed that Autodock program was able to generate a

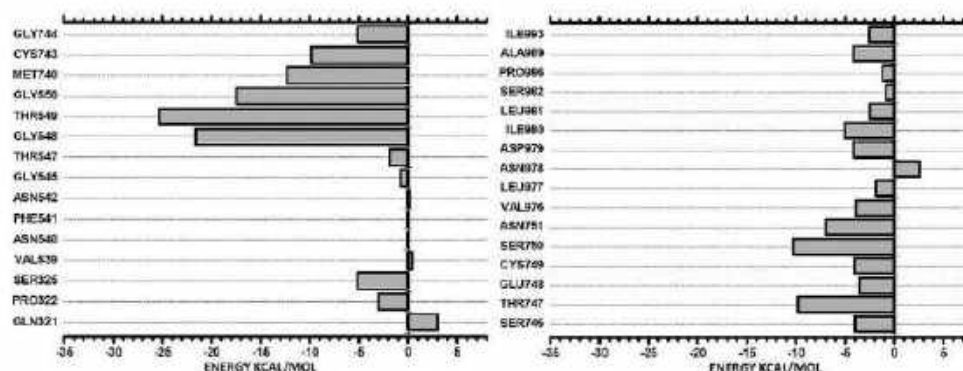


Figure 9. Energy for each spike glycoprotein (with a single receptor-binding domain up) amino acids in a radius of 15 Å from E40 ligand.

Table 7. Interaction energies between spike glycoprotein (with a single receptor-binding domain up) residues and E40.

Amino acids	Energy
GLN321	3.01
PRO322	-3.02
SER325	-5.13
VAL539	0.43
ASN540	0.01
PHE541	0.05
ASN542	0.18
GLY545	-0.78
THR547	-1.89
GLY548	-21.58
THR549	-25.31
GLY550	-17.44
MET740	-12.37
CYS743	-9.81
GLY744	-5.11
ASP745	-5.12
SER746	-4.01
THR747	-9.78
GLU748	-3.55
CYS749	-4.02
SER750	-10.24
ASN751	-7.03
VAL976	-3.89
LEU977	-1.85
ASN978	2.54
ASP979	-4.12
ILE980	-5.06
LEU981	-2.50
SER982	-0.84
PRO986	-1.22
ALA989	-4.10
ILE993	-2.56
Total energy	-1.65E +02 kcal/mol -7.14 eV

successful pose (RMSD = 1.9 Å) (Figure S6). It is assumed that scored poses with an RMSD of less 2.0 Å are considered to be successful (Bordogna et al., 2011; Razzaghi-Asl et al., 2020).

The docking analysis using iGemDock software revealed that the derivatives **A7**, **A8**, **B5**, **E35**, **E38**, **E39**, and **E40** showed favourable interactions energies for all the tested proteins from SARS-CoV-2 (Table 3). **E38** presented higher binding energy valued for spike protein in its closed state (Spike-C) (-116.6 kcal/mol) and spike protein in prefusion conformation (-107.1 kcal/mol). The higher binding energy

value for main protease (M^{pro}) was found for its interaction with **E38** (-117.2 kcal/mol). Among the tested compounds, **E39** presented highest affinity to RNA binding domain of nucleocapsid phosphoprotein with binding energy of -108.8 kcal/mol. For all ligand-protein complexes, the interaction energies were predominantly due to Van der Waals, followed by hydrogen bonds. No electrostatic interactions were observed for all interactions of compounds with SARS-CoV-2 proteins.

The use of molecular docking has been largely applied as a strategy for the selection of possible leader candidates against the new coronavirus (Karypidou et al., 2018). Since the SARS-CoV outbreak in 2002, *in silico* molecular docking and structural studies have demonstrated a relationship between the SARS-CoV-2 spike receptor-binding domain and the angiotensin-2 receptor (ACE2) in the host cells. This association is strictly linked to the infection and transmission of new corona virus in humans (Utomo & Meiyanto, 2020). The wide distribution of the ACE2 receptor in human tissues and organs could be related to the appearance of symptoms in the severe phase of the disease, mainly in the pulmonary system, where it is largely expressed at the surface of type II alveolar cells (Sanchis-Gomar et al., 2020). In our analysis we docked the selected compounds on different conformational states of SARS-CoV-2 spike protein. Overall, our results showed that all the selected compounds bound with high affinity to spike proteins independently of its conformational state (open, close, or in prefusion conformation). However, slight differences in binding energy values were observed. The lowest binding energy value found for the interaction of **E38**, and **E35** derivatives with different conformational states of spike protein, revealed the binding plasticity of 1,2,3-triazole-phthalimide derivatives towards this protein. Liu et al. (2004) described that the inhibition of peptides in the heptad repeat regions of the SARS-CoV spike protein results in the interference of the fusogenic mechanism. In this regard, it is possible that the selected Top-7 derivatives may interfere with the SARS-CoV-2 binding, entrance and replication processes in the host cells.

The predictive analysis of the inhibition constant values for best binding pose of protein-ligand complexes was

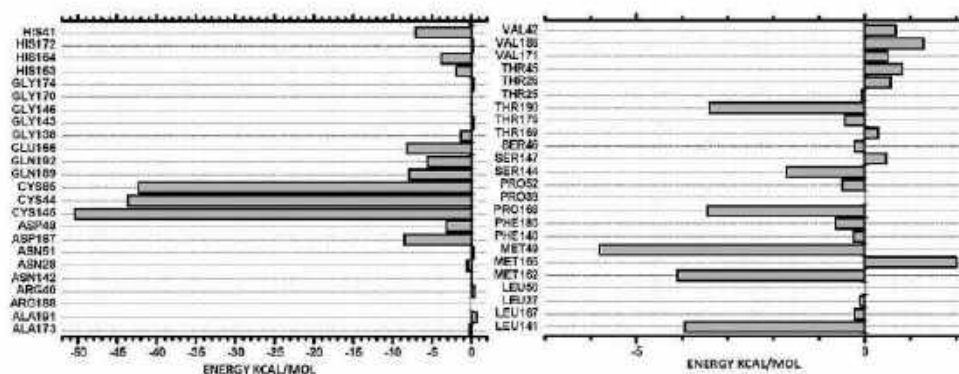


Figure 10. Energy for each main protease amino acids in a radius of 15 Å from E40 ligand.

Table 8. Interaction energies between main protease amino acid residues and E40.

Amino acids	Energy
ALA173	-0.25
ALA191	0.60
ARG188	0.00
ARG40	0.41
ASN142	0.02
ASN28	-0.50
ASN51	0.29
ASP187	-8.47
ASP48	-3.15
CYS145	-50.42
CYS44	-43.68
CYS85	-42.31
GLN189	-7.90
GLN192	-5.54
GLU166	-8.19
GLY138	-1.28
GLY143	0.26
GLY146	0.01
GLY170	0.03
GLY174	0.31
HIS103	-1.89
HIS164	-3.78
HIS172	0.23
HIS41	-7.06
ILE43	-0.22
LEU141	-3.94
LEU167	-0.23
LEU27	-0.11
LEU50	0.00
MET162	-4.10
MET165	2.00
MET49	-5.80
PHE140	-0.24
PHE185	-0.64
PRO168	-3.45
PRO39	0.00
PRO52	-0.50
SER144	-1.71
SER147	0.47
SER46	-0.23
THR169	0.29
THR175	-0.43
THR190	-3.40
THR25	-0.06
THR45	0.56
THR45	0.82
VAL171	0.50
VAL186	1.28
VAL42	0.67
Total energy	-2.01E + 02 kcal/mol -8.63 eV

performed using AutoDock software (Tables S11–S17). Our results showed that **A7**, **E35**, **E38** and **E40** presented higher binding affinity for at least one of the viral target SARS-CoV-2 proteins, with inhibition constant values (K_i) in the nanomolar range. All of these compounds showed high affinity for SARS-CoV-2 main protease. Comparing all ligand-protein interactions tested, the lowest values of inhibition constant (K_i) were found for the interaction of the compound **E38** with M^{pro} with $K_i = 23.85$ nM and binding energy of -10.4 kcal/mol for the best pose. The second-best value of K_i was obtained for the **E40**- M^{pro} complex with $K_i = 30.04$ nM and a binding energy of -10.26 kcal/mol. Compound **E40** also bound with high affinity to nucleocapsid protein, with $K_i = 91.09$ nM and binding energy of -9.6 kcal/mol (Figure 3).

In addition to the spike protein, coronavirus proteases are also essential for viral transmission and virulence by processing viral proteins involved in viral replication (Báez-Santos et al., 2015). Compounds that act as inhibitors of these proteins can be considered as promising antiviral agents by reducing the severity of the infection (Hall & Ji, 2020). All the top-7 compounds bound with high affinity to SARS-CoV-2 main protease, being **E38** and **E40** the compounds that presented the highest affinity (i.e. the lowest binding energy) towards this target. The analogous performance of these compounds toward M^{pro} can be attributed to chemical-structural similarities of the two derivatives, which are only distinguished from each other by the presence of the phenyl and pyridin-4-yl groups in the N1 of triazole ring. Molecules containing 1,2,3-triazole group have already been described as having anti-coronavirus activity, by interacting with viral proteases, corroborating the relevance of these proteins as the main target of these compounds (Karypidou et al., 2018). The main amino acids involved in **E40**-SARS-CoV-2 binding were MET165, MET49, HIS41, CYS145, GLU166 and GLN189 (M^{pro} —Figure 4) and ALA134, GLY69, VAL133, TRP132, ILE130, ALA125, ILE131, LYS65, PHE66, ARG68 and TYR123 (NCP—Figure 5). The most prevalent interaction was Van der Waals and hydrogen bonds. The electrostatic binding energy values were insignificant when compared to the contributions of van der Waals and hydrogen bonds.

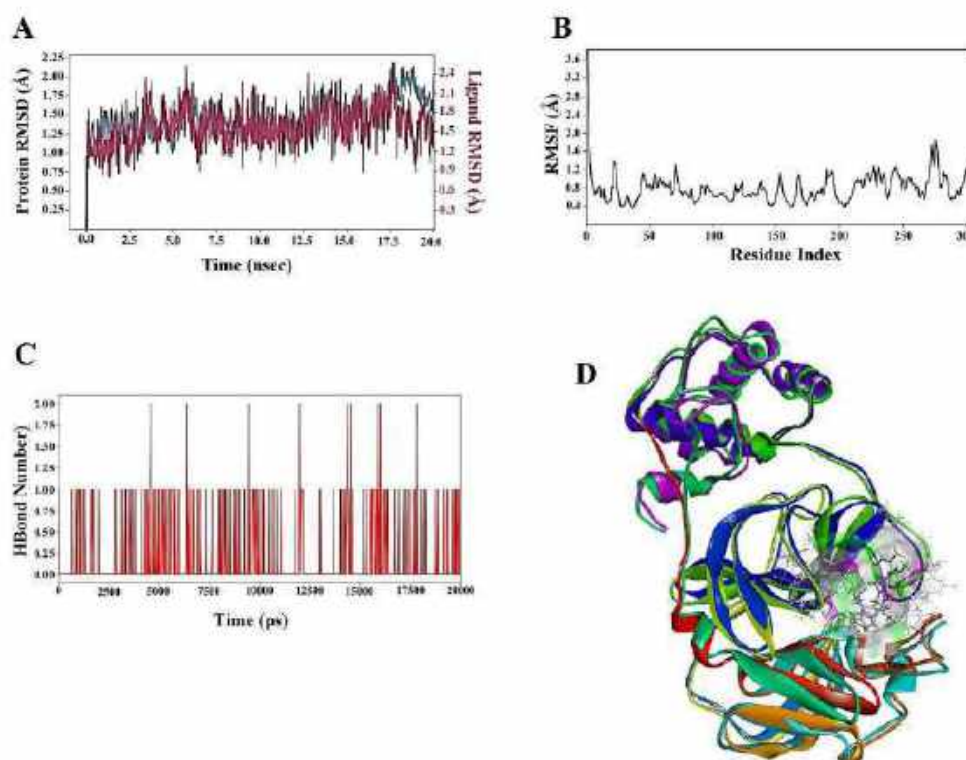


Figure 11. The molecular dynamic (MD) simulations of main protease bound to **E38**. (a) RMSD plot of C α -backbone (blue) and ligand (red) displayed stable convergence till 20 ns. (b) RMSF plot depicting fluctuations of each residual position of main protease amino acids. (c) Number of H-bonds formed during the entire course of simulation. (d) Superimposed initial and final frame structures of main protease complexed with **E38** (surface view) molecule before and after simulation.

In this study we also docked the Top-7 1,2,3-triazole-phthalimide derivatives on viral nucleocapsid protein. NCPS have been considered strategic targets to fight acute respiratory infections caused by SARS-CoV-2 due to their multiple functions in the viral replication cycle (Kang et al., 2020). All the compounds tested bound with high efficiency on NCP. Comparing the best-pose of selected molecules (1/10) for the NCP, the **E40** presented the most favorable binding energy (-9.6 kcal/mol) and was the only one that achieved an inhibition constant in the nanomolar range for this target. The interaction of these compounds with nucleocapsid proteins may interfere with essential functions of the viral cycle, such as the formation of the helical ribonucleoprotein complex, during the packaging of the viral genome, and the control of basic cellular functions in the host cell, like the deregulation of the cell cycle (Cong et al., 2020; Surjit et al., 2006). Although further *in vitro* analysis are needed to better understand the biological effects of the interaction of **E40**-SARS-CoV-2 nucleocapsid proteins complex, the high predicted binding affinity of this compound to these proteins suggests that **E40** may prevent nucleocapsid proteins from participating in the replication and release of viral particles (Zeng et al., 2008).

A characteristic of RNA viruses is the high rate of genetic mutation, which can result in the evolution of new viral strains and the inefficacy of treatment. Therefore, from a public-health perspective, the ability of our top-7 selected 1,2,3-triazole-phthalimide derivatives to bind to different viral proteins, may circumvent the drug resistance caused by single-target mutations or rare simultaneous mutations of several targets in different positions (Xu et al., 2020). Although some compounds were excluded from our study, it is important to take in mind that these molecules can be redesigned for more rational chemical-structural modifications, decreasing its toxic potential and/or improving their biological activity against target molecules.

Because our molecular docking analysis has pointed the compound **E40** as the most promising compound, the best complexes obtained between this compound and the targets were subjected to quantum energy calculations by the MFCC technique. The results of the interactions between **E40** and amino acids of the RNA binding domain of nucleocapsid phosphoprotein (PDB-ID 6VY0) are described in Figure 6 and Table 4.

Overall, 30 amino acids of NCP showed some kind of interaction with **E40**, 11 showing repulsion and 19 attraction

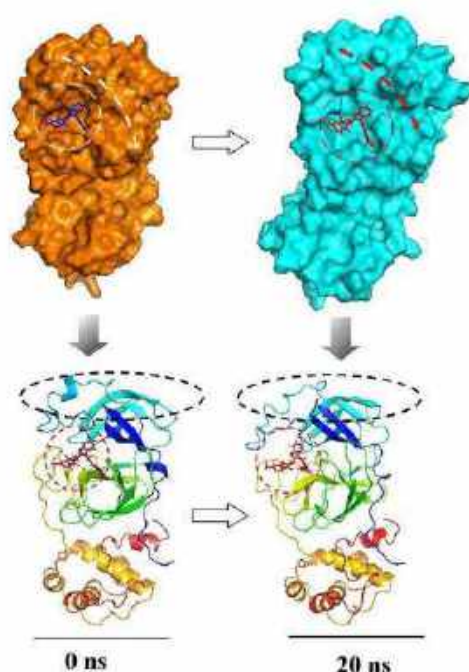


Figure 12: Surface view of main protease before MD simulation (orange) displaying little modifications in the E38-M^{pro} binding site (yellow ring) after simulation (cyan) (left). Lower panel depicted the conformational differences between the structures of E38 in the binding site provided orientation that is more accurate for higher binding.

with the ligand. The PRO67 residue presented energy value of 0.00. Consequently, it cannot be confirmed whether the interaction occurring with the ligand is repulsive or attractive. The general energy balance points to a stronger attraction (-8.03 eV) between the connection site of protein and the **E40** compound. The residues GLY85, TYR86, TYR87, LEU121, PRO122, TYR123 and GLY124 presented very low energies (negative values), suggesting very strong attractions by these amino acids, which are probably the anchor residues. Figure 7 and Table 5 show MFCC results between **E40** and the open state of SARS-CoV-2 spike ectodomain structure (PDB-ID 6VYB).

A total of 45 residues of spike-O showed energetic interactions with the drug, 13 of which are interacting repulsively and 32 attractively. The residues VAL1040, ASP1041, LEU1049, VAL1068, ASN907, and LYS1038 demonstrated a strong attraction for the ligand, possibly acting as anchoring residues. Figure 8 and Table 6 describe the results of the MFCC for interactions between spike glycoprotein in closed state (PDB-ID 6VXX) and **E40**.

There were 32 amino acids of spike protein interacting with **E40**, 5 with positive energies (repulsion), 26 with negative energies (attraction) and one with 0.0 energy (ASN394). The amino acids that stood out for having a strong attraction were: ALA522, THR523, VAL524 and PRO561. Although the

structures obtained at inputs spike-C and spike-O represented different conformational states of the same structural protein of SARS-CoV-2 (spike glycoprotein), the compound **E40** was strongly attached to both conformations but in different places. The results obtained for spike glycoprotein with a single receptor-binding domain up (6VSB) are shown in Figure 9 and Table 7.

A total of 32 residues of spike glycoprotein (with a single receptor-binding domain up) residues, interacted with the **E40** ligand, 6 of which presenting repulsion and 26 attraction interactions, with emphasis on the amino acids GLY548, THR549, GLY550, MET740 and SER750, which showed strong attraction for the compound. Figure 10 and Table 8 showed the MFCC results for **E40** interactions with the main protease in complex.

There are 49 amino acids of spike glycoprotein (with a single receptor-binding domain up) residues interacting with **E40**, 17 with slightly repulsive interactions, 3 with 0.0 energy and 29 with negative energy values. The CYS145, CYS44 and CYS85 amino acids residues, showed surprising attraction to **E40**, with energy values of -42.31 kcal/mol, -43.68 kcal/mol and -50.42 kcal/mol, respectively. These cysteine amino acids have already been identified as anchor residues in other studies on SARS-CoV-2 main protease (Komatsu et al., 2020). Taking in account the general energy balance, this protein also showed the lowest energy value in this study (-8.63 eV).

In a viral pandemic with rapid dispersion of cases, drug replacement strategy is promising to fight the disease, because substances that are commercially available in the pharmaceutical industry have already undergone several toxicity and safety tests. In chemistry, when atoms are held together, the energy needed to separate them is called binding energy. In covalent bonds, where the electrons are shared between atoms, the energy needed to break these bonds can vary, but energies less than -1.0 eV (Tkatchenko & Scheffler, 2009) suggest covalent bond among atoms. **E40** showed lower energy values in all analyses. Interestingly, but not surprisingly, **E40** showed affinity with all the three spike conformers (6VYB, 6VXX and 6VSB). Although, in this study, the ligand interacted with all Spike conformers, the anchorage residues were different in each interaction. This raises some concerns: would the **E40** function as an inhibitor of the Spike-ACE2 interaction in any of the three conformers? Is there a possibility of allosteric inhibition by the **E40**? Although our results suggest a drug repositioning for COVID-19, further *in vivo* and *in vitro* studies are needed to answer these questions.

3.4. MD simulation and MM-GBSA binding energy calculations

The final convergence and the stability of **E38** and **E40** bound with the main protease was assessed by MD simulation. The analysis of RMSD and RMSF plots showed that after 20 ns of convergence, **E3**, bound to the main protease, displayed a significant stability (Figure 11A and 11B). The interactions of this protein with **E38** displayed much less RMSD differences (~ 0.25 Å) showing its stable conformation (Figure 11A). The average of H-bonds formed between **E38** and the

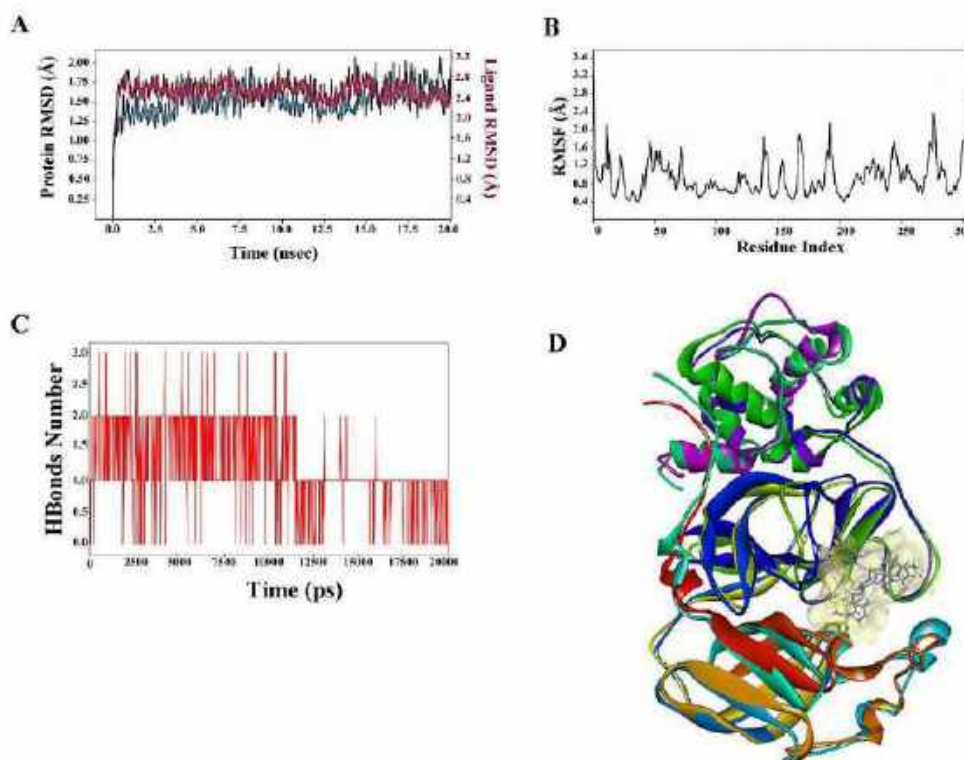


Figure 13. Molecular dynamic (MD) simulations of main protease bound to E40, (a) RMSD plot of C α -backbone (blue) and ligand (red) displayed stable convergence till 20 ns, (b) RMSF plot depicting fluctuations of each residual position of amino acids of main protease, (c) Number of H-bonds formed during the entire course of simulation, (d) Superimposed initial and final frame structures of main protease complexed with E40 (surface view) molecule before and after simulation.

main protease showed to be 1.0 throughout the simulation period for the stable conformation of both the main protease and E38 molecule (Figure 11C). The structural superimposition of the main protease-E38 complex before and after simulation displayed less significant changes in the overall conformation (Figure 11D). In addition, accommodation of E38 at the binding cavity of this protein showed small-altered orientation after simulation, which seems to be due to a change in the visible secondary structure at the E38-M^{pro} binding site (Figure 12).

After 20 ns of convergence, the E40-M^{pro} complex displayed significant stability as demonstrated by RMSD and RMSF plots (Figure 13A and 13B). The interactions of this protein with E40 displayed much less RMSD differences (~ 0.50 Å) conferring its stable conformation (Figure 13A). The average of H-bonds formed between E40 and the main protease showed to be 1.5 throughout the simulation period, for the stable conformation of both the main protease and the E40 molecule (Figure 13C). Structural superimposition of the main protease-E40 complex before and after simulation displayed less significant changes in the overall conformation (Figure 13D). In addition, accommodation of E40 at the binding cavity of M^{pro} showed small-altered orientation after the simulation which

appeared to be due to the change in the visible secondary structure at the E40-M^{pro} binding site (Figure 14).

The free energies of binding using MMGBSA were calculated for the E38 and E40-M^{pro} complexes of SARS-CoV-2. The properties of MMGBSA calculations are displayed in Table 9. The free energy average of binding for every 1 ns trajectory up to 20 ns, for the main protease-E38 complex displayed a $dG = -63.47$ kcal/mol, with standard deviation of 3.0 kcal/mol, coulombic force of -8.70 , solvent accessibility of 20.20 and high ligand binding affinity of -1.81 . On the other hand, the E40-main protease complex displayed a $dG = -63.31$ with standard deviation of 7.0 kcal/mol and high binding efficiency, solvent accessibility and significant coulombic as well covalent energies. All the properties calculated in the MD simulation point the significant role of E38 and E40 compounds as a potent inhibitor of the main protease of SARS-CoV-2.

4. Conclusion

Taken together, our results showed that the seven selected 1,2,3-triazole-phthalimide derivatives showed an excellent

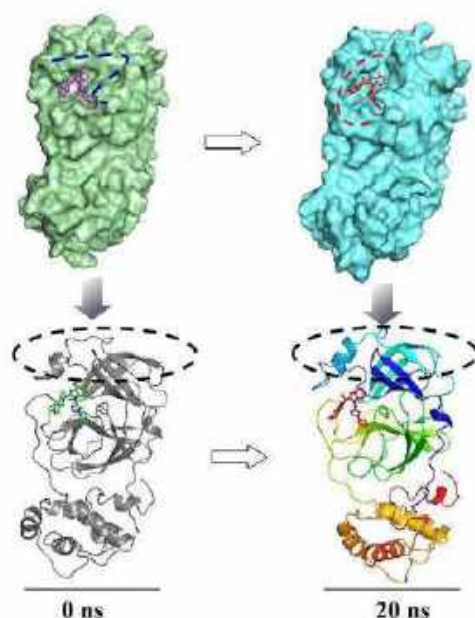


Figure 14. Surface view of main protease before MD simulation (green) displaying little modifications in the E40-MP66 binding site (yellow ring) after simulation (cyan teal). Lower panel represented the conformational differences between the structures of E40 in the binding site provided orientation that is more accurate for higher binding.

Table 9. Properties calculated after MMGBSA for E38 and E40 with main protease.

Molecule	dG _{bind} (kcal/mol)	dG _{coulombic} (kcal/mol)	dG _{covalent} (kcal/mol)	dG _{solvent} (kcal/mol)	Ligand efficiency (kcal/mol)
E38	-63.47 ± 3	-6.70	2.11	20.20	-1.81
E40	-63.31 ± 7	-13.22	1.098	25.07	-1.79

predicted pharmacokinetic and ADME properties, which are directly related to the solubility, permeability and toxicological profiles of such compounds. The *in silico* docking results showed that these selected compounds are potential multi-target ligands of essential SARS-CoV-2 spike, protease and nucleocapsid proteins suggesting the usefulness of 1,2,3-triazole-phthalimide derivatives for the development of drugs addressed to these proteins. Because the E40 presented predicted inhibition constant values in the nanomolar concentration range for both main protease and nucleocapsid proteins, this compound was considered the most promising as multi-target agent against SARS-CoV-2. Our MD simulation corroborated E40 and E38 as potent inhibitors of MP66 of SARS-CoV-2. However, we cannot rule out the possibility that other compounds, such as E38 can be included in further investigations alone or in combination with E40.

Acknowledgement

Vanderlan Nogueira Holanda would like to thank Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for the graduate scholarship (BPG-0333-2.08/18).

Disclosure statement

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.

Funding

This work was supported by the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE, BFT-109-2.08/18 and BIC-0489-2.13/20), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, PQ-400749/2019-0 and 312673/2018-6), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES code 001), Instituto Aggeu Magalhães and Inova-Fiocruz Program (VPPCB-007-FIO-18-2-85).

ORCID

Vanderlan Nogueira Holanda <http://orcid.org/0000-0001-5309-0036>
 Elton Marlon de Araújo Lima <http://orcid.org/0000-0003-0545-2447>
 Webon Vicente da Silva <http://orcid.org/0000-0001-5676-6863>
 Rafael Trindade Maia <http://orcid.org/0000-0002-5870-1091>
 Rafael de Lima Medeiros <http://orcid.org/0000-0003-2360-3595>
 Arambinda Ghosh <http://orcid.org/0000-0002-3891-5049>
 Vera Lúcia de Azevedo Lima <http://orcid.org/0000-0003-2581-9218>
 Regina Celia Bressan Queiroz de Figueiredo <http://orcid.org/0000-0001-5805-0944>

References

- Al-Masoudi, N. A., Abood, E., Al-Maliki, Z. T., Al-Masoudi, W. A., & Panmouque, C. (2016). Amino acid derivatives. Part 6. Synthesis, *In vitro* antiviral activity and molecular docking study of new N-aziridine acid derivatives conjugated spacer phthalimide backbone. *Medicinal Chemistry Research*, 25(11), 2578–2588. <https://doi.org/10.1007/s00044-016-1893-9>
- Assis, S. P. D. O., Silva, M. T. D., Oliveira, R. N., & Lima, V. L. D. M. (2012). Synthesis and anti-inflammatory activity of new alkyl-substituted phthalimide 1H-1,2,3-triazole derivatives. *The Scientific World Journal*, 2012, 7. <https://doi.org/10.1100/2012/925925>
- Assis, S. P. d. O., Silva, M. T. d., Silva, F. T. d., Sant'Anna, M. P., Tenório, C. M. B. d. A., Santos, C. F. B. D., Fonseca, C. S. M. d., Seabra, G., Lima, V. L. M., & Oliveira, R. N. d. (2019). Design and synthesis of triazole-phthalimide hybrids with anti-inflammatory activity. *Chemical & Pharmaceutical Bulletin*, 67(2), 96–105. <https://doi.org/10.1248/cpb.18-00607>
- Báez-Santos, Y. M., John, S. E. S., & Mesecar, A. D. (2015). The SARS-coronavirus papain-like protease: Structure, function and inhibition by designed antiviral compounds. *Antiviral Research*, 115, 21–38. <https://doi.org/10.1016/j.antiviral.2014.12.015>
- Bhatia, R., Narang, R. K., & Rawal, R. K. (2020). A summary of viral targets and recently released PDB IDs of SARS-CoV-2. *The OpenVirology Journal*, 14, 7–8.
- Bordogna, A., Pandini, A., & Bonati, L. (2011). Predicting the accuracy of protein-ligand docking on homology models. *Journal of Computational Chemistry*, 32(1), 81–98. <https://doi.org/10.1002/jcc.21601>
- Brito, M. A. D. (2011). Pharmacokinetic study with computational tools in the medicinal chemistry course. *Brazilian Journal of Pharmaceutical*

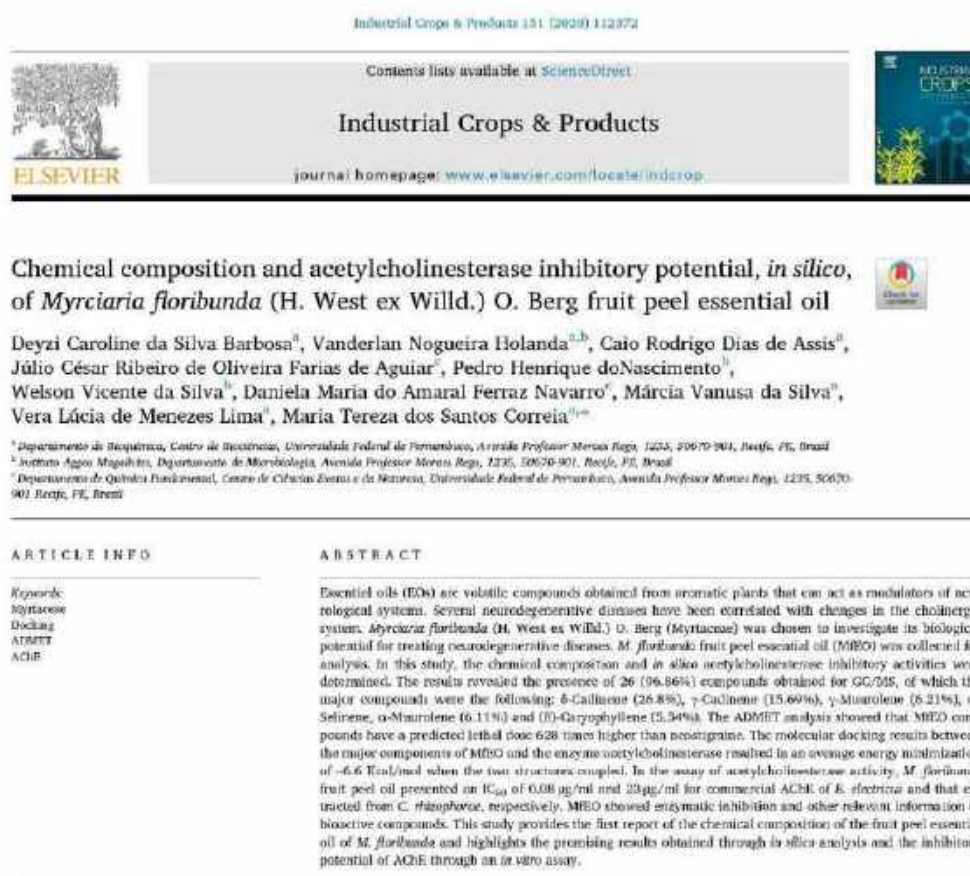
- Sciences, 47(4), 797–805. <https://doi.org/10.1590/S1984-82502011000400017>
- Butcher, G., Comer, J., & Avdeef, A. (2015). pKa-critical interpretations of solubility-pH profiles: PG-300995 and NSC-639829 case studies. *ADMET and DMPK*, 3(2), 131–140. <https://doi.org/10.5599/admet.3.2.182>
- Chen, Y. W., Yu, C. P., & Wong, K. Y. (2020). Prediction of the 2019-nCoV 3C-like protease (3CLpro) structure: Virtual screening reveals velipastat, ledipasvir, and other drug repurposing candidates. *ChemRxiv*, 2, 2–11. <https://doi.org/10.26434/chemrxiv.11831103.v1>
- Chowdhury, P. (2020). In silico investigation of phytoconstituents from Indian medicinal herb *Tinospora cordifolia* (giloy) against SARS-CoV-2 (COVID-19) by molecular dynamics approach. *Journal of Biomolecular Structure and Dynamics*, 1–18. <https://doi.org/10.1080/07391102.2020.1803968>
- Cong, Y., Ulasli, M., Schepers, H., Mauthe, M., Vukoski, P., Kriegenburg, F., ... Reggiori, F. (2020). Nucleocapsid protein recruitment to replication-transcription complexes plays a crucial role in coronavirus life cycle. *Journal of Virology*, 94(4), 1–21. <https://doi.org/10.1128/JVI.01925-19>
- Dayer, M. R., Taleb-Gassabi, S., & Dayer, M. S. (2017). Lopinavir: a potent drug against coronavirus infection: Insight from molecular docking study. *Archives of Clinical Infectious Diseases*, 12(4), e13823. <https://doi.org/10.5812/archicid.13823>
- Durán-Iribide, N. A., Díaz-Eufrasio, B. I., & Medina-Franco, J. L. (2020). In silico ADME/Tox profiling of natural products: A focus on BIOFACQUIM. *ACS Omega*, 5(26), 16076–16084. <https://doi.org/10.1021/acsomega.0c01581>
- Elkins, S., Honeycutt, J. D., & Metz, J. T. (2010). Evolving molecules using multi-objective optimization: Applying to ADME/Tox. *Drug Discovery Today*, 15(11–12), 451–460. <https://doi.org/10.1016/j.drudis.2010.04.003>
- Genheden, S., & Ryde, U. (2015). The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. *Expert Opinion on Drug Discovery*, 10(5), 449–461. <https://doi.org/10.1517/17460441.2015.1032936>
- Gonzaga, D. T. G., da Rocha, D. R., da Silva, F. d. C., & Ferreira, V. F. (2013). Recent advances in the synthesis of new antimicrobial agents based on the 1H-1,2,3-triazoles. *Current Topics in Medicinal Chemistry*, 13(22), 2850–2865. <https://doi.org/10.2174/15680266113136660202>
- Gümüş, F., Eren, G., Açık, L., Celebi, A., Öztürk, F., Yilmaz, S., Sağkan, R. I., Gür, S., Özkul, A., Elmali, A., & Elerman, Y. (2009). Synthesis, cytotoxicity, and DNA interactions of new cisplatin analogues containing substituted benzimidazole ligands. *Journal of Medicinal Chemistry*, 52(5), 1345–1357. <https://doi.org/10.1021/jm8009983>
- Hall, D. C., Jr., & Ji, H. F. (2020). A search for medications to treat COVID-19 via in silico molecular docking models of the SARS-CoV-2 spike glycoprotein and 3CL protease. *Travel Medicine and Infectious Disease*, 35, 101646. <https://doi.org/10.1016/j.tmaid.2020.101646>
- Homayun, B., Lin, X., & Choi, H.-J. (2019). Challenges and recent progress in oral drug delivery systems for biopharmaceuticals. *Pharmaceutics*, 11(3), 129. <https://doi.org/10.3390/pharmaceutics11030129>
- Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Gushui, F., Xu, J., Gu, J., Cheng, Z., Yu, T., Xia, J., Wei, Y., Wu, W., Xie, X., Yin, W., Li, H., Liu, M., ... Cheng, Z. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet*, 395(10223), 497–506. [https://doi.org/10.1016/S0140-6736\(20\)30183-5](https://doi.org/10.1016/S0140-6736(20)30183-5)
- Kaiser, J. (2005). Science resources: Chemists want NIH to curtail database. *Science*, 308(5725), 774. <https://doi.org/10.1126/science.308.5725.774a>
- Kamiya, Y., Tokaku, H., Yamada, R., Akase, C., Abe, Y., Sekiguchi, Y., Murayama, N., Shimizu, M., Kitajima, M., Shono, F., Funatsu, K., & Yamazaki, H. (2020). Determination and prediction of permeability across intestinal epithelial cell monolayer of a diverse range of industrial chemicals/drugs for estimation of oral absorption as a putative marker of hepatotoxicity. *Toxicology Reports*, 7, 149–154. <https://doi.org/10.1016/j.toxrep.2020.01.004>
- Kong, S., Yang, M., Hong, Z., Zhang, L., Huang, Z., Chen, X., He, S., Zhou, Z., Zhou, Z., Chen, Q., Yan, Y., Zhang, C., Shen, H., & Chen, S. (2020). Crystal structure of SARS-CoV-2 nucleocapsid protein RNA binding domain reveals potential unique drug targeting sites. *Acta Pharmaceutica Sinica B*, 10(7), 1228–1238. <https://doi.org/10.1016/j.apsb.2020.04.009>
- Karvath, A., & De Roedt, L. (2006). SMIRFP: Predicting chemical activity from SMILES. *Journal of Chemical Information and Modeling*, 46(6), 2432–2444. <https://doi.org/10.1021/ci060159g>
- Karypidou, K., Ribeiro, S. R., Quevedo, M. A., Persoons, L., Pannecouque, C., Helsen, C., Claessens, F., & Dehaen, W. (2018). Synthesis, biological evaluation and molecular modeling of a novel series of fused 1,2,3-triazoles as potential anti-coronavirus agents. *Bioorganic & Medicinal Chemistry Letters*, 28(21), 3472–3476. <https://doi.org/10.1016/j.bmcl.2018.09.019>
- Kauffmann, K., Grimm, L., Brandt, J., Schiley, A., Bluhm, K., Hollert, H., & Büche, J. (2020). Alternative type of Ames test allows for dynamic mutagenicity detection by online monitoring of respiration activity. *Science of the Total Environment*, 726, 137862. <https://doi.org/10.1016/j.scitotenv.2020.137862>
- Khan, R. J., Jha, R. K., Amera, G. M., Jain, M., Singh, E., Pathak, A., Singh, R. P., Muthukumar, J., & Singh, A. K. (2020). Targeting SARS-CoV-2: A systematic drug repurposing approach to identify promising inhibitors against 3C-like proteinase and 2'-O-ribose methyltransferase. *Journal of Biomolecular Structure and Dynamics*, 1–14. <https://doi.org/10.1080/07391102.2020.1753572>
- Komatsu, T. S., Okamoto, N., Koyama, Y. M., Hirano, Y., Morimoto, G., Ohno, Y., & Tajiri, M. (2020). Drug binding dynamics of the dimeric SARS-CoV-2 main protease, determined by molecular dynamics simulation. *Scientific Reports*, 10(1), 1–11. <https://doi.org/10.1038/s41598-020-74099-5>
- Kontagere, S., Elkins, S., & Welsh, W. J. (2008). Halogenated ligands and their interactions with amino acids: Implications for structure-activity and structure-toxicity relationships. *Journal of Molecular Graphics & Modelling*, 27(2), 170–177. <https://doi.org/10.1016/j.jmgm.2008.04.001>
- Laskowski, R. A., MacArthur, M. W., & Thornton, J. M. (2006). PROCHECK: Validation of protein structure coordinates.
- Lette, D. I., Fontes, F. d. V., Bastos, M. M., Hoelz, L. V. B., Blanco, V. d. C., A. D., de Oliveira, A. P., da Silva, P. B., da Silva, C. F., Batista, D. d. G. J., da Gama, A. N. S., Peres, R. B., Villar, J. D. F., Soares, M. d. N. C., & Boechat, N. (2018). New 1,2,3-triazole-based analogues of benzimidazole for use against *Trypanosoma cruzi* infection: In vitro and in vivo evaluations. *Chemical Biology & Drug Design*, 92(3), 1670–1682. <https://doi.org/10.1111/cbdd.13333>
- Li, D., Chen, L., Li, Y., Tian, S., Sun, H., & Hou, T. (2014). ADME evaluation in drug discovery. 13. Development of in silico prediction models for P-glycoprotein substrates. *Molecular Pharmacology*, 11(3), 715–726. <https://doi.org/10.1021/mp400450m>
- Li, Y., Meng, Q., Yang, M., Liu, D., Hou, X., Tang, L., Wang, X., Liu, Y., Chen, X., Liu, K., Yu, A., Zuo, Z., & Bi, H. (2019). Current trends in drug metabolism and pharmacokinetics. *Acta Pharmaceutica Sinica B*, 9(6), 1113–1144. <https://doi.org/10.1016/j.apsb.2019.10.001>
- Lipinski, C. A. (2004). Lead- and drug-like compounds: The rule-of-five revolution. *Drug Discovery Today: Technologies*, 1(4), 337–341. <https://doi.org/10.1016/j.ddtec.2004.11.007>
- Liu, S., Xiao, G., Chen, Y., He, Y., Niu, J., Escalante, C. R., Xiong, H., Farmer, J., Debnath, A. K., Tien, P., & Jiang, S. (2004). Interaction between heptad repeat 1 and 2 regions in spike protein of SARS-associated coronavirus: Implications for virus fusion mechanism and identification of fusion inhibitors. *The Lancet*, 363(9413), 938–947. [https://doi.org/10.1016/S0140-6736\(04\)15786-7](https://doi.org/10.1016/S0140-6736(04)15786-7)
- López-Camacho, E., Godoy, M. J. G., García-Nieto, J., Nebro, A. J., & Aidana-Montes, J. F. (2015). Solving molecular flexible docking problems with metaheuristics: A comparative study. *Applied Soft Computing*, 28, 379–393. <https://doi.org/10.1016/j.asoc.2014.10.049>
- López-González, R., Bautista-Renedo, J., Martínez-Otero, D., Reyes, H., González-Rivas, N., & Cuevas-Yañez, E. (2016). 1, 4-and 1, 5-di (N-phthalimido)methyl-1, 2, 3-triazoles: Crystal structures and density functional theory studies of the alkyne and azide precursors. *Journal of Chemical Research*, 40(5), 308–313. <https://doi.org/10.3184/174751916X14608096414015>
- Macrae, C. F., Bruno, I. J., Chisholm, J. A., Edgington, P. R., McCabe, P., Pidcock, E., Rodriguez-Monge, L., Taylor, R., van de Streek, J., & Wood,

- P. A. (2008). Mercury CSD 2.0-new features for the visualization and investigation of crystal structures. *Journal of Applied Crystallography*, 41(2), 466–470. <https://doi.org/10.1107/S0021889807067908>
- Mandić, L., Benić, P., Minarić-Majerski, K., Liekens, S., Snoeck, R., Andrei, G., Krajić, M., & Basić, N. (2020). Substituted adamantylphthalimides: Synthesis, antiviral and antiproliferative activity. *Archiv Der Pharmazie*, 353(5), 2000024. <https://doi.org/10.1002/ardp.202000024>
- Mehra, M. R., Desai, S. S., Ruschitzka, F., & Patel, A. N. (2020). Hydroxychloroquine or chloroquine with or without a macrolide for treatment of COVID-19: A multinational registry analysis. *The Lancet*, 1–10. [https://doi.org/10.1016/S0140-6736\(20\)31180-8](https://doi.org/10.1016/S0140-6736(20)31180-8)
- Meo, S. A., Al-Khlaiwi, T., Usmani, A. M., Meo, A. S., Klonoff, D. C., & Hoang, T. D. (2020). Biological and epidemiological trends in the prevalence and mortality due to outbreaks of novel coronavirus COVID-19. *Journal of King Saud University-Science*, 32(4), 2495–2499. <https://doi.org/10.1016/j.jksus.2020.04.004>
- Miao, R., Xia, L.-Y., Chen, H.-H., Huang, H.-H., & Liang, Y. (2019). Improved classification of blood-brain-barrier drugs using deep learning. *Scientific Reports*, 9(1), 18802. <https://doi.org/10.1038/s41598-019-44773-4>
- Myatt, G. J., Ahlberg, E., Akhazov, Y., Allen, D., Amberg, A., Anger, L. T., Aptula, A., Auerbach, S., Belke, L., Bellion, P., Benigni, R., Bercu, J., Booth, E. D., Bower, D., Brigo, A., Burdens, N., Cammerer, Z., Caporin, M. T. D., Cross, K. P., ..., Hasselgren, C. (2018). In silico toxicology protocols. *Regulatory Toxicology and Pharmacology*, 96, 1–17. <https://doi.org/10.1016/j.yrtph.2018.04.014>
- Ouyang, T., Liu, X., Ouyang, H., & Ren, L. (2018). Recent trends in click chemistry as a promising technology for virus-related research. *Virus Research*, 256, 21–28. <https://doi.org/10.1016/j.virusres.2018.08.003>
- Papadopoulos, M. V., Bloomer, W. D., Rosenzweig, H. S., Kaiser, M., Chatelain, E., & Islet, J. R. (2013). Novel 3-nitro-1H-1,2,4-triazole-based piperazines and 2-amino-1,3-benzothiazoles as antichagasic agents. *Bioorganic & Medicinal Chemistry*, 21(21), 6600–6607. <https://doi.org/10.1016/j.bmc.2013.08.022>
- Phatak, P. S., Bakale, B. D., Dhurmal, S. T., Dahiwalde, L. K., Choudhari, P. B., Siva Krishna, V., Sriram, D., & Haval, K. P. (2019). Synthesis, anti-tubercular evaluation and molecular docking studies of phthalimide bearing 1, 2, 3-triazoles. *Synthetic Communications*, 49(16), 2017–2028. <https://doi.org/10.1080/00397911.2019.1614530>
- Pfano, J., Montoro, J., Aznar, C., Lorenzo, L., Gómez, M. D., Guerrero, M., Carretero, C., González-Barberá, E. M., Balaguer-Rosello, A., Sanz, R., Salavert, M., Navarro, D., Sanz, M. A., Sanz, G., & Sanz, J. (2020). The clinical benefit of instituting a prospective clinical community-acquired respiratory virus surveillance program in allogeneic hematopoietic stem cell transplantation. *Journal of Infection*, 80(3), 333–341. <https://doi.org/10.1016/j.jinf.2019.12.022>
- Pires, D. E., Blundell, T. L., & Ascher, D. B. (2015). pkCSM: Predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures. *Journal of Medicinal Chemistry*, 58(9), 4066–4072. <https://doi.org/10.1021/acs.jmedchem.5b00104>
- Razzaghi-Asl, N., Ebadi, A., Shahabipour, S., & Gholami, D. (2020). Identification of a potential SARS-CoV2 inhibitor via molecular dynamics simulations and amino acid decomposition analysis. *Journal of Biomolecular Structure and Dynamics*, 1–16. <https://doi.org/10.1080/07391102.2020.1797536>
- Ronnebaum, J. M., & Luzzio, F. A. (2016). Synthesis of 1, 2, 3-triazole 'click' analogues of thalidomide. *Tetrahedron*, 72(40), 6136–6141. <https://doi.org/10.1016/j.tet.2016.07.019>
- Sadal-Ebrahimi, S. E., Rahmani, A., Mohammadi-Khanaposhtani, M., Jafari, N., Mojtavavi, S., Ali Faramarzi, M., Emadi, M., Yahya-Meymandi, A., Larjani, B., Biglar, M., & Mahdavi, M. (2020). New phthalimido-benzamide-1, 2, 3-triazole hybrids: design, synthesis, α -glucosidase inhibition assay, and docking study. *Medicinal Chemistry Research*, 29(5), 868–869. <https://doi.org/10.1007/s00044-020-02522-7>
- Sanchis-Gomar, F., Lavié, C. J., Perez-Quila, C., Henry, B. M., & Lippi, G. (2020). Angiotensin-Converting Enzyme 2 and antihypertensives (angiotensin receptor blockers and angiotensin-converting enzyme inhibitors) in Coronavirus Disease 2019. *Mayo Clinic Proceedings*, 95(6), 1222–1230. <https://doi.org/10.1016/j.mayocp.2020.03.026>
- Singh, S., El-Sakkary, N., Skinner, D. E., Sharma, P. P., Otiile, S., Antonova-Koch, Y., Kumar, P., Winzeler, E., Poonam, Caffrey, C., & Rath, B. (2020). Synthesis and bioactivity of phthalimide analogs as potential drugs to treat schistosomiasis, a neglected disease of poverty. *Pharmaceutics*, 13(2), 25. <https://doi.org/10.3390/ph13020025>
- Stewart, J. J. (1989). Optimization of parameters for semiempirical methods II. *Journal of Computational Chemistry*, 10(2), 221–264. <https://doi.org/10.1002/jcc.540100209>
- Sun, L., Huang, T., Dick, A., Meuser, M. E., Zalloum, W. A., Chen, C. H., Ding, X., Gao, P., Cocklin, S., Lee, K., Zhan, P., & Liu, X. (2020). Design, synthesis and structure-activity relationships of 4-phenyl-1H-1,2,3-triazole-phenylalanine derivatives as novel HIV-1 capsid inhibitors with promising antiviral activities. *European Journal of Medicinal Chemistry*, 190, 112085. <https://doi.org/10.1016/j.ejmech.2020.112085>
- Surjit, M., Liu, B., Chow, V. T., & Lal, S. K. (2006). The nucleocapsid protein of severe acute respiratory syndrome-coronavirus inhibits the activity of cyclin-cyclin-dependent kinase complex and blocks S phase progression in mammalian cells. *Journal of Biological Chemistry*, 281(16), 10669–10681. <https://doi.org/10.1074/jbc.M509233200>
- Tehrani, M. B., Emami, P., Rezaei, Z., Khoshneviszadeh, M., Ebrahimi, M., Edraki, N., Mahdavi, M., Larjani, B., Ranjbar, S., Foroumadi, A., & Khoshneviszadeh, M. (2019). Phthalimide-1, 2, 3-triazole hybrid compounds as tyrosinase inhibitors: synthesis, biological evaluation and molecular docking analysis. *Journal of Molecular Structure*, 1176, 86–93. <https://doi.org/10.1016/j.jmolstruc.2018.08.033>
- Terko, I. V., Varbanov, H. P., Galanski, M., Talmaci, M., Platts, J. A., Ravera, M., & Gabano, E. (2016). Prediction of logP for Pt(II) and Pt(IV) complexes: Comparison of statistical and quantum-chemistry based approaches. *Journal of Inorganic Biochemistry*, 156, 1–13. <https://doi.org/10.1016/j.jinorgbio.2015.12.005>
- Thanh, N. D., Hai, D. S., Ngoc Bich, V. T., Thu Hien, P. T., Ky Duyen, N. T., Mai, N. T., Dung, T. T., Toan, V. N., Kim Van, H. T., Dang, L. H., Toan, D. N., & Thanh Van, T. T. (2019). Efficient click chemistry towards novel 1H-1,2,3-triazole-tethered 4H-chromene-d-glucose conjugates: Design, synthesis and evaluation of in vitro antibacterial, MRSA and antifungal activities. *European Journal of Medicinal Chemistry*, 162, 454–471. <https://doi.org/10.1016/j.ejmech.2019.01.060>
- Tkatchenko, A., & Scheffler, M. (2009). Accurate molecular van der Waals interactions from ground-state electron density and free-atom reference data. *Physical Review Letters*, 102(7), 073005. <https://doi.org/10.1103/PhysRevLett.102.073005>
- Urtomo, R. Y., & Meiyanto, E. (2020). Revealing the potency of citrus and galangal constituents to halt SARS-CoV-2 infection. *Preprint*, 1, 1–8. <https://doi.org/10.20944/preprints202003.0214.v1>
- Wallis, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., & Vesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell*, 183(6), 1735. <https://doi.org/10.1016/j.cell.2020.02.058>
- Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., Graham, B. S., & McLellan, J. S. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science*, 367(6433), 1260–1263. <https://doi.org/10.1126/science.abb2507>
- Xu, L., Jiang, W., Jia, H., Zheng, L., Xing, J., Liu, A., & Du, G. (2020). Discovery of multitarget-directed ligands against influenza A virus from compound yizhihao through a predictive system for compound-protein interactions. *Frontiers in Cellular and Infection Microbiology*, 10, 16. <https://doi.org/10.3389/fcimb.2020.00016>
- Yadav, R., Imran, M., Dhamija, P., Suchal, K., & Handu, S. (2020). Virtual screening and dynamics of potential inhibitors targeting RNA binding domain of nucleocapsid phosphoprotein from SARS-CoV-2. *Journal of Biomolecular Structure and Dynamics*, 1–16. <https://doi.org/10.1080/07391102.2020.1778536>
- Yang, R., Liu, H., Bi, C., Wang, Y., Zhang, X., Guo, B., Wu, S., Wang, J., Leung, E., Chang, H., Li, P., Liu, T., & Wang, Y. (2020). Chemical composition and pharmacological mechanism of Qingfei Paidu Decoction and Ma Xing Shi Gan Decoction against Coronavirus Disease 2019 (COVID-19): In silico and experimental study. *Pharmacological Research*, 157, 104820. <https://doi.org/10.1016/j.phrs.2020.104820>
- Zeng, Y., Ye, L., Zhu, S., Zheng, H., Zhao, P., Cai, W., Su, L., Shi, Y., & Wu, Z. (2008). The nucleocapsid protein of SARS-associated coronavirus inhibits B23

- phosphorylation. *Biochemical and Biophysical Research Communications*, 369(2), 287–291. <https://doi.org/10.1016/j.bbrc.2008.01.096>
- Zhang, W., Zhao, Y., Zhang, F., Wang, Q., Li, T., Liu, Z., Wang, J., Qin, Y., Zhang, X., Yan, X., Zhang, X., Yan, Z., Zeng, X., & Zhang, S. (2020). The use of anti-inflammatory drugs in the treatment of people with severe coronavirus disease 2019 (COVID-19): The experience of clinical immunologists from China. *Clinical Immunology (Oxford, England)*, 214, 108393. <https://doi.org/10.1016/j.clim.2020.108393>
- Zhou, B., Liu, J., Wang, Q., Liu, X., Li, X., Li, P., Ma, Q., & Cao, C. (2020). The nucleocapsid protein of severe acute respiratory syndrome coronavirus inhibits cell cytokinesis and proliferation by interacting with translation elongation factor 1 α . *Journal of Virology*, 92(14), 6962–6971. <https://doi.org/10.1128/JVI.00133-20>
- Zhou, R., Zeng, R., von Brunn, A., & Li, J. (2020). Structural characterization of the C-terminal domain of SARS-CoV-2 nucleocapsid protein. *Molecular Biomedicine*, 1(1), 3–11. <https://doi.org/10.1186/s43550-020-00001-4>

5.6 ARTIGO 5

O artigo “Chemical composition and acetylcholinesterase inhibitory potential, *in silico*, of *Myrciaria floribunda* (H. West ex Willd.) O. Berg fruit peel essential oil” foi publicado no periódico *Industrial Crops and Products*, volume 151, 2020, link para acesso: <https://doi.org/10.1016/j.indcrop.2020.112372>



1. Introduction

Essential oils (EOs) are volatile substances of natural origin, including a variety of compounds and can be obtained from aromatic plants (Dakkali et al., 2000). Recently, the biological potential of EOs has been investigated, revealing broad potential for biomedical applications: with emphasis on antimicrobial and antibiofilm (Rahn et al., 2019), larvicidal (Pandey et al., 2019), leishmanicidal (Silva et al., 2018), trypanocidal (Azevedo and Soares, 2013; Costa et al., 2015) and fungicidal activities (Santos et al., 2018), as well as activities against autoimmune diseases (Cook and Chessman, 2019) and inflammation (Chou et al., 2018) and as a modulator of the neurological system

(Arruda et al., 2012; Zarraz et al., 2019; 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., 2017).

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 Mazzei, 2016). *Myrciaria floribunda* H. West ex Willd.) O. Berg (Myrtaceae) is native to Brazil and popularly known as *cambuin* or *fabaucabinha*; it is characteristic of

*Corresponding author at: Laboratório de Glicoproteínas, Departamento de Bioquímica, Centro de Biotecnologia, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, 1235, 50670-901 Recife, PE, Brazil.

E-mail address: miscorreia@gmail.com (M.T. dos Santos Correia).

<https://doi.org/10.1016/j.indcrop.2020.112372>

Received 19 November 2019; Received in revised form 12 February 2020; Accepted 17 March 2020
0926-6690/ © 2020 Elsevier B.V. All rights reserved.

coastal sandy areas (resingas) but has a wide geographical distribution (Amare 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 (Franceschini 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 (Teitobol 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 (V'Souza and Waldvogel, 2016), amyotrophic lateral sclerosis (Campanari et al., 2016), myasthenia gravis (Brammer et al., 2009; Hon 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-Fuot et al., 2016).

ACh is a neurotransmitter responsible for propagating an electrical impulse from a pre-synaptic neuron to a post-synaptic neuron (Sirlin 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 (Touga, 2000; 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; Mercoy et al., 2012).

Acetylcholinesterase (AChE) 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 Tacrine, 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 (Someein 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 (Noelky 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'-dithio-bis-2-nitrobenzoic acid (DTNB), 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 Ecu (Latitude: 7.355809029182327°S, Longitude: 39.887568331218304°W), state of Pernambuco, Brazil, in 2018. The species was collected and a specimen was herbarized 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 S (VascoBond 30 m $\times$ 0.25 mm $\times$ 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 μ l) 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 to 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 (SIT, Lausanne, Switzerland) PROTOX-II Server (Charité University, Berlin, Germany), Molinspiration (Molinspiration Cheminformatics, Nova Ulica, Slovakia), Osiris Property Explorer, Osiris Data Warrior (Actelion Pharmaceuticals Ltd., Allschwil, Switzerland) and pKCSM (Pires et al., 2015). Reference drug information (Neostigmine PubChem 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 $60 \times 60 \times 60$ grid with a spacing of $0.375 \text{ \AA}$, centered on the active site of acetylcholinesterase, with enzymatic residues within $8 \text{ \AA}$ 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 Waals-type bonds, hydrogen bridges and electrostatic energy.

2.5. Evaluation of acetylcholinesterase inhibitory activity (AChE)

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 *G. rhusaphorae* 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 $10 \mu\text{l}$ of AChE to $10 \mu\text{l}$ of oil at different concentrations (0.0001 to 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, $200 \mu\text{l}$ of the chromogenic reagent DNTB (0.25 mM) and $20 \mu\text{l}$ 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; Tietz 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	LR ^c calculated	LR: real	N ^d (%)	S.D. ^e
(E)- β -Caryophyllene	1050	1044	0.19	0.01
α -Cubebene	1352	1348	0.01	0.00
α -Mangonene	1374	1375	0.34	0.01
α -Cyperene	1378	1374	5.03	0.38
Solene	1392	1390	0.22	0.01
Bibacene	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	0.05
Aromadendrene	1442	1439	3.95	0.18
α -Humulene	1457	1452	1.81	0.03
Allo-Aromadendrene	1464	1458	1.47	0.02
trans-Cadinol-1(6),4-diene	1477	1475	0.01	0.00
γ -Muurolene	1486	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	0.28
γ -Cadinene	1516	1513	15.69	0.38
δ -Cadinene	1527	1522	26.84	0.50
trans-Cadinol-1,4-diene	1536	1533	0.12	0.00
α -Cadinene	1541	1537	1.96	0.05
α -Calacorene	1547	1544	2.21	0.10
Germacone B	1551	1559	0.31	0.08
β -Calacorene	1557	1564	0.31	0.06
α -Cadinol	1596	1652	4.57	0.91
		identified	96.86	

^a MfEO: Essential oil of *Myrcaria floribunda* peel fruit.

^b GC/MS: Gasose Chromatography/Mass Spectre.

^c LR: Retention Index.

^d A: Area.

^e S.D.: Standard 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 (Boudjedou 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 *Myrcaria jaboticaba* (Vell.) O. Berg (Plegmann 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 -pinene, α -caryophyllene, (Plegmann et al., 2012), d-limonene, β -caryophyllene (Franco and Shimamoto, 2000) and β -Myrcene (Fortes et al., 2011) are the most common. The compounds α -Selinene and α -Muurolene were identified as major components of the essential oil of the fruits of *Myrcaria 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

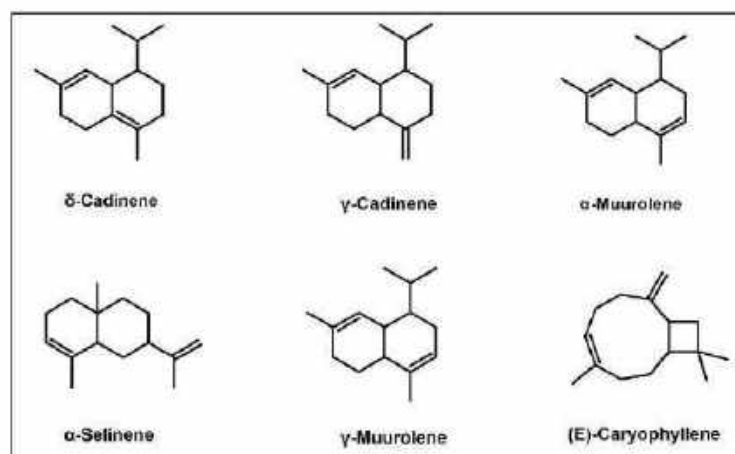


Fig. 1. Chemical structure of the major components of the essential oil of *M. floribunda*.

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 (Hanus, 2007).

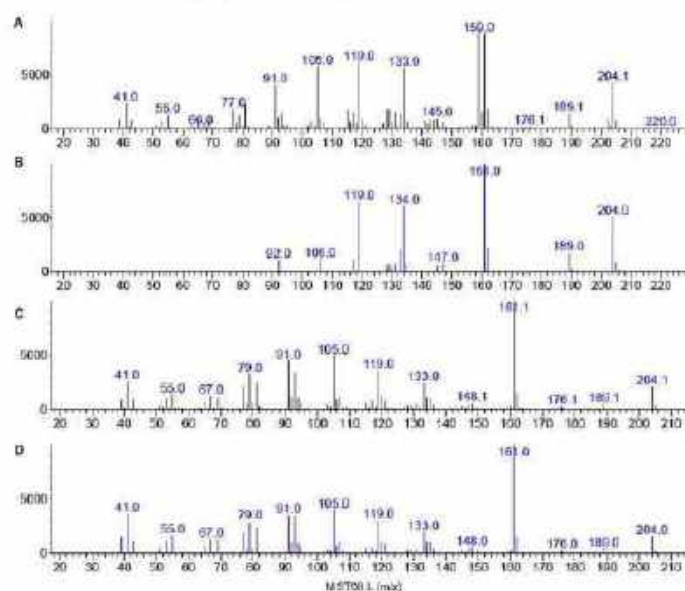


Fig. 2. Mass spectrometry of δ -Cadinene in the essential oil (A), reference standard of δ -Cadinene (B), γ -Cadinene in the essential oil (C), reference standard γ -Cadinene (D).

Table 2
In silico ADMET^a analysis of major constituents of MEO^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							
IRAN ^j	0	0	0	0	0	0	3
IRBD ^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	204.35	204.35	204.35	204.35	204.35	204.35	223.29
(g/mol)							
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 ^r 2D6 inhibitor	No	No	No	No	No	No	No
CYP450 ^r 2C19 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No
CYP450 ^r 3A4 inhibitor	No	No	No	No	No	No	No
CYP450 ^r 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	Little
LD ₅₀ ^w	4400	4400	4400	5000	4400	5000	7
mg/kg							

^a ADMET: absorption, distribution, metabolism, excretion and toxicity.

^b MEO: Essential oil of *Myrcia floribunda* peel fruit.

^c δ -Cad: δ -Cadinene.

^d γ -Cad: γ -Cadinene.

^e α -Mu: α -Muurolene.

^f α -Sel: α -Selinene.

^g γ -Mu: γ -Muurolene.

^h Car: (E)-Caryophyllene.

ⁱ Neo: neostigmine.

^j IRAN: Number of hydrogen bond acceptors.

^k IRBD: Number of hydrogen donors.

^l MW: Molecular Weight.

^m n-ROTB: Number of rotatory 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 Enzymes.

^s MUT: mutagenic.

^t TUM: tumorigenic.

^u IRR: irritant.

^v HEP: hepatotoxic.

^w LD₅₀: 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 MEO (Table 2).

The major MEO 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 MEO 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, 1983; Adhamah 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 EOM 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 (kcal/mol)	FE ^c (kcal/mol)	EE ^d (kcal/mol)	TIE ^e	E ^f
δ-Cad ^g	-6.77	16.99	-75.1	-0.01	-7.66	653.724
γ-Cad ^h	-7.35	4.10	71.7	0.03	-7.65	661.2
α-Mu ⁱ	-5.89	17.11	-71.4	-0.01	-6.33	591.832
α-Sa ^j	-6.37	6.33	-73.3	-0.00	-6.98	761.412
γ-Mu ^k	-6.23	8.91	-71.4	-0.02	-7.66	661.31
Car ^l	-6.99	12.33	-72.4	-0.00	-7.32	721.664
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: gamma-Cadinene.

ⁱ α-Mu: alpha-Muurolene.

^j α-Sa: alpha-Selinene.

^k γ-Mu: gamma-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) (Borrelli 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 Waals 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 (Shrivastava et al., 2019). The interactions dominated in the region of His447 and Trp286 amino acid residues due to the existence of the π-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 Waals 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; Ryler 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 (Karkosak 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 (Süchler et al., 2016).

In the assay of acetylcholinesterase activity, *M. floribunda* fruit peel oil presented a median inhibitory concentration (IC₅₀) of 0.08 µg/ml and 23 µg/ml for commercial AChE of *E. electricus* and that extracted from *C. rhizophora*, respectively. While neostigmine (the standard used) had an IC₅₀ of 23.3 µg/ml and 6.2 µg/ml, respectively, for the same enzymes (Fig. 4). The AChE action may be correlated to the presence of δ and γ cadinene compounds present in the oil, which are part of the sesquiterpene group.

The differences between the IC₅₀ values for the commercial enzyme (*E. electricus*) and *C. rhizophora* 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 (IC₅₀ 1583 µg / ml) and leaves (IC₅₀ 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., 2018; Scott and Scott, 2018).

Cyclic compounds such as 1,8-cineole (Petrachianan et al., 2019; Kalkstani et al., 2018; Abdelgaleil et al., 2019), α-pinene (Korakaya et al., 2019; Shahriari et al., 2018) and caryophyllene (Owokoteco et al., 2015; Xiang et al., 2017), found in essential oils, have been reported to have AChE inhibitory activity. According to Miyazawa et al.

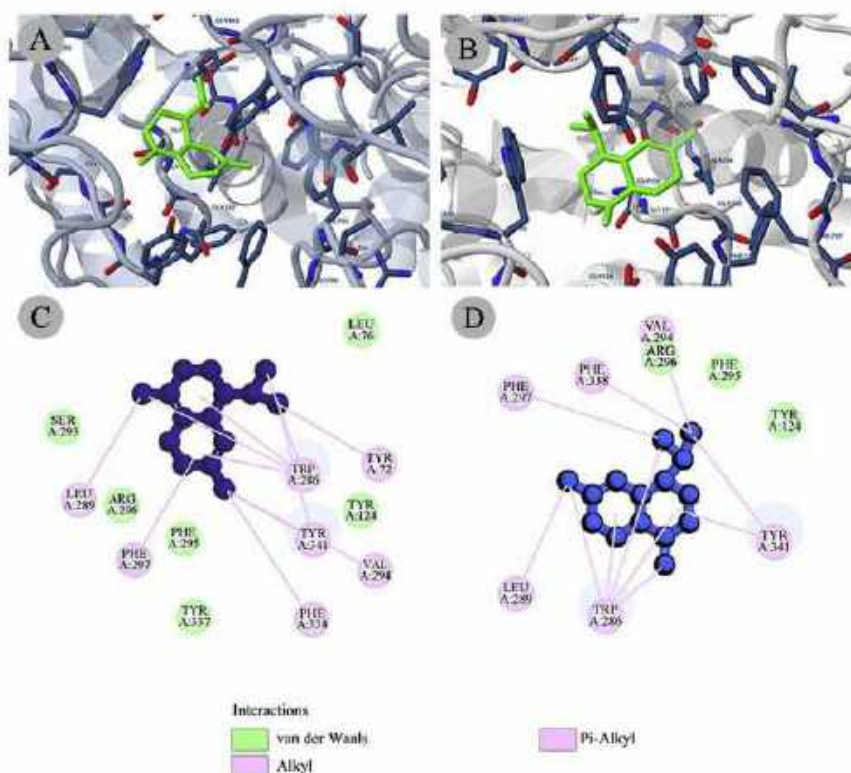


Fig. 3. Interaction of δ (A) and γ (B) cadinene with human AChE^a. Binding and related amino acid residue in molecular docking (C, D). AChE: Acetylcholinesterase.

(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 -Muurenone, α -Selinene, α -Muurenone, (*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 MEO 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 do Nascimento: Methodology, Validation, Resources, Investigation. Software, Writing - original draft. Welton 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.

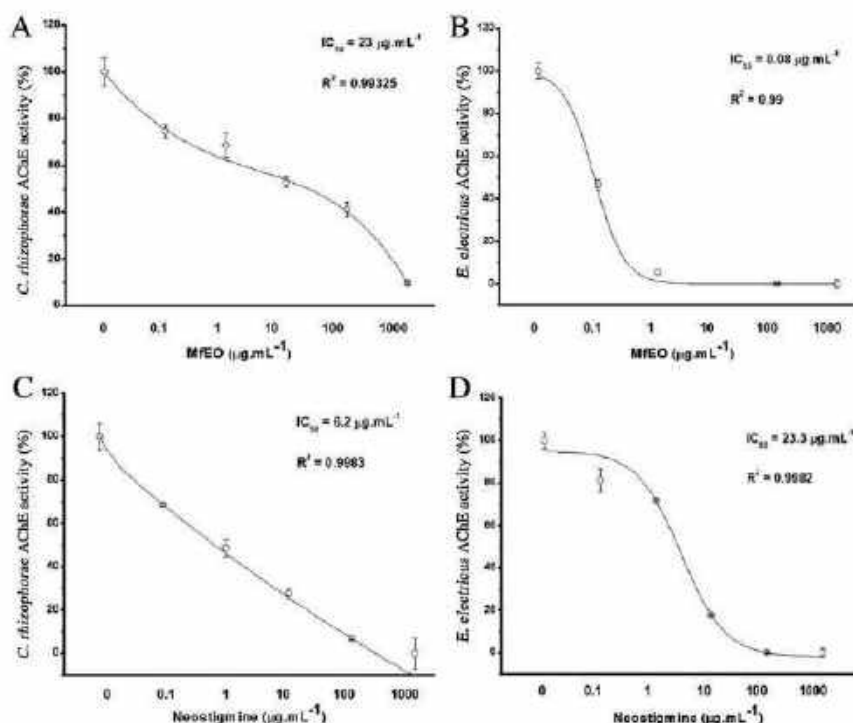


Fig. 4. Determination of inhibition activity (IC_{50}) of MEO^a (A, B) and neostigmine (C, D) on *Crossostrea rhizophorae* and *Electrophorus electricus* AChE^b respectively. IC_{50} : median inhibitory concentration. MEO: Essential oil of *Myrciaria floribunda* peel fruit. AChE: Acetylcholinesterase.

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.

Acknowledgments

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Amparo à Ciência e Tecnologia de Pernambuco (FACEPE) for supporting this research.

References

- Abdelgallil, S.A.M., Mohamed, F.E.B., Mohamed, M.R., Alai El-Salam, M.M., 2019. Acaricidal activity, biochemical effects and molecular docking of some monoterpene agonist (two-spotted spider mite (*Tetranychus urticae* Koch)). *Prote. Biochem. Physiol.* 156, 105–115. <https://doi.org/10.1016/j.probio.2019.02.009>.
- Abdulaziz, U., Uthairu, A., Uba, S., 2017. Quantitative structure-activity relationship and molecular docking studies of a series of quinoxalino[2,1-b]pyridine analogues as inhibitors of gamma amino butyric acid aminotransferase. *J. Adv. Res.* 8, 43–49. <https://doi.org/10.1016/j.jadvres.2016.10.004>.
- Adams, R.P., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry (Allured Publishing Corporation Carol Stream, IL). ISBN 978-1-892635-31-4.
- Adlanah, A., Johnson, S., Ho, A., Orbell, J., 2017. Neostigmine and glycopyrronium: a potential safe alternative for patients with pseudo obstruction without access to conventional methods of decompression. *BMJ Case Rep.* 2017 <https://doi.org/10.1136/bcr-2017-221249>. <https://doi.org/10.1136/bcr-2017-221249>.
- Ali, R., Subedi, M., Müller, S., Bontjens, A., Mezel, M., 2018. Molecular dynamics and docking studies on acetylcholinesterase (AChE) inhibitors. *Biophys. J.* 114 (3) 740a.
- Amend, R.E., Fernandes, C.P., Garamel, D., Tebbel, O.P., Santos, L.A.C., Carvalho, M.G., Rocha, J.C.T., 2013. Essential oils from fruits with different colors and leaves of *Neomitraria olivacea* (DC.) N. Silveira: an endemic species from Brazilian Atlantic Forest. *Braz. J. Bot.* 36, 2013. <https://doi.org/10.1590/2311-72391301>.
- Apel, M.A., Lima, M.R.L., Sousa, A., Cordeiro, I., Young, M.C.M., Sobral, M.R.G., Saffredini, L.B., Motono, P.J.L., 2006a. Screening of the biological activity from essential oils of native species from the Atlantic rain forest (São Paulo-Brazil). *Pharmazie* 61, 375–383.
- Apel, M.A., Sobral, M., Zanetti, J., Henriques, A.T., 2006b. Essential oil composition of four *Pithecia* species (Myristicaceae). *Flavour-Fragrance J.* 21, 565–567. <https://doi.org/10.1002/ffj.1538>.
- Arruda, M., Viana, H., Raithe, N., Neng, N.R., Rossi, J.S., Nogueira, J.M.F., Barreto, M.G., 2012. Anti-acetylcholinesterase and antioxidant activity of essential oils from *Hedyotis gardnerianus* cheppard ex hiergw. *Molecules* 17. <https://doi.org/10.3390/molecules17053082>.
- Assis, C.R.D., Linares, A.G., Oliveira, V.M., França, R.C.P., Carvalho, E.V.M.M., Bezerra, R.S., Carvalho Jr., J.B., 2012. Comparative effect of pesticides on brain acetylcholinesterase in tropical fish. *Sci. Total Environ.* 441, 141–150. <https://doi.org/10.1016/j.scitotenv.2012.09.058>.
- Azavedo, G.M.O., Soares, M.J., 2015. Combination of the essential oil constituents citral, eugenol and thymol enhance their inhibitory effect on *Cribellus fuscatus* and *Trypanosoma cruzi* growth. *Rev. Bras. Farmacogn.* 25, 762–768. <https://doi.org/10.1590/s0034-4719.201500000007>.
- Bakali, P., Averbeck, S., Averbeck, D., (da)mar, M., 2008. Biological effects of essential oils – a review. *Food Chem. Toxicol.* 46, 446–475. <https://doi.org/10.1016/j.fct.2007.09.006>.
- Beeman, H., Henrick, K., Nakamura, H., 2003. Announcing the worldwide protein data bank. *Nat. Struct. Biol.* 10, 900. <https://doi.org/10.1038/nstb.2003.900>.
- Barry, M.L., Claudio, A.G.S., Devi, S., 2018. *Griseb. triflorum* oil, a potential insecticide against the weevil with anti-acetylcholinesterase activity. *Ind. Crop. Prod.* 126, 434–439. <https://doi.org/10.1016/j.indcrop.2018.10.043>.

5.7 ARTIGO 6

O artigo “GC-MS Analysis and Hemolytic, Antipyretic and Antidiarrheal Potential of *Syzygium aromaticum* (Clove) Essential Oil” foi publicado no periódico *Separations*, volume 7, número 2, link para acesso: <https://doi.org/10.3390/separations7020035>



Article

GC-MS Analysis and Hemolytic, Antipyretic and Antidiarrheal Potential of *Syzygium aromaticum* (Clove) Essential Oil

Cristiane Marinho Uchôa Lopes ^{1,2}, João Ricardhis Saturnino de Oliveira ³, Vanderlan Nogueira Holanda ³, Antonio Yony Felipe Rodrigues ⁴, Caíque Silveira Martins da Fonseca ³, Fábio Fernandes Galvão Rodrigues ⁵, Cicera Janaine Camilo ⁵, Vera Lucia de Menezes Lima ³, Henrique Douglas Melo Coutinho ⁵, Radosław Kowalski ^{6,*} and José Galberto Martins da Costa ^{2,5}

¹ Department of Anatomy, Federal University of Cariri, Barbalha 63180-000, CE, Brazil; crismarinho@hotmail.com

² Northeast Network on Biotechnology, State University of Ceará, Fortaleza 60811-905, CE, Brazil; galberto.martins@gmail.com

³ Department of Biochemistry-Center of Biosciences, Federal University of Pernambuco, Recife 50670-420, PE, Brazil; ricardhis@gmail.com (J.R.S.d.O.); vanderlan.nogueira@gmail.com (V.N.H.); caiquesmfonseca@gmail.com (C.S.M.d.F.); lima.vera.ufpe@gmail.com (V.L.d.M.L.)

⁴ Department of Health Sciences, Medicine College of ABC, Santo André 09060-870, SP, Brazil; yonyrodrigues@hotmail.com

⁵ Department of Biological Chemistry, Program of Post-Graduation in Biological Chemistry, Regional University of Cariri, Crato 63105-000, CE, Brazil; fabiogalvaod1@hotmail.com (F.F.G.R.); janainecamilo@hotmail.com (C.J.C.); hdmcoutinho@gmail.com (H.D.M.C.)

⁶ Department of Analysis and Evaluation of Food Quality, University of Life Sciences in Lublin, 8 Skromna Street, 20-704 Lublin, Poland

* Correspondence: radoslaw.kowalski@up.lublin.pl

Received: 29 April 2020; Accepted: 16 June 2020; Published: 19 June 2020



Abstract: Clove (*Syzygium aromaticum*) is a spice widely used for its medical properties, though the species lacks scientific evidence regarding its toxicity and biologic effects. The aim of this study was the chemical identification by GC-MS analysis and evaluation of the hemolytic, anticoagulant, antidiarrheal and antipyretic activities of the essential oil from *S. aromaticum* (EOSa) in adult male mice. Essential oil was obtained by hydrodistillation and provided 9.8% *w/w* yield. GC-MS analyses allowed the identification of nine constituents, with eugenol (84.63%) as the majority. EOSa was diluted in several concentrations (0.005–2 mg/mL) for hemolytic assays, showing hemolytic activity above 20% in concentrations higher than 0.625 mg/mL. Different concentrations of EOSa induced a coagulation time 100% higher than control blood. 50 and 100 mg/kg of EOSa caused additional intestinal motility induced by castor oil by 90–100%. Fever, induced by *Saccharomyces cerevisiae* 15% (s.c.), was controlled by 50 and 100 mg/kg EOSa (p.o.), effects similar to 100 mg/kg dypirone. Results showed that when used orally, EOSa may have a certain degree of toxicity in high dosages, but with antipyretic and intestinal motility properties.

Keywords: essential oil; GC-MS; fever; intestinal motility; toxicity

1. Introduction

Plants are a rich source of potent and efficient compounds. These products can be extracted by several means and can play key roles in human health [1–4]. The main property found in natural products is the antioxidant activity and it is related to several adjusts in cell and tissue impairments,

such as hemolysis, apoptosis [5] and inflammatory and tumoral processes [6]. Moreover, studies have proved that specific roles, such as upregulating proteins responsible for stabilizing erythrocyte membranes [7,8] and reducing oxidative stress of chronic diseases [9,10]. Although industries tend to choose compounds due to commercial properties, several plants are commonly used in popular medicine [8].

Syzygium aromaticum (L.) Merr. & L.M. Perry—popularly known as the clove—is a Myrtaceae plant originally from Asia whose buds have been used worldwide for centuries as a spice in several dishes. In addition to this, clove buds are also used in popular medicine for a myriad of diseases and conditions, as a pain reliever and muscle relaxant, in fungal and bacterial infections, nausea and vomiting, allergies and for diabetes and hypertension [11]. In view of such variety, many doses, formulations, routes of administration and exposure times have been used, mainly without professional guidance [12]. This can considerably compromise the effects obtained and certainly cause unexpected side effects and toxicity [13].

Therefore, many researchers have been looking for scientific evidence for the use of clove extracts and essential oil. Some evidence of antioxidant, anti-inflammatory, analgesic, antipyretic, and sedative effects has been found [14]. Although these studies are of fundamental relevance, they lack similarities with the popular use since they have been performed *in vitro* or the products under test are applied intraperitoneally. This pathway excludes hepatic metabolism, which is fundamental in the effect and toxicity of biologically active molecules in animals [15].

Thus, this study aims to investigate hematologic, antipyretic and antidiarrheal biologic activities and possible side effects caused by the oral use of the *S. aromaticum* essential oil (EOSa). This strategy brings scientific evidence closer to popular use, widespread due to ease of access and low cost.

2. Materials and Methods

2.1. Essential Oil Obtention

Clove buds (500 g) were purchased at the town market of Barbalha—Ceará—Brazil. The samples were minced and submitted to hydrodistillation through a Clevenger-type apparatus for 3 h obtaining an essential oil for biologic research. To determine the content of essential oil in clove buds, quantitative distillation (according to European Pharmacopoeia, Strasbourg, France [16]) was performed in triplicate. Five grams of plant material was placed in a round-bottom flask (1000 mL) and 400 mL of distilled water was poured over it—the installation was provided with a reflux condenser. The flask was put into a distillation apparatus (steam distillation method) and distillation was carried out for 3 h from the moment of the distillation of the first drop of essential oil. Achieved data of the oil volumes were calculated to initial weight of vegetable material subjected to the distillation process, expressed as percentage of dry weight. In the end, oil was collected, dried with anhydrous Na_2SO_4 , measured, transferred to glass flasks, and kept at -18°C for further analysis.

2.2. Animals

This study has approval from the Committee of Ethics and Research with Animals of Regional University of Cariri, n° 00067/2017.1. Mice, *Mus musculus*, Swiss, male, 12 to 14 weeks old, 35 g of weight were chosen for this research. Animals were obtained from the Laboratory of Immunopathology Keizo Asami-LIKA of Federal University of Pernambuco (UFPE). Animals were kept in the Department of Biochemistry of UFPE, under light/dark cycle of 12 h/12 h, with food and water *ad libitum* before experiments.

2.3. GC-MS Analysis

EOSa was analyzed using a Shimadzu GC-QP2010 ultra series (Shimadzu, Kyoto, Japan) fitted with a fused silica Rtx-5MS (30 m $\times$ 0.25 mm I.D.; 0.25 μm film thickness) capillary column, with He as the carrier gas at the flow rate of 1.05 mL/min. GC oven temperature was programmed from 80–180 $^\circ\text{C}$

at 25 °C/min, then to 180–246 °C at 10 °C/min, ending with 246–280 °C at 10 °C/min. The injector was set to 220 °C, split ratio 1:20; injection volume 1 µL of 5-µg/mL solution in dichloromethane. Significant quadrupole MS operating parameters: interface temperature at 300 °C; electron impact ionization at 70 eV with scan mass range of 40–350 *m/z* at a sampling rate of 1.0 scan/s. Constituents were identified by computer search using digital libraries of mass spectral data (NIST 08) and by comparison of their authentic mass spectra. The retention index was obtained by injecting a C8–C40 linear hydrocarbon mixture under these same conditions [17]. Identity of the compounds was confirmed by their retention indices and mass spectra taken from literature [18,19] and own data for co-injection with standards (methyl salicylate ≥ 99% GC, chavicol ≥ 98.0% GC, (*E*)-cinnamaldehyde ≥ 99%, eugenol ≥ 98%, α-humulene ≥ 96.0% GC, eugenol acetate ≥ 98% GC from Sigma-Aldrich Chemie GmbH, Germany). The relative percentages of the separated compounds were calculated from integration of the peak areas in the GC chromatograms.

2.4. Anticoagulant Activity

Animals were anesthetized with xylazine (10 mg/kg) and ketamine (115 mg/kg) (i.m.) for blood extraction through the retroorbital plexus. Commercial anticoagulants ethylenediaminetetraacetic acid (EDTA) and sodium citrate 3.5% (Labor Import, Osasco, Brazil) were used as positive controls, samples of 500 µL of blood were added to tubes with several concentrations of EOSa (0.125 mg/mL; 0.250 mg/mL; 0.5 mg/mL; 1 mg/mL; 2 mg/mL and 4 mg/mL) and sodium chloride 0.9% and distilled water were negative controls.

Concentrations of EOSa in DMSO 0.4% (dimethyl sulfoxide) were based on a curve of concentrations, in which the concentration of sodium citrate was placed in the middle of the curve. Sodium citrate (3.5%), EOSa concentrations ranging from 0.222% to 14% of final volume. The samples' coagulation time (triplicates) were then observed, timed and noted [20].

2.5. Hemolytic Assay

Hemolytic activity was investigated according to the methodology described by [20]. An aliquot of 2 mL of blood was collected as described above and centrifuged at 1000 rpm for 10 min at −4 °C. After plasma removal, the pellet was resuspended and washed five times with PBS (phosphate buffered saline, pH = 7.4). 100 µL of red blood cells solution 8% (v/v) in PBS were added to tubes with EOSa in DMSO 0.4% diluted in several concentrations (0.009 mg/mL; 0.019 mg/mL; 0.039 mg/mL; 0.078 mg/mL; 0.156 mg/mL; 0.3125 mg/mL; 0.625 mg/mL; 1.25 mg/mL; 2.5 mg/mL). A set of three samples containing DMSO 0.4% and red blood cells was also included in order to subtract possible hemolysis caused by DMSO.

Samples were stirred for 60 min at 37 °C. After incubation, samples were centrifuged at 685 g for 2 min, and supernatant was used for absorbance analysis. Positive control of hemolysis was verified with Triton X-100 1% in PBS with red blood cells 4%. Negative control of hemolysis was performed by a solution of 4% red blood cells in DMSO 0.4% diluted in PBS. Supernatants were transferred to 96-well titer plate, and released hemoglobin was quantified by spectrophotometry in µQuant™ (Biotek, Winooski, VT, USA). Absorbances of samples were read in 540 nm and percentage of hemolysis was evaluated according to the following equation: (sample absorbance − negative control absorbance)/(positive control absorbance − negative control absorbance) × 100.

2.6. Antidiarrheal Activity In Vivo

For this test, 24 mice were fasted for 24 h previous to experiment. Animals were allowed only to drink water during this period. Mice were divided into 4 groups: negative control—100 µL of carboxymethylcellulose (CMC) 1%; positive control—loperamide hydrochloride (2 mg/kg in 100 µL of water); EOSa 50–50 mg/kg of EOSa diluted in 100 µL of CMC 1%; and EOSa 100–100 mg/kg of EOSa diluted in 100 µL of CMC 1%.

After 30 min, the animals received 2 mL of castor oil (p.o). After the following 30 min, 2 mL/animal (p.o) of 10% active carbon suspension solution in 5% gum arabic solution was administered. After 30 min

of rest, the mice were anesthetized, sacrificed and immediate bowel extirpation (from the pylorus to the onset of the cecum) was performed. The result of this test was assessed by the percentage of charcoal with gum arabic solution movement in intestines (distance of charcoal with gum arabic solution/intestine length) $\times$ 100 [21].

2.7. Antipyretic Activity

The methodology of antipyretic activity was based on Pasin [22]. Animals had their basal rectal temperature assessed before experiment. Only animals with rectal temperature ranging from 36 °C and 37 °C were used. 24 animals received 100 μ L of sodium chloride 0.9% containing *Saccharomyces cerevisiae* 15% subcutaneously. After 18 h, animals had rectal temperature assessed once again, and the ones with temperature equal to 38 °C or higher were divided into vehicle—DMSO (dimethyl sulfoxide 0.4%), metamizole (dipyrone 100 mg/kg), EOSa 50 (50 mg/kg of EOSa diluted in DMSO 0.4%) and EOSa 100 (100 mg/kg diluted in DMSO 0.4%). After administration of the material, the animals had their temperature evaluated hourly for 3 h.

2.8. Statistical Analysis

Results were expressed as mean standard deviation and analyzed with ANOVA, followed by Tukey's test (post hoc test). It was considered $p < 0.05$ for statistical difference. Data were analyzed in the software GraphPad Prism 5.0, USA.

3. Results

3.1. Yield and Composition of EOSa

The EOSa content was (on average) 9.8% *w/w* of total dry weight. Amelia et al. [23] reported that clove buds from Java and Manado contain about 4.99% *w/w* and 4.58% *w/w* of essential oil (steam distillation method), which is a content lower than those obtained in the present study. While Guan et al. [24], found a similar essential oil content for buds of clove from China—10.1% *w/w* (steam distillation) and 11.5% *w/w* (hydrodistillation). Kapadiya et al. [25] reported that clove buds contain about 11.93% and 13.11% *w/w* of essential oil (microwave assisted extraction method). Clove buds originated from the Persian Gulf contained only 0.7–0.92% *w/w* essential oil [26]. Other sources present the following yields of EOSa: 1.87% *w/w* [27], 7.05% *w/w* [28], 11.35% *w/w* [29] and 15.40% *w/w* [29]. The differences in the essential oil yield are influenced by origin of clove buds, variety, storage conditions, sample preparation, applied isolation method and the duration of the isolation process [23].

In the EOSa, 9 components were identified. The percentages of chemical constituents of essential oil are shown in Table 1. Eugenol, with retention index 1361, was the major component of EOSa—84.63%. Other studies have also reported high concentrations of eugenol in EOSa 90.3% [27], 82.47% [30], 83.75% [31], and 77.58% [32], according to place and time of the year floral buttons were collected. The second major constituent of EOSa was eugenol acetate 11.37% with retention index 1530. Unlike eugenol, studies do not present important information about other constituents of EOSa. eugenol acetate is part of eugenol metabolism, which increases phenylpropanoid representation in this essential oil. eugenol is also found in other species, but it is not reported in such high concentrations as in clove [33].

Table 1. Compounds identified in the essential oil of *S. anomala* using gas chromatography-mass spectrometry (GC-MS).

Compounds	RI	RI ^a	RI ^b	RI ^c	RI ^d	RI ^e	RI ^f	Mass Fragment Ions with Relative (%)	Identification **	(%)
methyleugenol	1228	1190	1197–1234	1191–1234	1182–1225	1185–1193	—	152 (84%), 129 (100%), 92 (65%) and 121 (75%)	1, 2, 3	0.55
chavicol	1257	1227	—	—	1249–1254	—	1251–1265	154 (100%), 133 (91%), 107 (45%) and 77 (64%)	1, 2, 3	0.08
<i>o</i> -Ethymethylcyclo	1272	1267	—	—	1266–1277	—	1253–1282	152 (72%), 131 (100%), 105 (82%) and 77 (70%)	1, 2, 3	0.06
eugenol	1301	1256	1248–1270	1246–1279	1291–1316	1246–1274	1332–1347	164 (100%), 143 (33%), 140 (37%) and 151 (42%)	1, 2, 3	84.65
isohumulol	1446	1452	1425–1486	1425–1489	1431–1495	1408–1469	1414–1495	205 (151.45) (100%), 91 (36%) and 80 (29%)	1, 2, 3	0.32
9-epi-7-oxabicyclolene	1456	1464	1451–1476	1467	1453–1465	—	1467–1470	164 (94%), 41 (100%), 92 (37%), 164 (75%)	1, 2	2.34
eugenol acetate	1536	1523	1525	—	1522–1533	—	1526–1534	205 (8%), 164 (100%), 149 (21%), 151 (17%) and 141 (14%)	1, 2, 3	11.37
caryophyllol (2E,4E)-B-dien-5E-ol	1628	1639	—	—	—	—	1590–1641	41 (100%), 156 (70%), 91 (32%) and 78 (30%)	1, 2	0.67
α-bisabolol	1762	1760	1755	—	1751–1766	1764–1761	1738–1762	225 (153), 45 (100%), 90 (24%), 41 (75%) and 124 (20%)	1, 2	0.13
Total										99.26

RI—retention indices from computerized programming using database of Van Den Dool and Korte [17]; RI^a—retention indices reported in literature [18]; RI^b—retention indices for nonpolar column (5% phenyl and 95% dimethylpolysiloxane), temperature ramp [19]; RI^c—retention indices for nonpolar column (5% phenyl and 95% dimethylpolysiloxane), custom temperature program [19]; RI^d—Van Den Dool and Korte retention indices, nonpolar column (5% phenyl and 95% dimethylpolysiloxane), temperature ramp [19]; RI^e—Van Den Dool and Korte retention indices, nonpolar column (5% phenyl and 95% dimethylpolysiloxane), custom temperature program [19]; RI^f—Shimali-Khate retention indices, nonpolar column (5% phenyl and 95% dimethylpolysiloxane), temperature ramp [19]; ** Relative relative intensities; %—retention indices, 2—core spectrum, 3—co-spectrum with standard.

3.2. Anticoagulant Activity

Different concentrations of EOSa (0.0625 mg/mL to 4 mg/mL) provoked similar coagulation times (Figure 1), around 2 min ($p > 0.05$). Results of EOSa effect compared to negative controls (saline 0.9% and distilled water) showed an effect of 1 min retardation to coagulation ($p < 0.0001$). Anticoagulant test, performed thrice, showed delay in coagulation during 1 min (Figure 1). Studies, commonly, are performed in vivo and evaluate the time of protrombine activation. For these studies, there are several aqueous extracts that have anticoagulant potential [34–37].

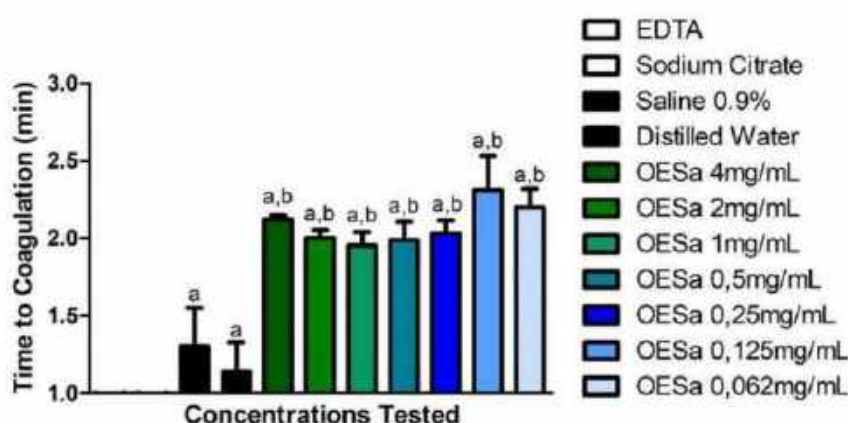


Figure 1. Anticoagulation potential of *S. aromaticum* essential oil. a $p < 0.0001$ compared to coagulant compounds (ethylenediaminetetraacetic acid (EDTA) and sodium citrate); b $p < 0.001$ compared to non-anticoagulant compounds (saline 0.9% and distilled water), one-way ANOVA.

With the same test used in this work, a study with aqueous extract of *Cesalpinia ferrea* retarded coagulation during 30 min [20]. This same study indicated that, even though 30 min seems a short period, the purification of aqueous extract of *C. ferrea* constituents was needed to attest which compounds had such activity, and if there is a way to enhance their anticoagulant potential [20].

Literature concerning essential oils that inhibit coagulation is vague, but one study with essential oil of *Tropaeolum majus* also presented a retardation in coagulation time, despite this activity being related to the intrinsic pathway of coagulation, these authors suggested that utilization of their material in in vivo experiments was needed to confirm the potential of *T. majus* as anticoagulant [34].

Phenylpropanoids have not been tested for anticoagulant potential. Although eugenol is the major compound of EOSa, it is possible that the retardation seen was caused by the minor compounds of EOSa. Purification of these compounds could elevate the time to coagulate or stop coagulation process.

3.3. Hemolytic Activity

Figure 2 shows that higher concentrations of EOSa presented hemolytic effect. This characterizes danger to EOSa utilization in high concentrations—and in high frequency—due to its toxicity to red blood cells. The second test with blood content showed that EOSa is toxic to red blood cells if used in higher concentrations. Results of this hemolytic test demonstrate that acute over exposition to EOSa can lead to more than 70% of hemolysis (Figure 2). Valente and collaborators found similar results with EOSa used in acute toxicity test in rodents [13].

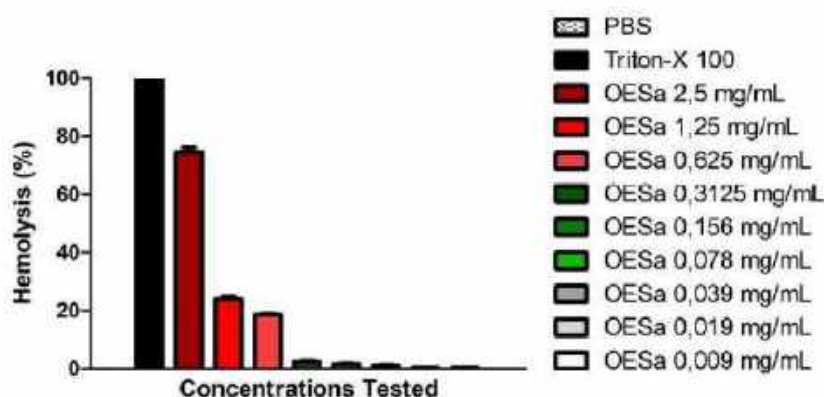


Figure 2. Hemolytic activity of *S. aromaticum* essential oil. Black bars indicate 100% of hemolysis; red bars indicate moderate and high hemolysis; green bars indicate low hemolysis; White bars indicate no hemolysis, $p < 0.001$, one-way ANOVA.

Hemolytic tests provide evidence to the possibility of a substance causing damage to mammals' cell. Erythrocytes possess all essential structures of a human cell except for their lack of nuclei. Studies often demonstrate that compounds with antioxidant properties have protector effects on cells due to their free radical reducing potential [20,38,39]. Nonetheless, EOSa, with predominance of eugenol, showed toxicity in higher concentrations, even though its antioxidant potential has been well described in the literature [31,40]. EOSa presented up to 70% of hemolysis in the 2,5-mg/mL concentration; almost the same effect of Triton-X 100. This potential for cell lysis may be related to eugenol being capable of interaction with the cell membrane, diminishing some of its properties.

Extract of *Syzygium cumini* (seeds) tested for cytotoxicity in a study by Mathur and collaborators [41] showed maximum cytotoxicity. However, seeds of *S. cumini* also showed anti-hemolytic potential in the study of Saha and collaborators [42] and this was related to the concentration of polyphenols and flavonoids found in the solvent extracts of *S. cumini*. The present study did not evaluate the anti-hemolytic potential of EOSa, which could balance the essential oil's toxicity factor.

3.4. Antidiarrheal Activity

EOSa 50 mg/kg and 100 mg/kg obtained different results from negative control ($p < 0.01$) and from positive control ($p < 0.0001$) (Figure 3). These results were unexpected compared to this study's hypothesis. EOSa showed additive effect to castor oil. Figure 3 illustrates that EOSa treated animals, independently of concentration, presented 90–100% of mark solution movement. 50 mg/kg of EOSa and 100 mg/kg had the same effect ($p > 0.05$).

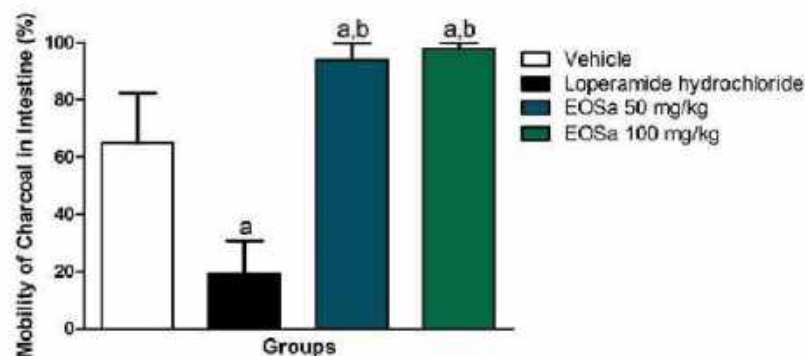


Figure 3. Evaluation of antidiarrheal activity of *S. aromaticum* essential oil. vehicle; 100 μ L of carboxymethylcellulose (CMC) 1%; loperamide hydrochloride: positive control for diarrhea reduction; EOSa: essential oil of *S. aromaticum*. a $p < 0.01$ compared to vehicle; b $p < 0.0001$ compared to loperamide hydrochloride, one-way ANOVA.

For antidiarrheal effect, essential oils are divergent. Some essential oils have been reported for their antidiarrheal capacities, such as rosemary (*Rosmarinus officinalis*) and oregano (*Origanum vulgare*). However, this effect is related to diarrhea caused by bacterial infection [43]. A couple of studies with other plants that possess eugenol showed the same results and associated the substance's capacity to balance microorganisms [44]. In general, essential oils help intestinal motility, besides enhancing the digestive process due to their capability to increase enzymatic activity and nitrogen absorption [43,45]. Regarding this effect, eugenol and other substances found in essential oil also play a key role through their neurotransmitter regulation action, such as histamine and dopamine [46].

EOSa did not present diarrheic potential when administered in the mice used for the antipyretic test. However, mice that were treated with castor oil before EOSa administration presented higher intestinal motility than those who only received castor oil, about 35% (Figure 3). This represents an additive effect of EOSa to castor oil.

Results suggest that EOSa induces diarrhea, contrasting with other study that indicates that extracts of *S. aromaticum* have antidiarrheal effect, which differs from what is reported about its essential oil [47]. Malik and Ahmad [48] tested *S. polyanthum*, another *Syzygium* species, with mice and reported antidiarrheal activity in the same model of diarrhea assessment.

Literature shows that extracts of *S. aromaticum* are reported with different results and activities of its essential oil. This is related to the concentration of secondary metabolites found in essential oil, which differs completely from the compounds found in the extracts [40]. Aqueous extract of *S. cumini* (125 mg/kg, 250 mg/kg, 500 mg/kg) was tested for antidiarrheal potential in the same protocol [49] and presented antidiarrheal activity through intestinal motility and secretion reduction. It is observed that extracts from *Syzygium* species combat diarrhea, and their essential oils produce the opposite effect, possibly, because of their constituents. Thus, the process to obtain substances from clove are related to the activity desired.

Interestingly, isolated eugenol has been reported as an inhibitor of the Ca^{2+} -Activated Cl^- Channel TMEM16A, causing intestinal motility reduction in mice [30]. Another study has pointed to a possible activity of eugenol in reducing the capacity of *Campylobacter jejuni* to cause gastroenteritis on human intestinal epithelial cells [51]. For that we have no explanation, but we can hypothesize that charcoal-induced diarrhea acts through a divergent mechanism from what has been observed by others using isolated eugenol.

3.5. Antipyretic Activity

Treatment groups presented antipyretic effect in the first hour after administration and kept animals out of fever state during the time observed ($p < 0.01$). There was no difference between groups 50 mg/kg and 100 mg/kg, neither between EOSa groups and positive control.

Regarding antipyretic effect, our results were similar to others [15] that analyzed antipyretic potential of EOSa, and it significantly reduced fever ($p < 0.001$) between 30 to 180 min after intraperitoneal administration. Data from our study (Table 2) provides evidence that EOSa is well absorbed in the liver and does not lose its potential since low concentrations such 50 mg/kg are as effective via oral administration as higher concentrations were when administered intraperitoneally.

Table 2. Effect of *S. aromaticum* essential oil on fungi-induced fever.

Group	0 h	1 h	2 h	3 h
Vehicle	38.28 ± 0.18	38.36 ± 0.24	38.38 ± 0.3	38.35 ± 0.6
Metamizole	38.18 ± 0.17	36.98 ± 0.29 *	36.86 ± 0.51 *	36.65 ± 0.43 **
EOSa 50 mg/kg	38.21 ± 0.14	37.06 ± 1.2 *	36.53 ± 0.7 *	36.18 ± 1.08 **
EOSa 100 mg/kg	38.5 ± 0.33	36.5 ± 1.07 *	36.65 ± 0.74 *	36.25 ± 0.96 **

vehicle: DMSO 0.4%; metamizole: 100 mg/kg of dipyrene; EOSa: Essential oil of *S. aromaticum*. Results expressed as mean ± SD, n = 6. * $p < 0.01$ compared to vehicle; ** $p < 0.001$ compared to vehicle, two-way ANOVA.

In another study using *S. cumini* extract, it was observed that the antipyretic potential was as effective as the one presented by the positive control [52]. This was also found in a study with *S. polyanthum*, which had paracetamol as positive control [53]. In this study, it was used metamizole, but *S. aromaticum* also showed similar potential to control fever. Isolated eugenol showed antipyretic activity when intragastrically or intravenously injected in rabbits, probably through central action [54].

As with many other biologic materials, the capability to combat fever was related to these materials' antioxidant effect. Clove materials applications have been related to their high antioxidant activity [15,27,33,55]. Antioxidants reduce fever through reducing chemotaxis and inhibiting cyclooxygenases COX-1 and COX-2 [40,55].

It is possible that the antipyretic activity of EOSa is due to eugenol presence. Data from previous studies with rabbits demonstrate treatment of fever with eugenol [54]. In addition, it has been reported that eugenol can inhibit COX-2, the main enzyme in the fever process [56].

4. Conclusions

Clove essential oil is already reported as a good antioxidant, anti-inflammatory and antipyretic. GCMS analysis confirmed that eugenol is the major chemical component of EOSa. Moreover, results proved antipyretic effect when orally administered, indicating that the essential oil is not affected by liver metabolism. In addition, essential oil presented an additive effect with castor oil to increase digestive motility. Besides this, essential oil of clove showed a brief retarded time for coagulation that indicates a possible area for studies of its minority compounds.

Author Contributions: Conceptualization, C.M.U.L.; methodology, C.M.U.L., J.R.S.d.O., F.F.G.R., C.J.C., V.N.H.; software, A.Y.F.R.; writing—original draft preparation, C.S.M.d.F.; writing—review and editing V.L.d.M.L., C.M.U.L.; visualization, C.M.U.L., J.R.S.d.O., F.F.G.R.; resources, R.K. and H.D.M.C.; supervision, J.G.M.d.C.; project administration, J.G.M.d.C., R.K. and H.D.M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: Cariri Regional University, Funcap an Caps.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Johnson, A.; Cowtham, J.; Janakiraman, N.; Malar, T.R.J.J.; Rocha, J.E.; Coutinho, H.D.M. Phytochemical profile of *Asplenium aethiopicum* (Burm. f.) Becherer using HPTLC. *Separations* **2020**, *7*, 8. [CrossRef]
- Fontanals, N.; Marcé, R.M.; Borull, F. Materials for solid-phase extraction of organic compounds. *Separations* **2019**, *6*, 56. [CrossRef]
- Salvatore, M.M.; Ciaravolo, M.; Cirino, P.; Toscano, A.; Salvatore, F.; Fallo, M.; Naviglio, D.; Andolfi, A. Fatty acids from *Paracentrotus lividus* sea urchin shells obtained via rapid solid liquid dynamix extraction (RSLDE). *Separations* **2019**, *6*, 50. [CrossRef]
- Ngo, T.V.; Scarlett, C.J.; Bowyer, M.C.; Vuong, Q.V. Isolation and maximisation of extraction of mangiferin from the root of *Salacia chinensis* L. *Separations* **2019**, *6*, 44. [CrossRef]
- Li, H.T.; Wu, M.; Wang, J.; Qin, C.J.; Long, J.; Zhou, S.S.; Yuan, P.; Jing, X.Q. Protective role of *Angelica sinensis* extract on trichlorfon-induced oxidative damage and apoptosis in gills and erythrocytes of fish. *Aquaculture* **2020**, *519*, 734895. [CrossRef]
- Carmo, M.C.L.; Martins, L.M.; Magalhães, A.E.R.; Maróstica Júnior, M.R.; Macedo, J.A. Passion fruit (*Passiflora edulis*) leaf aqueous extract ameliorates intestinal epithelial barrier dysfunction and reverts inflammatory parameters in Caco-2 cells monolayer. *Food Res. Int.* **2020**, *133*, 109162. [CrossRef]
- Remigante, A.; Morabito, R.; Marino, A. Natural antioxidants beneficial effects on anion exchange through band 3 protein in human erythrocytes. *Antioxidants* **2020**, *9*, 25. [CrossRef]
- Anosike, C.A.; Igboegwu, O.N.; Nwodo, O.F.C. Antioxidant properties and membrane stabilization effects of methanol extract of *Mucuna pruriens* leaves on normal and sickle erythrocytes. *J. Tradit. Complement. Med.* **2019**, *9*, 278–284. [CrossRef]
- Prihatin, J.; Narulita, E.; Mufidah, L.; Kurniawan, A.; Wulandari, D.; Hariyadi, S. Antihyperglycaemic and tissue-repair effects of *Myrmecodia formicarius* extract in streptozotocin-induced diabetic mice. *J. Taibah Univ. Med. Sci.* **2019**, *14*, 149–155. [CrossRef]
- Aljuhani, N.; Elkahlawy, M.A.; Elbadawy, H.M.; Alahmadi, A.M.; Aloufi, A.M.; Farsi, S.H.; Alhubayshi, B.S.; Alhejaili, S.S.; Alhejaili, J.M.; Abdel-Halim, O.B. Protective effect of Ajwa date extract against tissue damage induced by acute diclofenac toxicity. *J. Taibah Univ. Med. Sci.* **2019**, *14*, 553–559. [CrossRef]
- Bhowmik, D.; Sampath, K.K.P.; Yadav, A.; Srivastava, S.; Paswan, S.; Dutta, A.S. Recent trends in Indian traditional herbs *Syzygium aromaticum* and its health benefits. *J. Pharmacogn. Phytochem* **2010**, *1*, 6–17.
- Sasidharan, S.; Chen, Y.; Saravan, D.; Sundram, K.M.; Latha, L.Y. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *Afr. J. Tradit. Complement. Altern. Med.* **2011**, *8*, 1–10. [CrossRef] [PubMed]
- Valente, R.O.H.; Sampaio, F.C.; Souza, L.A.; Higino, J.S. Estudo toxicológico pré-clínico (agudo) do extrato do *Syzygium aromaticum* (L.) em roedores. *Rev. Bras. Farmacogn.* **2009**, *19*, 557–560. [CrossRef]
- Cortés-Rojas, D.F.; Souza, C.R.F.; Oliveira, W.P. Clove (*Syzygium aromaticum*): A precious spice. *Asian. Pac. J. Trop. Biomed.* **2014**, *4*, 90–96. [CrossRef]
- Taher, Y.A.; Samad, A.M.; El-Taher, F.E.; ben-Hussin, G.J.; Elmezog, S.B.; Al-Mehdawi, F.; Sale, H.A. Experimental evaluation of anti-inflammatory, antinociceptive and antipyretic activities of clove oil in mice. *Libyan J. Med.* **2015**, *10*, 28685. [CrossRef] [PubMed]
- EDQM Council of Europe. *European Pharmacopoeia*, 8th ed.; EDQM Council of Europe: Strasbourg, France, 2014; pp. 273–274, Essential oils in herbal drugs, Monograph 2. 8.12.
- Van Den Dool, H.; Kratz, P.D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr. A* **1963**, *11*, 463–471. [CrossRef]
- Adams, R.P. *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy*; Allured Publishing Corporation: Carol Stream, IL, USA, 2012.
- NIST Mass Spectrometry Data Center. Available online: <https://www.nist.gov/mml/biomolecular-measurement/mass-spectrometry-data-center> (accessed on 27 April 2020).
- Cavalheiro, M.G.; Farias, D.F.; Fernandes, G.S.; Nunes, E.P.; Cavalcanti, E.S.; Vasconcelos, L.M.; Melo, V.M.M.; Carvalho, A.E.U. Atividades biológicas e enzimáticas do extrato aquoso de sementes de *Caesalpinia ferrea* Mart., Leguminosae. *Rev. Bras. Farmacogn.* **2009**, *19*, 586–591. [CrossRef]

21. Prestes, L.S. Atividade antimicrobiana in vitro e anti-diarreica em modelo experimental de extratos de folhas de plantas da família Myrtaceae. Ph.D. Thesis, Programa de Pós-graduação, Federal University of Pelotas, Pelotas, Brazil, 2011.
22. Pasin, J.S.M.; Ferreira, A.P.O.; Saraiva, A.L.L.; Ratzlaff, V.; Andrighetto, R.; Machado, S.; Marchesan, S.; Zanette, R.A.; Bonacorso, H.G.; Zanatta, N.; et al. Antipyretic and antioxidant activities of 5-trifluoromethyl-4,5-dihydro-1H-pyrazoles in rats. *Braz. J. Med. Biol. Res.* **2010**, *43*, 1193–1202. [\[CrossRef\]](#)
23. Amelia, B.; Saepudin, E.; Cahyana, A.H.; Rahayu, D.U.; Sulistyoningrum, A.S.; Haib, J. GC-MS analysis of clove (*Syzygium aromaticum*) bud essential oil from Java and Manado. In Proceedings of the AIP Conference Proceedings 1862, International Symposium on Current Progress in Mathematics and Sciences 2016 (ISCPMS 2016), Jawa Barat, Indonesia, 1–2 November 2016; 2017; p. 030082. [\[CrossRef\]](#)
24. Guan, W.; Li, S.; Yan, R.; Tang, S.; Quan, C. Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. *Food Chem.* **2007**, *101*, 1538–1564. [\[CrossRef\]](#)
25. Kapadiya, S.M.; Parikh, J.; Desai, M.A. A greener approach towards isolating clove oil from buds of *Syzygium aromaticum* using microwave radiation. *Ind. Crop. Prod.* **2018**, *112*, 626–632. [\[CrossRef\]](#)
26. Hossain, M.A.; Harbi, S.R.; Weli, A.M.; Al-Riyami, Q.; Al-Sabahi, J.N. Comparison of chemical constituents and antimicrobial activities of three essential oils from three different brands' clove samples collected from Gulf region. *Asian Pac. J. Trop. Dis.* **2014**, *4*, 262–268. [\[CrossRef\]](#)
27. Silvestri, J.D.F.; Paroul, N.; Czyewski, E.; Lerin, L.; Rotava, L.; Casian, R.L.; Mossi, A.; Toniazzi, G.; Oliveira, D.; Treichel, H. Perfil da composição química e atividades antibacteriana e antioxidante do óleo essencial do cravo-da-índia (*Eugenia caryophyllata* Thunb.). *Rev. Ceres* **2010**, *57*, 589–594. [\[CrossRef\]](#)
28. Sohailat, H.J. Chemical composition of the essential oils in *Eugenia caryophyllata*, Thunb from Amboina Island. *Sci. J. Chem.* **2015**, *3*, 95–99. [\[CrossRef\]](#)
29. Kapadiya, S.; Desai, M.A. Desai Isolation of essential oil from buds of *Syzygium Aromaticum* using hydrodistillation: Multi-response optimization and predictive modelling. *Int. J. Adv. Res. Sci. Eng.* **2017**, *6*, 405–418.
30. Oliveira, R.A.; Reis, T.V.; Sacramento, C.K.; Duarte, L.P.; Oliveira, F.F. Constituintes químicos voláteis de especiarias ricas em eugenol. *Rev. Bra. Farmacogn.* **2009**, *19*, 771–775. [\[CrossRef\]](#)
31. Scherer, R.; Wagner, R.; Duarte, M.C.T.; Godoy, H.T. Composição e atividades antioxidante e antimicrobiana dos óleos essenciais de cravo-da-índia, citronela e palmarosa. *Rev. Bra. Plan. Med.* **2009**, *4*, 442–449. [\[CrossRef\]](#)
32. Beraldo, C.; Daneluzzi, N.S.; Scanavacca, J.; Doyama, J.T.; Fernandez, A., Jr.; Moritz, C.M.F. Eficiência de óleos essenciais de canela e cravo-da-índia como sanitizantes na indústria de alimentos. *Pesq. Agropecuária Trop.* **2013**, *43*, 436–440. [\[CrossRef\]](#)
33. Chaieb, K.; Hajlaoui, H.; Zmantar, T.; Kahla-Nakbi, A.B.; Rouabbia, M.; Mahdouni, K.; Nakhoul, A. The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzygium aromaticum* L. Myrtaceae): A short review. *Phytother. Res.* **2007**, *21*, 501–506. [\[CrossRef\]](#)
34. Ostrowski, A.A.; Valentini, S.A.; Pavanelli, M.F. Atividade anticoagulante de extrato aquoso, hidroetanólico e óleo essencial das folhas de *Tropaeolum majus*. *Rev. Saúde Biol.* **2014**, *9*, 46–53.
35. Melo, K.R.T.; Almeida-Lima, J.; Gomes, D.L.; Dantas-Santos, N.; Camara, R.G.B.; Rocha, H.A.O. Caracterização e atividade anticoagulante de polissacarídeos sulfatados extraídos de alga marrom *Dictyopteris justii*. *Holos* **2012**, *28*, 29–40. [\[CrossRef\]](#)
36. Rodrigues, J.A.C.; Farias, W.R.L. Purificação e atividade anticoagulante in vitro de galactanas sulfatadas extraídas da alga marinha vermelha *Halymenia pseudofloresia*. *Rev. Bra. Eng. Pesca.* **2008**, *2*, 16–29.
37. Rodrigues, J.A.C.; Vanderlei, E.S.O.; Quindere, A.L.G.; Coura, C.O.; Benevides, N.M.B. Avaliação do potencial anticoagulante de polissacarídeos sulfatados de macroalgas marinhas. *Rev. Bra. Eng. Pesca.* **2010**, *5*, 56–69.
38. Elgebaly, H.A.; Mosa, N.M.; Allach, M.; El-massry, K.F.; El-Ghorab, A.H.; Al-Hroob, A.M.; Mahmoud, A.M. Olive oil and leaf extract prevent fluoxetine-induced hepatotoxicity by attenuating oxidative stress, inflammation and apoptosis. *Biomed. Pharmacother.* **2018**, *98*, 446–453. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Zhao, Y.; Tang, H.; Zeng, X.; Ye, D.; Liu, J. Resveratrol inhibits proliferation, migration and invasion via Akt and ERK1/2 signaling pathways in renal cell carcinoma cells. *Biomed. Pharmacother.* **2018**, *98*, 36–44. [\[CrossRef\]](#) [\[PubMed\]](#)

40. Alfonso, R.S.; Rennó, M.N.; Slana, G.B.C.A.; França, T.C.C. Aspectos químicos e biológicos do óleo essencial de cravo da Índia. *Rev. Virtual Quím.* **2012**, *4*, 146–161. [\[CrossRef\]](#)
41. Mathur, A.; Purohit, R.; Mathur, D.; Prasad, G.B.K.S.; Dua, V.K. Pharmacological investigation of methanol extract of *Syzygium cumini* seeds and *Crataeva nurcula* bark on the basis of antimicrobial, antioxidant and anti-inflammatory properties. *Der Chem. Sin.* **2011**, *2*, 174–181.
42. Saha, R.K. Comparative evaluation of the medicinal activities of methanolic extract of seeds, fruit pulps and fresh juice of *Syzygium cumini* in vitro. *J. Coast. Life Med.* **2013**, *1*, 300–308. [\[CrossRef\]](#)
43. Luchese, F.C. Óleos essenciais de orégano e alecrim na prevenção e no tratamento da diarreia neonatal em leitões. Master's Thesis, Programa de Pós-graduação em Medicina Veterinária, Federal University of Santa Maria, Santa Maria, Brazil, 2009.
44. Gairola, S.; Sharma, J.; Gaur, R.D.; Siddiqi, T.O.; Painuli, R.M. Plants used for treatment of dysentery and diarrhoea by the Bhoxa community of district Dehradun, Uttarakhand, India. *J. Ethnopharmacol.* **2013**, *140*, 969–1006. [\[CrossRef\]](#)
45. Darroz, J.V.; Fuso, L.C.; Borges, N.M.; Gomes, J.P.S. Utilização de fitoterápicos no tratamento de constipação intestinal. *Arg. Cienc. Saúde Unipar* **2014**, *18*, 113–119. [\[CrossRef\]](#)
46. Maheswari, D.U.; Anand, T.; Padma, A.; Ilaiyaraaja, N.; Khanum, F. Evaluation of effect of herbal extracts and their bioactive compounds against motion sickness by regulating neurotransmitter levels in vitro and in vivo. *S. Afr. J. Bot.* **2020**, *130*, 130–140. [\[CrossRef\]](#)
47. Hamad, A.; Mahardika, M.G.P.; Yuliani, I.; Hartanti, D. Chemical constituents and antimicrobial activities of essential oils of *Syzygium polyanthum* and *Syzygium aromaticum*. *Rasayan J. Chem.* **2017**, *10*, 564–569. [\[CrossRef\]](#)
48. Malik, A.; Ahmad, A.R. Antidiarrheal activity of ethanolic extract of bay leaves (*Syzygium polyanthum* [Wight.] Walp.). *Int. Res. J. Pharm.* **2013**, *4*, 106–108. [\[CrossRef\]](#)
49. Shamkuwar, P.B.; Pawar, D.P.; Chauhan, S.S. Antidiarrhoeal activity of seeds of *Syzygium cumini* L. *J. Pharm. Res.* **2012**, *5*, 5537–5539.
50. Yao, Z.; Nankung, W.; Ko, E.A.; Park, I.; Tradtrantip, L.; Verkman, A.S. Fractionation of a herbal antidiarrheal medicine reveals eugenol as an inhibitor of Ca²⁺-Activated Cl⁻ Channel TMEM16A. *PLoS ONE* **2012**, *7*, 38030. [\[CrossRef\]](#)
51. Upadhyay, A.; Arsi, K.; Wagle, B.R.; Upadhyaya, I.; Shrestha, S.; Donoghue, A.M.; Donoghue, D.J. Trans-Cinnamaldehyde, carvacrol, and eugenol reduce *Campylobacter jejuni* colonization factors and expression of virulence genes in vitro. *Front. Microbiol.* **2017**, *8*, 713. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Srivastava, S.; Chandra, D. Pharmacological potentials of *Syzygium cumini*: A review. *J. Sci. Food Agric.* **2013**, *93*, 2084–2093. [\[CrossRef\]](#)
53. Purnomo, S.J.; Sikni, R.K.; Anita, D.J. Antipyretic effect test of *Syzygium polyanthum* [Wight.] Walp. leaves infusion on male white rats of wistar strain. *J. Farm. Dan Obet Alam.* **2012**, *1*, 22–28.
54. Feng, J.; Lipton, J.M. Eugenol: Antipyretic activity in rabbits. *Neuropharmacology* **1987**, *26*, 1775–1778. [\[CrossRef\]](#)
55. Ramos, M.F.S.; Siani, A.C.; Souza, M.C.; Rosas, E.C.; Henriques, M.G.M.O. Avaliação da atividade antiinflamatória dos óleos essenciais de cinco espécies de Myrtaceae. *Rev. Fitos* **2006**, *2*, 58–66.
56. Nikoui, V.; Ostadhadi, S.; Bakhtarian, A.; Abbasi-Goujani, E.; Habibian-Dehkordi, S.; Rezaei-Roshan, M.; Foroozandeh, M.; Giorgi, M. The anti-inflammatory and antipyretic effects of clove oil in healthy dogs after surgery. *PharmaNutrition* **2017**, *5*, 52–57. [\[CrossRef\]](#)



5.8 ARTIGO 7

O artigo “Leishmanicidal and cytotoxic activity of essential oil from the fruit peel of *Myrciaria floribunda* (H. West ex Willd.) O. Berg: Molecular docking and molecular dynamics simulations of its major constituent onto *Leishmania* enzyme targets” foi publicado no periódico *Journal of Biomolecular Structure and Dynamics*, 2021, link para acesso: 10.1080/07391102.2021.1978320



Journal of Biomolecular Structure and Dynamics



ISSN: (Print) (Online) Journal homepage: <https://www.tandfonline.com/loi/tbsd20>

Leishmanicidal and cytotoxic activity of essential oil from the fruit peel of *Myrciaria floribunda* (H. West ex Willd.) O. Berg: Molecular docking and molecular dynamics simulations of its major constituent onto *Leishmania* enzyme targets

Deyzi Caroline da Silva Barbosa, Vanderlan Nogueira Holanda, Arabinda Ghosh, Rafael Trindade Maia, Welson Vicente da Silva, Vera Lúcia de Menezes Lima, Márcia Vanusa da Silva, Maria Tereza dos Santos Correia & Regina Celia Bressan Queiroz de Figueiredo

To cite this article: Deyzi Caroline da Silva Barbosa, Vanderlan Nogueira Holanda, Arabinda Ghosh, Rafael Trindade Maia, Welson Vicente da Silva, Vera Lúcia de Menezes Lima, Márcia Vanusa da Silva, Maria Tereza dos Santos Correia & Regina Celia Bressan Queiroz de Figueiredo (2021): Leishmanicidal and cytotoxic activity of essential oil from the fruit peel of *Myrciaria floribunda* (H. West ex Willd.) O. Berg: Molecular docking and molecular dynamics simulations of its major constituent onto *Leishmania* enzyme targets, *Journal of Biomolecular Structure and Dynamics*, DOI: 10.1080/07391102.2021.1978320

To link to this article: <https://doi.org/10.1080/07391102.2021.1978320>



View supplementary material.



Published online: 11 Oct 2021.



Submit your article to this journal.



View related articles.



View Crossmark data.

6 CONCLUSÃO

A aplicação de técnicas experimentais na investigação do potencial terapêutico de compostos bioativos de origem natural ou sintética se constitui uma importante ferramenta para a área da bioquímica e fisiologia, sobretudo no contexto do desenvolvimento de novos fármacos. Em conjunto, os resultados da presente tese de doutorado demonstram o amplo potencial farmacológico dos derivados de triazol e ftalimida obtidos por *click chemistry* no campo das doenças infecciosas e parasitárias.

As modificações estruturais presentes na série FT1-FT7 não interferiram nas propriedades *druglikness* das moléculas e isto resultou no perfil físico-químico, farmacocinético e toxicológico adequado quando se considera moléculas candidatas a fármacos. O ensaio de citotoxicidade e efeito leishmanicida, revelaram que os derivados contendo o grupo naftoquinona (FT6 e FT7) se mostraram mais tóxicos e as moléculas contendo ftalimida (FT1 e FT2), apresentaram maior seletividade quando levado em consideração as células de mamífero desse estudo, o macrófago e o fibroblasto.

Adicionalmente à caracterização da toxicidade *in vitro*, os derivados FT1 e FT2 não apresentaram atividade hemolítica em hemácias de diferentes fenótipos de sangue humano. Os dados do estudo de mecanismo de ação demonstram que a mitocôndria é o principal alvo envolvido na atividade leishmanicida de FT1 e FT2 e que as enzimas esterol 14 α -demetilase e tripanonitona redutase podem ser susceptíveis à ação inibitória desses compostos. Esses resultados apoiam a tese de que a produção de moléculas híbridas derivadas de ftalimida e 1,2,3-triazol através da *click chemistry* pode ser uma ferramenta útil na descoberta de potentes agentes leishmanicidas. O efeito anti-*Leishmania* dos compostos FT1 e FT2, descrito neste estudo, destaca o alto potencial terapêutico na produção de fármacos mais seletivos e menos tóxicos, possíveis de serem utilizados de forma isolada ou conjunta no tratamento das leishmanioses.

REFERÊNCIAS

- ABADÍAS-GRANADO, *et al.* Leishmaniasis Cutánea y Mucocutánea. **Actas Dermo-Sifiliográficas**, 2021.
- ABIDIN, B.; *et al.* Infection-adapted emergency hematopoiesis promotes visceral leishmaniasis. **PLoS Pathogens**, v. 13, n. 8, p. e1006422, 2017.
- ABRAÃO, L.; *et al.* Perfil epidemiológico dos casos de leishmaniose tegumentar americana no estado do Pará, Brasil, entre 2008 e 2017. **Revista Pan-Amazônica de Saúde**, v. 11, 2020.
- ABUZOID, A. A. *et al.* Visceral leishmaniasis in Saudi Arabia: From hundreds of cases to zero. **Acta Tropica**, p. 105707, 2020.
- AFONSO, L. *et al.* Interactions with apoptotic but not with necrotic neutrophils increase parasite burden in human macrophages infected with *Leishmania amazonensis*. **Journal of Leukocyte Biology**, v. 84, n. 2, p. 389-396, 2008.
- AGALAVE, S. G.; M., S. R.; PORE, V. S. Click chemistry: 1, 2, 3-triazoles as pharmacophores. **Chemistry–An Asian Journal**, v. 6, n. 10, p. 2696-2718, 2011.
- AGUDELO, S.; RESTREPO, S.; VÉLEZ, I. D. Cutaneous New World leishmaniasis-sporotrichosis coinfection: report of 3 cases. **Journal of the American Academy of Dermatology**, v. 40, n. 6, p. 1002-1004, 1999.
- ALIANÇA, A. S. *et al.* *In vitro* evaluation of cytotoxicity and leishmanicidal activity of phthalimido-thiazole derivatives. **European Journal of Pharmaceutical Sciences**, v. 105, p. 1-10, 2017.
- AL-SALEM, W.; HERRICKS, J. R.; HOTEZ, P. J. A review of visceral leishmaniasis during the conflict in South Sudan and the consequences for East African countries. **Parasites & Vectors**, v. 9, n. 1, p. 1-11, 2016.
- ALMEIDA-SOUZA, F. *et al.* 1, 4-Disubstituted-1, 2, 3-Triazole compounds induce ultrastructural alterations in *Leishmania amazonensis* promastigote: An *in vitro* antileishmanial and *in silico* pharmacokinetic study. **International Journal of Molecular Sciences**, v. 21, n. 18, p. 6839, 2020.
- ALVAR, J. *et al.* Leishmaniasis worldwide and global estimates of its incidence. **PloS One**, v. 7, n. 5, p. e35671, 2012.
- ALY, M. R.; SAAD, H. A.; MOHAMED, M. Click reaction-based synthesis, antimicrobial, and cytotoxic activities of new 1, 2, 3-triazoles. **Bioorganic & Medicinal Chemistry Letters**, v. 25, n. 14, p. 2824-2830, 2015.
- ANDERSEN, E. M. *et al.* **Comparison of meglumine antimoniate and pentamidine for Peruvian cutaneous leishmaniasis.** NAVY ENVIRONMENTAL AND PREVENTIVE MEDICINE UNIT NO. 5 SAN DIEGO CA, 2005.

ANDRIAMIFIDY, R. *et al.* Do we know how mosquito disease vectors will respond to climate change? **Emerging Topics in Life Sciences**, v. 3, n. 2, p. 115-132, 2019.

ANTINORI, S. *et al.* Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. **Clinical Infectious Diseases**, v. 44, n. 12, p. 1602-1610, 2007.

ANTONIO, L. *et al.* Montenegro skin test and age of skin lesion as predictors of treatment failure in cutaneous leishmaniasis. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 56, p. 375-380, 2014.

ARAUJO FILHO, N. A. Leishmaniose Tegumentar Americana e o desmatamento da Amazônia. **Acta Amazônica**, v. 11, n. 1, p. 187-189, 1981.

ARAÚJO, I. *et al.* Efficacy of lapachol on treatment of cutaneous and visceral leishmaniasis. **Experimental parasitology**, v. 199, p. 67-73, 2019.

ARAÚJO, F. F. *et al.* Chemokines in Leishmaniasis: Map of cell movements highlights the landscape of infection and pathogenesis. **Cytokine**, p. 155339, 2020.

AREVALO, I. *et al.* Role of imiquimod and parenteral meglumine antimoniate in the initial treatment of cutaneous leishmaniasis. **Clinical Infectious Diseases**, v. 44, n. 12, p. 1549-1554, 2007.

ARIF, R. *et al.* Synthesis, molecular docking and DNA binding studies of phthalimide-based copper (II) complex: in vitro antibacterial, hemolytic and antioxidant assessment. **Journal of Molecular Structure**, v. 1160, p. 142-153, 2018.

ARORA, P. *et al.* Importance of heterocyclic chemistry: a review. **International Journal of Pharmaceutical Sciences and Research**, v. 3, n. 9, p. 2947, 2012.

ASSIS, S. P.; ARAÚJO, T. G.; SENA, V. L. M.; CATANHO, M. T.; RAMOS, M. N.; SRIVASTAVA, R.; LIMA, V. L. M. Synthesis, hypolipidemic, and anti-inflammatory activities of arylphthalimides. **Medicinal Chemistry Research**, v. 23, n. 2, p. 708-716, 2014.

ASSIS, S. P.; SILVA, M. T.; OLIVEIRA, R. N.; LIMA, V. L. M. Synthesis and anti-inflammatory activity of new alkyl-substituted phthalimide 1H-1, 2, 3-triazole derivatives. **The Scientific World Journal**, v. 2012, 2012.

ATO, M.; STÄGER, S.; ENGWERDA, C.; KAYE, P. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. **Nature immunology**, v. 3, n. 12, p. 1185-1191, 2002.

AU, W. Y.; MA, E. S.; KWONG, Y. L. Intravenous pentamidine induced megaloblastic anaemia. **Haematologica**, v. 87, n. 1, p. ECR06-ECR06, 2002.

AZIM, M. *et al.* Therapeutic advances in the topical treatment of cutaneous leishmaniasis: A review. **PLoS Neglected Tropical Diseases**, v. 15, n. 3, p. e0009099, 2021.

BABALOO, Z.; KAYE, P. M.; ESLAMI, M. B. Interleukin-13 in Iranian patients with visceral leishmaniasis: relationship to other Th2 and Th1 cytokines. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 95, n. 1, p. 85-88, 2001.

BACELLAR, O. *et al.* Up-regulation of Th1-type responses in mucosal leishmaniasis patients. **Infection and Immunity**, v. 70, n. 12, p. 6734-6740, 2002.

BALASEGARAM, M. *et al.* Liposomal amphotericin B as a treatment for human leishmaniasis. **Expert Opinion on Emerging Drugs**, v. 17, n. 4, p. 493-510, 2012.

BANAROUËI, N. *et al.* N-arylmethylideneaminophthalimide: Design, Synthesis and Evaluation as Analgesic and Anti-inflammatory Agents. **Mini reviews in medicinal chemistry**, v. 19, n. 8, p. 679-687, 2019.

BANERJEE, Priyanka *et al.* ProTox-II: a webserver for the prediction of toxicity of chemicals. **Nucleic acids research**, v. 46, n. W1, p. W257-W263, 2018.

BANGERT, M. *et al.* Validation of rK39 immunochromatographic test and direct agglutination test for the diagnosis of Mediterranean visceral leishmaniasis in Spain. **PLoS Neglected Tropical Diseases**, v. 12, n. 3, p. e0006277, 2018.

BARDOSH, K. Global aspirations, local realities: the role of social science research in controlling neglected tropical diseases. **Infectious Diseases of Poverty**, v. 3, n. 1, p. 1-15, 2014.

BASANO, S. A.; CAMARGO, L. M. A. Leishmaniose tegumentar americana: histórico, epidemiologia e perspectivas de controle. **Revista Brasileira de Epidemiologia**, v. 7, p. 328-337, 2004.

BASSELIN, M. *et al.* Resistance to pentamidine in *Leishmania mexicana* involves exclusion of the drug from the mitochondrion. **Antimicrobial Agents and Chemotherapy**, v. 46, n. 12, p. 3731-3738, 2002.

BEDOYA, S. J. *et al.* Estigmatização social pela leishmaniose cutânea no estado do Rio de Janeiro, Brasil. **Revista Eletrônica de Comunicação, Informação e Inovação em Saúde**, v. 11, n. 3, 2017.

BENCHIMOL, J. **Medicina e Ambiente: articulação e desafios no passado, presente e futuro**. 1. 2019. ISBN: 978-85-8054-399-5.

BENCHIMOL, J. L. *et al.* Leishmanioses: sua configuração histórica no Brasil com ênfase na doença visceral nos anos 1930 a 1960. **Boletim do Museu Paraense Emílio Goeldi. Ciências Humanas**, v. 14, p. 611-626, 2019.

BENNIS, I. *et al.* Accuracy of a rapid diagnostic test based on antigen detection for the diagnosis of cutaneous leishmaniasis in patients with suggestive skin lesions in

Morocco. **The American Journal of Tropical Medicine and Hygiene**, v. 99, n. 3, p. 716-722, 2018.

BHAT, M. A. *et al.* Biginelli Synthesis of Novel Dihydropyrimidinone Derivatives Containing Phthalimide Moiety. **Journal of Chemistry**, v. 2020, 2020.

BI, K. *et al.* Current visceral leishmaniasis research: a research review to inspire future study. **BioMed Research International**, v. 2018, 2018.

BORBÓN, T. Y. *et al.* Coinfection with *Leishmania major* and *Staphylococcus aureus* enhances the pathologic responses to both microbes through a pathway involving IL-17A. **PLoS Neglected Tropical Diseases**, v. 13, n. 5, p. e0007247, 2019.

BORGES, S. L. C. *et al.* A Percepção de uma Comunidade Quilombola a Respeito de Leishmaniose Visceral, em Mato Grosso do Sul e a Educação em Saúde como Ferramenta de Controle Desta Endemia. **Revista de Ensino, Educação e Ciências Humanas**, v. 21, n. 2, p. 191-199, 2020.

BOZOROV, K.; ZHAO, J.; AISA, H. A. 1, 2, 3-Triazole-containing hybrids as leads in medicinal chemistry: A recent overview. **Bioorganic & Medicinal Chemistry**, v. 27, n. 16, p. 3511-3531, 2019.

BRACAMONTE, M. E. *et al.* High performance of an enzyme linked immunosorbent assay for American tegumentary leishmaniasis diagnosis with *Leishmania (Viannia) braziliensis* amastigotes membrane crude antigens. **PloS One**, v. 15, n. 5, p. e0232829, 2020.

BRAGA, S. S. Multi-target drugs active against leishmaniasis: A paradigm of drug repurposing. **European Journal of Medicinal Chemistry**, v. 183, p. 111660, 2019. ISSN 0223-5234.

BRASIL. **Decreto Nº 51.838, de 14 de março de 1963. Baixa Normas Técnicas Especiais para o Combate às Leishmanioses.**

_____. **Portaria Interministerial Nº 1.426, de 11 de julho de 2008. Proíbe o tratamento de leishmaniose visceral canina com produtos de uso humano ou não registrados no Ministério da Agricultura, Pecuária e Abastecimento.** SAÚDE, M. D. 2008.

_____. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. **Sistema de Informação de Agravos de Notificação - SINAN.** Brasília: Editora do Ministério da Saúde, 2021.

_____. **Portaria Interministerial nº 1.426, de 11 de jul. de 2008.** Cães com leishmaniose. Proibição do tratamento de leishmaniose visceral canina com produtos de uso humano ou não registrados no Ministério da Agricultura, Pecuária e Abastecimento. ANVISA, p. 1-2, jul. 2008.

_____. **NOTA INFORMATIVA Nº 13/2020.** Coordenação-Geral de Vigilância de Zoonoses e Doenças de Transmissão Vetorial. Departamento de Imunização e

Doenças Transmissíveis, Secretaria de Vigilância em Saúde/Ministério da Saúde. 2020.

_____. **Boletim Epidemiológico - Doenças Tropicais Negligenciadas**. Secretaria de Vigilância em Saúde. Ministério da Saúde. 2021.

_____. **Guia de Vigilância em Saúde**. Secretaria de Vigilância em Saúde. Ministério da Saúde. 2017a.

_____. **Manual de Vigilância da Leishmaniose Tegumentar**: Secretaria de Vigilância em Saúde/Ministério da Saúde Brasília, 2017b.

BRAY, P. G. *et al.* Pentamidine uptake and resistance in pathogenic protozoa: past, present and future. **Trends in Parasitology**, v. 19, n. 5, p. 232-239, 2003.

BRIANTI, E. *et al.* Efficacy of a slow-release imidacloprid (10%)/flumethrin (4.5%) collar for the prevention of canine leishmaniosis. **Parasites & Vectors**, v. 7, n. 1, p. 1-10, 2014.

BUTCHER, G.; COMER, J.; AVDEEF, A. pKa-critical interpretations of solubility–pH profiles: PG-300995 and NSC-639829 case studies. **ADMET and DMPK**, v. 3, n. 2, p. 131-140, 2015.

CALVOPINA, M. *et al.* Leishmaniasis recidiva cutis due to *Leishmania (Viannia) panamensis* in subtropical Ecuador: isoenzymatic characterization. **International Journal of Dermatology**, v. 45, n. 2, p. 116-120, 2006.

CAMPO, V. L. *et al.* Design, synthesis and the effect of 1, 2, 3-triazole sialylmimetic neoglycoconjugates on *Trypanosoma cruzi* and its cell surface trans-sialidase. **Bioorganic & Medicinal Chemistry**, v. 20, n. 1, p. 145-156, 2012.

CAPELLA, D.; CHO, M.; LIMA, R. D. S. A segurança do paciente e a qualidade em serviços de saúde no contexto da América Latina e Caribe. **Brasil Ministério da Saúde. Agência Nacional de Vigilância Sanitária. Assistência segura: uma reflexão teórica aplicada à prática**. Brasília, p. 13-7, 2013.

CARMO, E. Leishmaniose Visceral no Brasil: situação atual, principais aspectos epidemiológicos, clínicos e medidas de controle. **Revista-Sociedade Brasileira de Medicina Tropical**, v. 35, p. 41-41, 2003.

CARNIELLI, J. B. *et al.* Natural resistance of *Leishmania infantum* to miltefosine contributes to the low efficacy in the treatment of visceral leishmaniasis in Brazil. **The American journal of tropical medicine and hygiene**, v. 101, n. 4, p. 789-794, 2019.

CARVALHO, S. F. G. *et al.* Should an intersection between visceral leishmaniasis endemicity and the COVID-19 pandemic be considered? **Medical Hypotheses**, v. 144, p. 110289, 2020/11/01/ 2020.

CASTELLANO, L. R. *et al.* Th1/Th2 immune responses are associated with active cutaneous leishmaniasis and clinical cure is associated with strong interferon- γ production. **Human Immunology**, v. 70, n. 6, p. 383-390, 2009.

CERUTTI, P. H. P. *et al.* Métodos diagnósticos da leishmaniose tegumentar americana: uma revisão de literatura. **Revista de Patologia do Tocantins**, v. 4, n. 4, p. 55-59, 2017.

CHAKRABORTY, A. K.; MAJUMDER, H. K. Mode of action of pentavalent antimonials: Specific inhibition of type I DNA topoisomerase of *Leishmania donovani*. **Biochemical and Biophysical Research Communications**, v. 152, n. 2, p. 605-611, 1988/04/29/ 1988.

CHARMOY, M. *et al.* Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of *Leishmania major* inoculation in resistant mice. **PLoS Pathog**, v. 6, n. 2, p. e1000755, 2010.

CHATTOPADHYAY, A.; JAFURULLA, M. A novel mechanism for an old drug: Amphotericin B in the treatment of visceral leishmaniasis. **Biochemical and Biophysical Research Communications**, v. 416, n. 1, p. 7-12, 2011/12/09/ 2011.

CHEN, Y. *et al.* Bioactive-guided structural optimization of 1, 2, 3-triazole phenylhydrazones as potential fungicides against *Fusarium graminearum*. **Pesticide biochemistry and physiology**, v. 164, p. 26-32, 2020.

CHRISTENSEN, S. M. *et al.* Host and parasite responses in human diffuse cutaneous leishmaniasis caused by *L. amazonensis*. **PLoS Neglected Tropical Diseases**, v. 13, n. 3, p. e0007152, 2019.

CLABORN, D. M. The biology and control of leishmaniasis vectors. **Journal of global Infectious Diseases**, v. 2, n. 2, p. 127, 2010.

CLEMONS, K. V.; STEVANS, D. A. Efficacies of amphotericin B lipid complex (ABLC) and conventional amphotericin B against marine coccidioidomycosis. **Journal of Antimicrobial Chemotherapy**, v. 30, n. 3, p. 353-363, 1992.

COMANDOLLI-WYREPKOWSKI, C. D. *et al.* Topical treatment of experimental cutaneous leishmaniasis in golden hamster (*Mesocricetus auratus*) with formulations containing pentamidine. **Acta Amazonica**, v. 47, n. 1, p. 39-46, 2017.

CONCEIÇÃO, J. *et al.* Characterization of neutrophil function in human cutaneous leishmaniasis caused by *Leishmania braziliensis*. **PLoS Neglected Tropical Diseases**, v. 10, n. 5, p. e0004715, 2016. ISSN 1935-2735.

COSER, E. M. *et al.* Activity of paromomycin against *Leishmania amazonensis*: Direct correlation between susceptibility *in vitro* and the treatment outcome *in vivo*. **International Journal for Parasitology: Drugs and Drug Resistance**, v. 14, p. 91-98, 2020.

COSTA, D. N. C. C. *et al.* Leishmaniose visceral em humanos e relação com medidas de controle vetorial e canino. **Revista de Saúde Pública**, v. 52, 2018.

COSTA, J. M. L. Leishmaniose tegumentar americana: origens e histórico no Brasil. **Acta Amazônica**, v. 22, n. 1, p. 71-77, 1992.

COSTA, J. M. L. *et al.* Leishmaniose cutânea difusa (LCD) no Brasil após 60 anos de sua primeira descrição. **Arca Fiocruz**, 2009.

COSTA, J. M. L. *et al.* Aspectos psicossociais e estigmatizantes da leishmaniose cutâneo-mucosa. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 20, n. 2, p. 77-82, 1987.

CROFT, S. *et al.* The activity of alkyl phosphorylcholines and related derivatives against *Leishmania donovani*. **Biochemical Pharmacology**, v. 36, n. 16, p. 2633-2636, 1987. ISSN 0006-2952.

CROFT, S. L.; SUNDAR, S.; FAIRLAMB, A. H. Drug Resistance in Leishmaniasis. **Clinical Microbiology Reviews**, v. 19, n. 1, p. 111-126, 2006.

ROCHA, G. P.; PETRONI, T. F. Leishmaniose Visceral e Tegumentar. **Revista Saúde UniToledo**, v. 1, n. 2, 2017.

CAVALCANTE, L. R. *et al.* Diagnóstico diferencial para infecção cutânea crônica em região endêmica de leishmaniose. **Brazilian Journal of Health Review**, v. 2, n. 2, p. 888-894, 2019.

DAI, X. *et al.* Design, Synthesis, and *In Vitro/In Vivo* Anti-Cancer Activities of Novel (20S)-10, 11-Methylenedioxy-Camptothecin Heterocyclic Derivatives. **International Journal of Molecular Sciences**, v. 21, n. 22, p. 8495, 2020.

DANESHBOD, Y. *et al.* Clinical, histopathologic, and cytologic diagnosis of mucosal leishmaniasis and literature review. **Archives of Pathology & Laboratory Medicine**, v. 135, n. 4, p. 478-482, 2011.

DAVIDSON, R. N.; DEN BOER, M.; RITMEIJER, K. Paromomycin. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 103, n. 7, p. 653-660, 2009/07/01/ 2009.

DAYAKAR, A. *et al.* Cytokines: key determinants of resistance or disease progression in visceral leishmaniasis: opportunities for novel diagnostics and immunotherapy. **Frontiers in Immunology**, v. 10, p. 670, 2019.

DEANE, L. D. M. Leishmaniose visceral no Brasil: estudos sobre reservatórios e transmissores realizados no Estado do Ceará. In: (Ed.). **Leishmaniose visceral no Brasil: estudos sobre reservatórios e transmissores realizados no estado do Ceará**, 1956.

DELGADO, J. *et al.* High frequency of serious side effects from meglumine antimoniate given without an upper limit dose for the treatment of visceral

leishmaniasis in human immunodeficiency virus type-1-infected patients. **The American Journal of Tropical Medicine and Hygiene**, v. 61, n. 5, p. 766-769, 1999.

DEMICHELI, C. *et al.* Antimony(V) complex formation with adenine nucleosides in aqueous solution. **Biochimica et Biophysica Acta (BBA) - General Subjects**, v. 1570, n. 3, p. 192-198, 2002.

DENTON, H.; MCGREGOR, JOANNE C.; COOMBS, GRAHAM H. Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1. **Biochemical Journal**, v. 381, n. 2, p. 405-412, 2004.

DEPS, P. D. *et al.* Avaliação comparativa da eficácia e toxicidade do antimoniato de N-metil-glucamina e do Estibogluconato de Sódio BP88® no tratamento da leishmaniose cutânea localizada. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 33, n. 6, p. 535-543, 2000.

DESJEUX, P. Leishmaniasis: public health aspects and control. **Clinics in Dermatology**, v. 14, n. 5, p. 417-423, 1996.

DHEER, D.; SINGH, V.; SHANKAR, R. Medicinal attributes of 1,2,3-triazoles: Current developments. **Bioorganic Chemistry**, v. 71, p. 30-54, 2017.

DI-CRISTINA, G.; CARONIA, G. Sulla terapia della leishmaniosi interna. **Pathologica**, v. 7, p. 82-83, 1915.

DI-LELLA, F. *et al.* Mucocutaneous leishmaniasis: report of a case with massive involvement of nasal, pharyngeal and laryngeal mucosa. **International Journal of Oral and Maxillofacial Surgery**, v. 35, n. 9, p. 870-872, 2006.

DORLO, T. P. *et al.* Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. **Journal of Antimicrobial Chemotherapy**, v. 67, n. 11, p. 2576-2597, 2012.

DUQUE, G. A. *et al.* The host cell secretory pathway mediates the export of *Leishmania* virulence factors out of the parasitophorous vacuole. **PLoS Pathogens**, v. 15, n. 7, p. e1007982, 2019.

EHRENBERG, J. P. *et al.* Strategies supporting the prevention and control of neglected tropical diseases during and beyond the COVID-19 pandemic. **Infectious Diseases of Poverty**, v. 9, n. 1, p. 1-7, 2020.

EL-SAGHEER, A. H.; BROWN, T. Click nucleic acid ligation: applications in biology and nanotechnology. **Accounts of Chemical Research**, v. 45, n. 8, p. 1258-1267, 2012.

EL AZAB, I. H. *et al.* New 1, 2, 3-Triazole-Containing Hybrids as Antitumor Candidates: Design, Click Reaction Synthesis, DFT Calculations, and Molecular Docking Study. **Molecules**, v. 26, n. 3, p. 708, 2021.

ENGWERDA, C. R.; ATO, M.; KAYE, P. M. Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. **Trends in Parasitology**, v. 20, n. 11, p. 524-530, 2004.

ERASMUS, C. et al. Synthesis and comparison of in vitro dual anti-infective activities of novel Naphthoquinone hybrids and Atovaquone. **Bioorganic Chemistry**, p. 105118, 2021. ISSN 0045-2068.

ESTUMANO, J. C.; SÁ, L. L.; MACÊDO, C. G. Leishmaniose tegumentar americana: Análise epidemiológica de uma década no interior da Amazônia, Brasil. **Brazilian Journal of Development**, v. 6, n. 6, p. 36311-36325, 2020.

FALCI, D. R.; PASQUALOTTO, A. C. Anfotericina B: uma revisão sobre suas diferentes formulações, efeitos adversos e toxicidade. **Clinical & Biomedical Research**, v. 35, n. 2, 2015.

FÉLIX, I. L. C. et al. Leishmaniose cutânea difusa em membro inferior: relato de caso. **Anais do Seminário Científico do UNIFACIG**, n. 4, 2019.

FERNANDES, F. S. et al. Discovery of highly potent and selective antiparasitic new oxadiazole and hydroxy-oxindole small molecule hybrids. **European Journal of Medicinal Chemistry**, v. 201, p. 112418, 2020.

FERNÁNDEZ, O. L. et al. Miltefosine and antimonial drug susceptibility of *Leishmania Viannia* species and populations in regions of high transmission in Colombia. **PLoS Neglected Tropical Diseases**, v. 8, n. 5, p. e2871, 2014. ISSN 1935-2735.

FERREIRA, C. D. S. et al. Thiol-induced reduction of antimony (V) into antimony (III): a comparative study with trypanothione, cysteinyl-glycine, cysteine and glutathione. **Biometals**, v. 16, n. 3, p. 441-446, 2003.

FICHTALI, I. et al. Synthesis, characterization and antimicrobial activity of novel benzophenone derived 1, 2, 3-triazoles. **Journal of Materials and Environmental Science**, v. 7, n. 5, p. 1633-1641, 2016.

FIDALGO, L. M.; GILLE, L. Mitochondria and trypanosomatids: targets and drugs. **Pharmaceutical Research**, v. 28, n. 11, p. 2758-2770, 2011.

FILIPPIN, F. B.; SOUZA, L. C. Eficiência terapêutica das formulações lipídicas de anfotericina B. **Revista Brasileira de Ciências Farmacêuticas**, v. 42, n. 2, p. 167-194, 2006.

FRANC, M. et al. Efficacy of dinotefuran, permethrin and pyriproxyfen combination spot-on against *Aedes aegypti* mosquitoes on dogs. **Veterinary Parasitology**, v. 189, n. 2-4, p. 333-337, 2012.

FRANC, M. et al. Efficacy of a new combination of fipronil and permethrin (Effitix®) against *Phlebotomus perniciosus* in dogs. **Veterinary Parasitology**, v. 212, n. 3-4, p. 156-160, 2015.

FRÉZARD, F.; DEMICHELI, C.; RIBEIRO, R. R. Pentavalent antimonials: new perspectives for old drugs. **Molecules**, v. 14, n. 7, p. 2317-2336, 2009.

GALLIS, H. A.; DREW, R. H.; PICKARD, W. W. Amphotericin B: 30 years of clinical experience. **Reviews of Infectious Diseases**, v. 12, n. 2, p. 308-329, 1990.

GARCIA, L. P. *et al.* **Epidemiologia das doenças negligenciadas no Brasil e gastos federais com medicamentos**. ECONSTOR. 2011

GE, R.; SUN, H. Bioinorganic Chemistry of Bismuth and Antimony: Target Sites of Metallodrugs. **Accounts of Chemical Research**, v. 40, n. 4, p. 267-274, 2007.

GILBERT, I. H. Drug Discovery for Neglected Diseases: Molecular Target-Based and Phenotypic Approaches. **Journal of Medicinal Chemistry**, v. 56, n. 20, p. 7719-7726, 2013.

GIUDICE, A. *et al.* Macrophages participate in host protection and the disease pathology associated with *Leishmania braziliensis* infection. **BMC infectious diseases**, v. 12, n. 1, p. 1-9, 2012.

GLANZMANN, N. *et al.* Synthesis and biological activity of novel 4-aminoquinoline/1,2,3-triazole hybrids against *Leishmania amazonensis*. **Biomedicine & Pharmacotherapy**, v. 141, p. 111857, 2021.

GOA, K. L.; CAMPOLI-RICHARDS, D. M. Pentamidine isethionate. **Drugs**, v. 33, n. 3, p. 242-258, 1987.

GOMTSYAN, A. Heterocycles in drugs and drug discovery. **Chemistry of Heterocyclic Compounds**, v. 48, n. 1, p. 7-10, 2012.

GONTIJO, B.; CARVALHO, M. D. L. R. D. Leishmaniose tegumentar americana. **Revista da Sociedade Brasileira de Medicina Tropical**, p. 71-80, 2003.

GONTIJO, C. M. F.; MELO, M. N. Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. **Revista Brasileira de Epidemiologia**, v. 7, n. 3, p. 338-349, 2004.

GONTIJO, V. S. *et al.* Leishmanicidal, antiproteolytic, and mutagenic evaluation of alkyltriazoles and alkylphosphocholines. **European Journal of Medicinal Chemistry**, v. 101, p. 24-33, 2015.

GONZÁLEZ, G. M. *et al.* Efficacies of amphotericin B (AMB) lipid complex, AMB colloidal dispersion, liposomal AMB, and conventional AMB in treatment of murine coccidioidomycosis. **Antimicrobial Agents and Chemotherapy**, v. 48, n. 6, p. 2140-2143, 2004.

GUIMARÃES, T. T. *et al.* Potent naphthoquinones against antimony-sensitive and -resistant *Leishmania* parasites: Synthesis of novel α - and nor- α -lapachone-based

1,2,3-triazoles by copper-catalyzed azide–alkyne cycloaddition. **European Journal of Medicinal Chemistry**, v. 63, p. 523-530, 2013.

HANDLER, M. Z. *et al.* Cutaneous and mucocutaneous leishmaniasis: Clinical perspectives. **Journal of the American Academy of Dermatology**, v. 73, n. 6, p. 897-908, 2015.

HARGROVE, T. Y. *et al.* Substrate preferences and catalytic parameters determined by structural characteristics of sterol 14 α -demethylase (CYP51) from *Leishmania infantum*. **Journal of Biological Chemistry**, v. 286, n. 30, p. 26838-26848, 2011.

HENKE, O. *et al.* **Skin maculae, chronic diarrhea, cachexia, and splenomegaly—Late presentation of the first autochthonous case of visceral leishmaniasis in Tanzania**: Public Library of Science San Francisco, CA USA 2021.

HENN, G. A. D. L. *et al.* Is Visceral Leishmaniasis the same in HIV-coinfected adults? **Brazilian Journal of Infectious Diseases**, v. 22, p. 92-98, 2018.

HOLANDA, V. N. One step before the workbench: the use of cheminformatics tools in drug discovery. **Revista Interfaces: Saúde, Humanas e Tecnologia**, v. 9, n. 2, p. 995-996, 2021.

HOLANDA, V. N. *et al.* Desafios e perspectivas no tratamento da Leishmaniose Tegumentar: revisão de literatura. **Revista Interfaces: Saúde, Humanas e Tecnologia**, v. 6, n. 17, p. 140-157, 2019.

HOLANDA, V. N. *et al.* Antileishmanial activity of 4-phenyl-1-[2-(phthalimido-2-yl) ethyl]-1H-1, 2, 3-triazole (PT4) derivative on *Leishmania amazonensis* and *Leishmania braziliensis*: In silico ADMET, *in vitro* activity, docking and molecular dynamic simulations. **Bioorganic Chemistry**, v. 105, p. 104437, 2020.

HOLANDA, V. N. *et al.* Identification of 1, 2, 3-triazole-phthalimide derivatives as potential drugs against COVID-19: a virtual screening, docking and molecular dynamic study. **Journal of Biomolecular Structure and Dynamics**, p. 1-19, 2021.

HOTEZ, P. J. Ten failings in global neglected tropical diseases control. **PLOS Neglected Tropical Diseases**, v. 11, n. 12, p. e0005896, 2017.

HOTEZ, P. J. *et al.* What constitutes a neglected tropical disease? **PLOS Neglected Tropical Diseases**, v. 14, n. 1, p. e0008001, 2020.

HOU, J. *et al.* The impact of click chemistry in medicinal chemistry. **Expert Opinion on Drug Discovery**, v. 7, n. 6, p. 489-501, 2012.

IHMAID, S. K. *et al.* Design of molecular hybrids of phthalimide-triazole agents with potent selective MCF-7/HepG2 cytotoxicity: Synthesis, EGFR inhibitory effect, and metabolic stability. **Bioorganic Chemistry**, v. 111, p. 104835, 2021.

JEAN-MORENO, V. *et al.* *Leishmania donovani*: Differential activities of classical topoisomerase inhibitors and antileishmanials against parasite and host cells at the

level of DNA topoisomerase I and in cytotoxicity assays. **Experimental Parasitology**, v. 112, n. 1, p. 21-30, 2006.

KANEZAKI, R. M. *et al.* Leishmaniose mucocutânea diagnosticada através de lesões em mucosa oral. **71ª Reunião Anual da SBPC**, 2019.

KARUNAWEEERA, N. D.; FERREIRA, M. U. Leishmaniasis: current challenges and prospects for elimination with special focus on the South Asian region. **Parasitology**, v. 145, n. 4, p. 425-429, 2018.

KASSARDJIAN, A. A. *et al.* Diffuse Cutaneous Leishmaniasis and HIV Co-infection: A Case Report and Review of the Literature. **Journal of Cutaneous Pathology**, 2021.

KAYE, P. M.; BEATTIE, L. Lessons from other diseases: granulomatous inflammation in leishmaniasis. **Seminars in Immunopathology**, Springer. p.249-260. 2016.

KEVRIC, I.; CAPPEL, M. A.; KEELING, J. H. New world and old world *Leishmania* infections: a practical review. **Dermatologic Clinics**, v. 33, n. 3, p. 579-593, 2015.

KILLICK-KENDRICK, R. *et al.* Protection of dogs from bites of phlebotomine sandflies by deltamethrin collars for control of canine leishmaniasis. **Medical and Veterinary Entomology**, v. 11, n. 2, p. 105-111, 1997.

KROLEWIECKI, A. *et al.* A randomized clinical trial comparing oral azithromycin and meglumine antimoniate for the treatment of American cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis*. **The American Journal of Tropical Medicine and Hygiene**, v. 77 4, p. 640-6, 2007.

KUMAR, S.; NARASIMHAN, B. Therapeutic potential of heterocyclic pyrimidine scaffolds. **Chemistry Central Journal**, v. 12, n. 1, p. 1-29, 2018.

LAGUARDIA, J. *et al.* Sistema de informação de agravos de notificação em saúde (SINAN): desafios no desenvolvimento de um sistema de informação em saúde. **Epidemiologia e Serviços de Saúde**, v. 13, n. 3, p. 135-146, 2004.

LANDMANN-SZWARCWALD, C.; MACINKO, J. A panorama of health inequalities in Brazil. **International Journal for Equity in Health**, v. 15, n. 1, p. 1-3, 2016.

LANIADO-LABORÍN, R.; CABRALES-VARGAS, M. N. Amphotericin B: side effects and toxicity. **Revista Iberoamericana de Micología**, v. 26, n. 4, p. 223-227, 2009.

LASKOWSKI, R.; MACARTHUR, M.; THORNTON, J. PROCHECK: validation of protein-structure coordinates. **International Tables for Crystallography**, v. F, ch. 25.2, pp. 722-725, 2006.

LEITE, C. E. A.; VIDAL, S. A.; DE QUENTAL, O. B. Análise espacial dos casos humanos de leishmaniose visceral no Estado de Pernambuco, Brasil. **Brazilian Journal of Production Engineering**, p. 149-157, 2020.

LI, H.; ANEJA, R.; CHAIKEN, I. Click chemistry in peptide-based drug design. **Molecules**, v. 18, n. 8, p. 9797-9817, 2013.

LIMA, M. V. N. D. *et al.* Leishmaniose cutânea com desfecho fatal durante tratamento com antimonial pentavalente. **Anais Brasileiros de Dermatologia**, v. 82, n. 3, p. 269-271, 2007.

LINDOSO, J. A. L.; LINDOSO, A. A. B. Neglected tropical diseases in Brazil. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 51, n. 5, p. 247-253, 2009.

LIPINSKI, C. A. Lead-and drug-like compounds: the rule-of-five revolution. **Drug discovery today: Technologies**, v. 1, n. 4, p. 337-341, 2004.

LIU, N.-N. *et al.* Covid-19 pandemic: Experiences in china and implications for its prevention and treatment worldwide. **Current Cancer Drug Targets**, v. 20, n. 6, p. 410-416, 2020.

LÓPEZ-CAMACHO, E. *et al.* Solving molecular flexible docking problems with metaheuristics: A comparative study. **Applied Soft Computing**, v. 28, p. 379-393, 2015.

LORÍA-CERVERA, E. N.; ANDRADE-NARVAEZ, F. The role of monocytes/macrophages in *Leishmania* infection: a glance at the human response. **Acta Tropica**, v. 207, p. 105456, 2020.

LUCUMI, A. *et al.* Sensitivity of *Leishmania viannia panamensis* to pentavalent antimony is correlated with the formation of cleavable DNA-protein complexes. **Antimicrobial Agents and Chemotherapy**, v. 42, n. 8, p. 1990-1995, 1998.

MACHADO, C. A. L. *et al.* Spatial analysis and epidemiological profile of visceral leishmaniasis, northeastern Brazil: A cross-sectional study. **Acta Tropica**, v. 208, p. 105520, 2020.

MACHADO, C. J. S.; SILVA, E. G.; VILANI, R. M. O uso de um instrumento de política de saúde pública controverso: a eutanásia de cães contaminados por leishmaniose no Brasil. **Saúde e Sociedade**, v. 25, p. 247-258, 2016.

MACRAE, C. F. *et al.* Mercury CSD 2.0—new features for the visualization and investigation of crystal structures. **Journal of Applied Crystallography**, v. 41, n. 2, p. 466-470, 2008.

MANTRAVADI, P. K.; PARTHASARATHY, A.; KALESH, K. Antileishmanial Drug Development: A Review of Modern Molecular Chemical Tools and Research Strategies. **Current Medicinal Chemistry**, 2020.

LOURDES, M. G. F. *et al.* Design, synthesis, and antiviral activity of new 1 H-1, 2, 3-triazole nucleoside ribavirin analogs. **Medicinal Chemistry Research**, v. 23, n. 3, p. 1501-1511, 2014.

BOSCH, M. *et al.* Neurological toxicity due to antimonial treatment for refractory visceral leishmaniasis. **Clinical Neurophysiology Practice**, v. 6, p. 164-167, 2021.

MARQUES, S. A. *et al.* American tegumentary leishmaniasis: severe side effects of pentavalent antimonial in a patient with chronic renal failure. **Anais Brasileiros de Dermatologia**, v. 94, n. 3, p. 355-357, 2019.

MÁRQUEZ, M. *et al.* *Leishmania* amastigotes in the central nervous system of a naturally infected dog. **Journal of Veterinary Diagnostic Investigation**, v. 25, n. 1, p. 142-146, 2013.

MARRA, F.; CHIAPPETTA, M. C.; VINCENTI, V. Ear, nose and throat manifestations of mucocutaneous Leishmaniasis: a literature review. **Acta Biomed**, v. 85, n. 1, p. 3-7, 2014.

MARSDEN, P. D. Pentavalent antimonials: old drugs for new diseases. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 18, n. 3, p. 187-198, 1985.

MASPI, N.; ABDOLI, A.; GHAFARIFAR, F. Pro- and anti-inflammatory cytokines in cutaneous leishmaniasis: a review. **Pathogens and Global Health**, v. 110, n. 6, p. 247-260, 2016.

MAURYA, R. *et al.* Evaluation of blood agar microtiter plates for culturing leishmania parasites to titrate parasite burden in spleen and peripheral blood of patients with visceral leishmaniasis. **Journal of clinical microbiology**, v. 48, n. 5, p. 1932, 2010.

MEINEL, R. S. *et al.* Novel functionalized 1,2,3-triazole derivatives exhibit antileishmanial activity, increase in total and mitochondrial-ROS and depolarization of mitochondrial membrane potential of *Leishmania amazonensis*. **Chemico-Biological Interactions**, v. 315, p. 108850, 2020/01/05/ 2020.

MEIRELES, C. B. *et al.* Atypical presentations of cutaneous leishmaniasis: a systematic review. **Acta Tropica**, v. 172, p. 240-254, 2017.

MENDGEN, T.; STEUER, C.; KLEIN, C. D. Privileged scaffolds or promiscuous binders: a comparative study on rhodanines and related heterocycles in medicinal chemistry. **Journal of Medicinal Chemistry**, v. 55, n. 2, p. 743-753, 2012.

MENU, E. *et al.* Immunoblot for the Diagnosis of Cutaneous Leishmaniasis in French Guiana. **The American Journal of Tropical Medicine and Hygiene**, v. 1, n. A p, 2021.

MERMER, A.; KELES, T.; SIRIN, Y. Recent studies of nitrogen containing heterocyclic compounds as novel antiviral agents: A review. **Bioorganic Chemistry**, v. 114, p. 105076, 2021.

MESA-ARANGO, A. C.; SCORZONI, L.; ZARAGOZA, O. It only takes one to do many jobs: Amphotericin B as antifungal and immunomodulatory drug. **Frontiers in microbiology**, v. 3, p. 286, 2012.

MIOTTI, A. *et al.* COVID-19 in a Patient with Visceral Leishmaniasis. **J Infect Dis Ther**, v. 8, p. 430, 2020.

MIRZAEI, A. *et al.* A historical review of the role of cytokines involved in leishmaniasis. **Cytokine**, p. 155297, 2020.

MISHRA, J. *et al.* Evaluation of toxicity & therapeutic efficacy of a new liposomal formulation of amphotericin B in a mouse model. **The Indian Journal of Medical Research**, v. 137, n. 4, p. 767, 2013.

MNIOUIL, M. *et al.* Comparative evaluation of immunochromatographic dipstick test (ICT) rk39, soluble antigen ELISA and IFAT for the sero-diagnosis of visceral leishmaniasis in Morocco. **Acta Tropica**, v. 182, p. 185-189, 2018.

MOEN, M. D.; LYSENG-WILLIAMSON, K. A.; SCOTT, L. J. Liposomal amphotericin B. **Drugs**, v. 69, n. 3, p. 361-392, 2009.

MOHAMMED, A. S. A. *et al.* *Leishmania* lipophosphoglycan components: A potent target for synthetic neoglycoproteins as a vaccine candidate for leishmaniasis. **Carbohydrate Polymers**, v. 237, p. 116120, 2020.

MOLLINEDO, J. S. *et al.* Visceral Leishmaniasis in Bolivia: Current Status. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 53, 2020.

MOLYNEUX, D. H.; SAVIOLI, L.; ENGELS, D. Neglected tropical diseases: progress towards addressing the chronic pandemic. **The Lancet**, v. 389, n. 10066, p. 312-325, 2017.

MONZOTE, L.; GILLE, L. Mitochondria as a promising antiparasitic target. **Current Clinical Pharmacology**, v. 5, n. 1, p. 55-66, 2010.

MOSMANN, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **Journal of Immunological Methods**, v. 65, n. 1-2, p. 55-63, 1983.

MOURA, N. M. M.; TOMÉ, A. C. 1,2,3-Triazoles. In: (Ed.). **Reference Module in Chemistry, Molecular Sciences and Chemical Engineering**: Elsevier, 2021. ISBN 978-0-12-409547-2.

MUKBEL, R. M. *et al.* Macrophage killing of *Leishmania amazonensis* amastigotes requires both nitric oxide and superoxide. **The American journal of tropical medicine and hygiene**, v. 76, n. 4, p. 669-675, 2007.

MUKHOPADHYAY, R. *et al.* Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. **Proceedings of the National Academy of Sciences**, v. 93, n. 19, p. 10383-10387, 1996.

MURBACK, N. D. N. *et al.* Leishmaniose tegumentar americana: estudo clínico, epidemiológico e laboratorial realizado no Hospital Universitário de Campo Grande,

Mato Grosso do Sul, Brasil. **Anais Brasileiros de Dermatologia**, v. 86, n. 1, p. 55-63, 2011.

MWENECHANYA, R. *et al.* Sterol 14 α -demethylase mutation leads to amphotericin B resistance in *Leishmania mexicana*. **PLoS Neglected Tropical Diseases**, v. 11, n. 6, p. e0005649, 2017.

NAIR, M. *et al.* Quality of life perceptions amongst patients co-infected with Visceral Leishmaniasis and HIV: A qualitative study from Bihar, India. **PloS One**, v. 15, n. 2, p. e0227911, 2020.

NASCIMENTO, W. S.; CAMARA, C. A.; DE OLIVEIRA, R. N. Synthesis of 2-(1H-1, 2, 3-Triazol-1-yl)-1, 4-naphthoquinones from 2-Azido-1, 4-naphthoquinone and Terminal Alkynes. **Synthesis**, v. 2011, n. 20, p. 3220-3224, 2011.

NAVARRETE-DECHENT, C. *et al.* Leishmaniasis cutánea causada por *Leishmania braziliensis* y uso de anfotericina B liposomal. Comunicación de un caso clínico importado. **Revista chilena de infectología**, v. 35, p. 612-616, 2018.

NEVES, D. B. D. J.; CALDAS, E. D.; SAMPAIO, R. N. R. Antimony in plasma and skin of patients with cutaneous leishmaniasis—relationship with side effects after treatment with meglumine antimoniate. **Tropical Medicine & International Health**, v. 14, n. 12, p. 1515-1522, 2009.

NEVES, L. O. *et al.* Estudo clínico randomizado comparando antimoniato de meglumina, pentamidina e anfotericina B para o tratamento da leishmaniose cutânea ocasionada por *Leishmania guyanensis*. **Anais Brasileiros de Dermatologia**, v. 86, n. 6, p. 1092-1101, 2011.

NILFOROUSHZADEH, M. A. *et al.* A comparison of the effects of topical injection of glucantime plus glycolic acid 70% and salicylic acid 20% with topical injection of glucantime alone in the treatment of acute cutaneous leishmaniasis. **Journal of Isfahan Medical School**, v. 34, n. 416, p. 1693-1698. 2017.

NJOGU, P. M. *et al.* Computer-Aided Drug Discovery Approaches against the Tropical Infectious Diseases Malaria, Tuberculosis, Trypanosomiasis, and Leishmaniasis. **ACS Infectious Diseases**, v. 2, n. 1, p. 8-31, 2016.

NYLÉN, S.; SACKS, D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. **Trends in Immunology**, v. 28, n. 9, p. 378-384, 2007.

OLIVEIRA-NETO, M. P. *et al.* A Low-Dose Antimony Treatment in 159 Patients with American Cutaneous Leishmaniasis: Extensive Follow-up Studies (Up to 10 Years). **The American Journal of Tropical Medicine and Hygiene**, Arlington VA, USA, v. 57, n. 6, p. 651-655, 1997.

OLIVEIRA, F. *et al.* Lesion size correlates with *Leishmania* antigen-stimulated TNF-levels in human cutaneous leishmaniasis. **The American journal of tropical Medicine and Hygiene**, v. 85, n. 1, p. 70-73, 2011.

OLIVEIRA, L. F. *et al.* Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. **Acta Tropica**, v. 118, n. 2, p. 87-96, 2011.

OLIVEIRA, M. C. *et al.* Óbito em caso de leishmaniose cutâneomucosa após o uso de antimonial pentavalente. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 38, n. 3, p. 258-260, 2005.

OLIVEIRA-NETO, M. P.; MATTOS MDA, S. An alternative antimonial schedule to be used in cutaneous leishmaniasis when high doses of antimony are undesirable. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 39, n. 4, p. 323-6, 2006.

OLIVEIRA, S. S. D.; ARAÚJO, T. M. D. Avaliação das ações de controle da leishmaniose visceral (calazar) em uma área endêmica do Estado da Bahia, Brasil (1995-2000). **Cadernos de Saúde Pública**, v. 19, p. 1681-1690, 2003.

OLIVEIRA, W. N. *et al.* The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of human tegumentary leishmaniasis. **Cytokine**, v. 66, n. 2, p. 127-132, 2014.

OPAS, Organização Panamericana da Saúde: **Leishmanioses: Informe Epidemiológico nas Américas**. 2016.

_____. **Plano de ação para fortalecer a vigilância e o controle da leishmaniose nas Américas 2017-2022**: 1-69 p. 2017.

ORAMAS-ROYO, S. *et al.* Synthesis and antiparasitic activity of 1, 2, 3-triazole-naphthoquinone conjugates. **Molecules**, v. 24, n. 21, p. 3917, 2019.

WHO, World Health Organization. **Working to overcome the global impact of neglected tropical diseases: first WHO report on neglected tropical diseases**. 2010.

OTHMAN, I. M. *et al.* Novel phthalimide based analogues: Design, synthesis, biological evaluation, and molecular docking studies. **Journal of Enzyme Inhibition and Medicinal Chemistry**, v. 34, n. 1, p. 1259-1270, 2019.

OTRANTO, D.; DANTAS-TORRES, F. The prevention of canine leishmaniasis and its impact on public health. **Trends in Parasitology**, v. 29, n. 7, p. 339-345, 2013.

OTRANTO, D. *et al.* Prevention of canine leishmaniosis in a hyper-endemic area using a combination of 10% imidacloprid/4.5% flumethrin. **PLoS One**, v. 8, n. 2, p. e56374, 2013.

OTRANTO, D. *et al.* Prevention of endemic canine vector-borne diseases using imidacloprid 10% and permethrin 50% in young dogs: a longitudinal field study. **Veterinary Parasitology**, v. 172, n. 3-4, p. 323-332, 2010.

OYAMA, J. *et al.* American tegumentary leishmaniasis: diagnostic and treatment challenges in a clinical case. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 60, 2018. ISSN 0036-4665.

OPAS, Organização Panamericana da Saúde: **Leishmaniasis: Epidemiological Report in the Americas** 2020.

PALOMINO, D. C. T.; MARTI, L. C. Quimiocinas e imunidade. **Einstein (São Paulo)**, v. 13, n. 3, p. 469-473, 2015. ISSN 1679-4508.

PARK, M. *et al.* A systematic review of COVID-19 epidemiology based on current evidence. **Journal of Clinical Medicine**, v. 9, n. 4, p. 967, 2020.

PAULA, C. D. R. D. *et al.* Estudo comparativo da eficácia de isotionato de pentamidina administrada em três doses durante uma semana e de N-metil-glucamina 20mgSbV/kg/dia durante 20 dias para o tratamento da forma cutânea da leishmaniose tegumentar americana. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 36, n. 3, p. 365-371, 2003.

PEREIRA, M. D.; LOPES, J. D.; NEVES, M. D. G. C. Leishmaniose Visceral em criança: um relato de caso sobre a recidiva da doença. **Comunicação em Ciências da Saúde**, p. 145-150, 2015.

PERNAMBUCO, S. E. D. S. **Plano Integrado de Ações para o Enfretamento às Doenças Negligenciadas no Estado de Pernambuco/SANAR 2015-2018**. SAÚDE., S. E. D. V. E.: 1-48 p. 2015.

_____. **Situação Epidemiológica da Leishmaniose Visceral em Pernambuco 1º semestre de 2018** 2018.

PERTINO, M. W. *et al.* Synthesis, trypanocidal and anti-leishmania activity of new triazole-lapachol and nor-lapachol hybrids. **Bioorganic Chemistry**, v. 103, p. 104122, 2020.

PHATAK, P. S. *et al.* Synthesis, antitubercular evaluation and molecular docking studies of phthalimide bearing 1, 2, 3-triazoles. **Synthetic Communications**, v. 49, n. 16, p. 2017-2028, 2019.

PICCICA, M. *et al.* Efficacy and safety of Pentamidine isethionate for tegumentary and visceral human leishmaniasis: a systematic review. **Journal of Travel Medicine**, 2021.

PINAZO, M. J. *et al.* Tolerance of benznidazole in treatment of Chagas' disease in adults. **Antimicrobial Agents and Chemotherapy**, v. 54, n. 11, p. 4896-4899, 2010.

PINTO, L.; VARGAS, E. Leishmania: reflexões sobre o manejo do mundo em contextos de risco sanitário. **Vivência: Revista de Antropologia**, v. 1, n. 49, p. 121-134, 2017.

PIRES, D. E.; BLUNDELL, T. L.; ASCHER, D. B. pkCSM: predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures. **Journal of Medicinal Chemistry**, v. 58, n. 9, p. 4066-4072, 2015.

PORTA, E. O. *et al.* Click chemistry decoration of amino sterols as promising strategy to developed new leishmanicidal drugs. **Steroids**, v. 79, p. 28-36, 2014.

POTVIN, J.-E. *et al.* Mutations in an aquaglyceroporin as a proven marker of antimony clinical resistance in the parasite *Leishmania donovani*. **Clinical Infectious Diseases**, v. 72, n. 10, p. e526-e532, 2021.

POUNTAIN, A. W. *et al.* Genomic instability at the locus of sterol C24-methyltransferase promotes amphotericin B resistance in *Leishmania* parasites. **PLoS Neglected Tropical Diseases**, v. 13, n. 2, p. e0007052, 2019.

POURMOHAMMADI, B. *et al.* Comparison of three methods for diagnosis of cutaneous leishmaniasis. **Iranian Journal of Parasitology**, v. 5, n. 4, p. 1, 2010.

QUEIRÃO, A. L. *et al.* A toxicidade das drogas empregadas no tratamento de leishmaniose e tripanossomíase americana. **Mostra Científica em Biomedicina**, v. 4, n. 1, 2019.

RANGEL, E. F.; VILELA, M. L. *Lutzomyia longipalpis* (Diptera, Psychodidae, Phlebotominae) and urbanization of visceral leishmaniasis in Brazil. **Cadernos de Saúde Pública**, v. 24, p. 2948-2952, 2008.

RASTI, S. *et al.* Comparison of molecular, microscopic, and culture methods for diagnosis of cutaneous leishmaniasis. **Journal of Clinical Laboratory Analysis**, v. 30, n. 5, p. 610-615, 2016.

RATH, S. *et al.* Antimoniais empregados no tratamento da leishmaniose: estado da arte. **Química Nova**, v. 26, n. 4, p. 550-555, 2003..

REGUERA, R. M. *et al.* Current status on prevention and treatment of canine leishmaniasis. **Veterinary Parasitology**, v. 227, p. 98-114, 2016.

REITHINGER, R. *et al.* Cutaneous leishmaniasis. **The Lancet Infectious Diseases**, v. 7, n. 9, p. 581-596, 2007.

REVUELTO, A. *et al.* Efficient Dimerization Disruption of *Leishmania infantum* Trypanothione Reductase by Triazole-phenyl-thiazoles. **Journal of Medicinal Chemistry**, v. 64, n. 9, p. 6137-6160, 2021.

REX, J. H.; WALSH, T. J. Editorial response: estimating the true cost of amphotericin B. **Clinical Infectious Diseases**, v. 29, n. 6, p. 1408-1410, 1999.

RIBEIRO-GOMES, F.; SACKS, D. The influence of early neutrophil-*Leishmania* interactions on the host immune response to infection. **Frontiers in Cellular and Infection Microbiology**, v. 2, p. 59, 2012.

ROATT, B. M. *et al.* Recent advances and new strategies on leishmaniasis treatment. **Applied Microbiology and Biotechnology**, p. 1-13, 2020.

ROCHAEL, N. C. *et al.* Classical ROS-dependent and early/rapid ROS-independent release of neutrophil extracellular traps triggered by *Leishmania* parasites. **Scientific reports**, v. 5, n. 1, p. 1-11, 2015.

RODRIGUES, A. C. M. *et al.* Epidemiologia da leishmaniose visceral no município de Fortaleza, Ceará. **Pesquisa Veterinária Brasileira**, v. 37, n. 10, p. 1119-1124, 2017.

RODRIGUES, A. M. *et al.* Fatores associados ao insucesso do tratamento da leishmaniose cutânea com antimoniato de meglumina. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 39, n. 2, p. 139-145, 2006.

RODRIGUES, B. C. *et al.* A retrospective cohort study of the effectiveness and adverse events of intralesional pentavalent antimonials in the treatment of cutaneous leishmaniasis. **International Journal for Parasitology: Drugs and Drug Resistance**, v. 14, p. 257-263, 2020.

ROQUE, A. L. R.; JANSEN, A. M. Wild and synanthropic reservoirs of *Leishmania* species in the Americas. **International Journal for Parasitology: Parasites and Wildlife**, v. 3, n. 3, p. 251-262, 2014.

RUFINO, R. A. A relação entre o desmatamento e a incidência de leishmaniose no município de Mesquita, RJ. **Revista GEOMAE**, v. 2, n. especial, p. 245-262, 2011.

SADAT-EBRAHIMI, S. E. *et al.* New phthalimide-benzamide-1, 2, 3-triazole hybrids; design, synthesis, α -glucosidase inhibition assay, and docking study. **Medicinal Chemistry Research**, p. 1-9, 2020.

SALDANHA, A. C. R. *et al.* Estudo comparativo entre estibogluconato de sódio BP 88® e antimoniato de meglumina no tratamento da leishmaniose cutânea II. Toxicidade bioquímica e cardíaca. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 33, n. 4, p. 383-388, 2000.

SAMPAIO, R. N. R. *et al.* Morte súbita causada por glucantime. **Anais Brasileiros de Dermatologia**, p. 35-7, 1988.

SARASWAT, P. *et al.* Review of synthesis and various biological activities of spiro heterocyclic compounds comprising oxindole and pyrrolidine moities. **Synthetic Communications**, v. 46, n. 20, p. 1643-1664, 2016.

SARIGOL, D. *et al.* Novel thiazolo [3, 2-b]-1, 2, 4-triazoles derived from naproxen with analgesic/anti-inflammatory properties: Synthesis, biological evaluation and molecular modeling studies. **Bioorganic & Medicinal Chemistry**, v. 23, n. 10, p. 2518-2528, 2015.

SASSI, A. *et al.* *Leishmania* skin test lymphoproliferative responses and cytokine production after symptomatic or asymptomatic *Leishmania major* infection in Tunisia. **Clinical & Experimental Immunology**, v. 116, n. 1, p. 127-132, 1999.

SCHELLEKENS, P.; SOURROUILLE, D. M. COVID-19 mortality in rich and poor countries: a tale of two pandemics? **World Bank Policy Research Working Paper**, n. 9260, 2020.

SCORZA, B. M.; CARVALHO, E. M.; WILSON, M. E. Cutaneous manifestations of human and murine leishmaniasis. **International Journal of Molecular Sciences**, v. 18, n. 6, p. 1296, 2017.

SEATON, R. A. *et al.* Out-patient parenteral antimicrobial therapy—a viable option for the management of cutaneous leishmaniasis. **QJM: An International Journal of Medicine**, v. 92, n. 11, p. 659-667, 1999.

SECHI, A. *et al.* Ultrasound patterns of localized cutaneous leishmaniasis and clinical correlations. **Journal of Ultrasound**, p. 1-6, 2021.

SEIFERT, K. *et al.* *In vitro* interactions between sitamaquine and amphotericin B, sodium stibogluconate, miltefosine, paromomycin and pentamidine against *Leishmania donovani*. **Journal of Antimicrobial Chemotherapy**, v. 66, n. 4, p. 850-854, 2011.

SENA, V. L. *et al.* Synthesis and hypolipidemic activity of N-substituted phthalimides. Part V. **Il Farmaco**, v. 58, n. 12, p. 1283-1288, 2003.

SERENO, D.; HOLZMULLER, P.; LEMESRE, J. Efficacy of second line drugs on antimonyl-resistant amastigotes of *Leishmania infantum*. **Acta Tropica**, v. 74, n. 1, p. 25-31, 2000.

SERENO, D. *et al.* DNA transformation of *Leishmania infantum* axenic amastigotes and their use in drug screening. **Antimicrobial Agents and Chemotherapy**, v. 45, n. 4, p. 1168-1173, 2001.

SHAKED-MISHAN, P. *et al.* Novel intracellular SbV reducing activity correlates with antimony susceptibility in *Leishmania donovani*. **Journal of Biological Chemistry**, v. 276, n. 6, p. 3971-3976, 2001.

SHARMA, U. *et al.* Recent advances in the chemistry of phthalimide analogues and their therapeutic potential. **Mini Reviews in Medicinal Chemistry**, v. 10, n. 8, p. 678-704, 2010.

SHIRZADI, M. R. Liposomal amphotericin B: a review of its properties, function, and use for treatment of cutaneous leishmaniasis. **Research and Reports in Tropical Medicine**, v. 10, p. 11, 2019.

SHOWLER, A. J.; BOGGILD, A. K. Protozoan Diseases: Leishmaniasis. In: QUAH, S. R. (Ed.). **International Encyclopedia of Public Health (Second Edition)**. Oxford: Academic Press, 2017. p.97-102. ISBN 978-0-12-803708-9.

SILVA, M. T. D. *et al.* Synthesis of N-substituted phthalimidoalkyl 1H-1, 2, 3-triazoles: a molecular diversity combining click chemistry and ultrasound irradiation. **Journal of the Brazilian Chemical Society**, v. 23, p. 1839-1843, 2012.

- SILVA, G. F. *et al.* Anti-hypersensitivity effects of the phthalimide derivative N-(4methyl-phenyl)-4-methylphthalimide in different pain models in mice. **Biomedicine & Pharmacotherapy**, v. 96, p. 503-512, 2017.
- SILVA JUNIOR, S. V. *et al.* Qualidade de vida relacionada à saúde de pessoas com Leishmaniose Tegumentar Americana. **Revista Eletrônica de Enfermagem**, v. 22, 2020.
- SILVA, M. S.; CANO, M. I. N. **Molecular and Cellular Biology of Pathogenic Trypanosomatids**. Bentham Science Publishers, 2017.
- SILVA, M. T. *et al.* Design and Synthesis of Triazole-Phthalimide Hybrids with Anti-inflammatory Activity. **Chemical and Pharmaceutical Bulletin**, v. 67, n. 2, p. 96-105, 2019.
- SILVA, S. M. *et al.* First report of infection of *Lutzomyia longipalpis* by *Leishmania (Leishmania) infantum* from a naturally infected cat of Brazil. **Veterinary Parasitology**, v. 174, n. 1-2, p. 150-154, 2010.
- SILVEIRA, B. P. *et al.* Parto prematuro após uso de antimonial pentavalente: relato de um caso. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 36, n. 4, p. 523-525, 2003.
- SILVEIRA, F. T. Leishmaniose cutânea difusa (LCD) na Amazônia, Brasil: aspectos clínicos e epidemiológicos. **Gazeta médica da Bahia**, n. 79, 2009.
- SINGH, G. *et al.* Synthesis, characterization, electronic absorption and antimicrobial studies of N-(silatranylpropyl) phthalimide derived from phthalic anhydride. **Inorganica Chimica Acta**, v. 427, p. 232-239, 2015.
- SINGH, N.; SUNDAR, S. Inflammatory chemokines and their receptors in human visceral leishmaniasis: gene expression profile in peripheral blood, splenic cellular sources and their impact on trafficking of inflammatory cells. **Molecular Immunology**, v. 85, p. 111-119, 2017.
- SOARES, G. H. C. *et al.* Case Report: Coinfection by *Leishmania amazonensis* and HIV in a Brazilian Diffuse Cutaneous Leishmaniasis Patient. **The American journal of tropical medicine and hygiene**, v. 103, n. 3, p. 1076-1080, 2020.
- SOUSA, C. M. H.; WINCK, C. A. Leishmaniose visceral canina: revisão de literatura. **Revista da Universidade Vale do Rio Verde**, v. 16, n. 1, 2018.
- SOTO, J. *et al.* Comparison of generic to branded pentavalent antimony for treatment of new world cutaneous leishmaniasis. **The American Journal of Tropical Medicine and Hygiene**, v. 71, n. 5, p. 577-581, 2004.
- SRINIVASARAO, S. *et al.* Discovery of 1, 2, 3-triazole based quinoxaline-1, 4-di-N-oxide derivatives as potential anti-tubercular agents. **Bioorganic Chemistry**, v. 100, p. 103955, 2020.

SRIVASTAVA, P. *et al.* Diagnosis of visceral leishmaniasis. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 105, n. 1, p. 1-6, 2011.

STEVERDING, D. The history of leishmaniasis. **Parasites & Vectors**, v. 10, n. 1, p. 1-10, 2017.

STEWART, J. J. Optimization of parameters for semiempirical methods II. Applications. **Journal of Computational Chemistry**, v. 10, n. 2, p. 221-264, 1989.

STRAZZULLA, A. *et al.* Mucosal leishmaniasis: an underestimated presentation of a neglected disease. **BioMed Research International**, v. 2013, 2013.

STROPPIA, P. H. F. *et al.* Effect of 1,2,3-triazole salts, non-classical bioisosteres of miltefosine, on *Leishmania amazonensis*. **Bioorganic & Medicinal Chemistry**, v. 25, n. 12, p. 3034-3045, 2017.

SUNDAR, S. *et al.* Oral miltefosine for Indian visceral leishmaniasis. **New England Journal of Medicine**, v. 347, n. 22, p. 1739-1746, 2002.

SUNDAR, S.; RAI, M. Laboratory diagnosis of visceral leishmaniasis. **Clinical and Diagnostic Laboratory Immunology**, v. 9, n. 5, p. 951, 2002.

SWIFT, T. **Daylight**. New York: Republic Records, 2019. 1 CD (61:48).

SWINNEY, D. C.; ANTHONY, J. How were new medicines discovered? **Nature Reviews Drug Discovery**, v. 10, n. 7, p. 507-519, 2011.

TALBI, F. Z. *et al.* Cartography and epidemiological study of leishmaniasis disease in Sefrou province (2007–2010), Central North of Morocco. **Interdisciplinary Perspectives on Infectious Diseases**, v. 2020, 2020.

TEHRANI, M. B. *et al.* Phthalimide-1, 2, 3-triazole hybrid compounds as tyrosinase inhibitors; synthesis, biological evaluation and molecular docking analysis. **Journal of Molecular Structure**, v. 1176, p. 86-93, 2019.

TEIXEIRA, D. E. *et al.* The cell biology of *Leishmania*: how to teach using animations. **PLOS Pathogens**, v. 9, n. 10, p. e1003594, 2013. ISSN 1553-7374.

THAKUR, L. *et al.* Atypical leishmaniasis: A global perspective with emphasis on the Indian subcontinent. **PLOS Neglected Tropical Diseases**, v. 12, n. 9, p. e0006659, 2018. ISSN 1935-2735.

THAKUR, S.; JOSHI, J.; KAUR, S. Leishmaniasis diagnosis: An update on the use of parasitological, immunological and molecular methods. **Journal of Parasitic Diseases**, v. 44, n. 2, p. 253-272, 2020.

TIUMAN, T. S. *et al.* Recent advances in leishmaniasis treatment. **International Journal of Infectious Diseases**, v. 15, n. 8, p. e525-e532, 2011.

UNICEF; ORGANIZATION, W. H. **TDR at a glance: fostering an effective global research effort on diseases of poverty**. World Health Organization. 2010

VALE, E. C. S. D.; FURTADO, T. Leishmaniose tegumentar no Brasil: revisão histórica da origem, expansão e etiologia. **Anais brasileiros de dermatologia**, v. 80, n. 4, p. 421-428, 2005.

VALENÇA, W. O.; DE FREITAS, J. J. R.; OLIVEIRA, R. N. D. Synthesis of aryl-1H-1, 2, 3-triazoles via 1, 3-dipolar cycloaddition. **Orbital: The Electronic Journal of Chemistry**, v. 4, n. 1, p. 31-32, 2012.

VAN-GRIENSVEN, J. *et al.* Leishmaniasis in immunosuppressed individuals. **Clinical Microbiology and Infection**, v. 20, n. 4, p. 286-299, 2014.

VAN-GRIENSVEN, J.; DIRO, E. Visceral Leishmaniasis: Recent Advances in Diagnostics and Treatment Regimens. **Infectious Disease Clinics of North America**, v. 33, n. 1, p. 79-99, 2019.

VAN-GRIENSVEN, Johan *et al.* Tuberculosis in visceral leishmaniasis-human immunodeficiency virus coinfection: an evidence gap in improving patient outcomes?. In: **Open Forum Infectious Diseases**. US: Oxford University Press, 2018.

VAN-HENTEN, S. *et al.* Cutaneous Leishmaniasis Due to *Leishmania aethiopica*. **EClinicalMedicine**, v. 6, p. 69-81, 2018.

VANLIER, C. *et al.* A case report of cutaneous leishmaniasis: a misleading clinical presentation. **Infection**, v. 49, n. 1, p. 177-180, 2021.

VEGA-LÓPEZ, F. Diagnosis of cutaneous leishmaniasis. **Current Opinion in Infectious Diseases**, v. 16, n. 2, p. 97-101, 2003.

VEREVKIN, S. P. *et al.* Thermodynamic analysis of strain in the five-membered oxygen and nitrogen heterocyclic compounds. **The Journal of Physical Chemistry A**, v. 115, n. 10, p. 1992-2004, 2011.

VERMA, N. K.; DEY, C. S. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. **Antimicrobial Agents and Chemotherapy**, v. 48, n. 8, p. 3010-3015, 2004.

VIANNA, G. Tratamento da leishmaniose tegumentar por injeções intravenosas de tártaro emético. **Arquivo Brasileiro de Medicina**, v. 4, p. 426-8, 1912.

VRIES, H. J. C.; REEDIJK, S. H.; SCHALLIG, H. D. F. H. Cutaneous Leishmaniasis: Recent Developments in Diagnosis and Management. **American Journal of Clinical Dermatology**, v. 16, n. 2, p. 99-109, 2015/04/01 2015.

WERNECK, G. L. **Expansão geográfica da leishmaniose visceral no Brasil**: SciELO Public Health 2010.

WHO, World Health Organization. **The Leishmaniasis: Report of a WHO Expert Committee**. 1984.

WHO, World Health Organization. **Report of the scientific working group meeting on leishmaniasis**, Geneva, 2-4 February, 2004.

WIJNANT, G. J. *et al.* Local skin inflammation in cutaneous leishmaniasis as a source of variable pharmacokinetics and therapeutic efficacy of liposomal amphotericin B. **Antimicrobial Agents and Chemotherapy**, v. 62, n. 10, 2018.

WYLIE, C. *et al.* A systematic review of the efficacy of prophylactic control measures for naturally-occurring canine leishmaniosis, part I: vaccinations. **Preventive Veterinary Medicine**, v. 117, n. 1, p. 7-18, 2014.

XU, Z. 1, 2, 3-Triazole-containing hybrids with potential antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). **European Journal of Medicinal Chemistry**, p. 112686, 2020.

YANG, T. *et al.* Recent advances in the rational drug design based on multi-target ligands. **Current Medicinal Chemistry**, v. 27, n. 28, p. 4720-4740, 2020.

YANG, Z.-G.; SUN, H.-X.; FANG, W.-H. Haemolytic activities and adjuvant effect of *Astragalus membranaceus* saponins (AMS) on the immune responses to ovalbumin in mice. **Vaccine**, v. 23, n. 44, p. 5196-5203, 2005.

ZHANG, W.-W. *et al.* Comparison of the A2 gene locus in *Leishmania donovani* and *Leishmania major* and its control over cutaneous infection. **Journal of Biological Chemistry**, v. 278, n. 37, p. 35508-35515, 2003.

ZHOU, D. B.; IPP, E. Pentamidine–Induced Beta Cell Toxicity Is Not Preventable by High Glucose. **The American Journal of the Medical Sciences**, v. 298, n. 2, p. 89-92, 1989.

ZHOU, Y. *et al.* *Leishmania major* LmACR2 Is a Pentavalent Antimony Reductase That Confers Sensitivity to the Drug Pentostam*. **Journal of Biological Chemistry**, v. 279, n. 36, p. 37445-37451, 2004.

ZULFIQAR, B.; SHELPER, T. B.; AVERY, V. M. Leishmaniasis drug discovery: recent progress and challenges in assay development. **Drug Discovery Today**, v. 22, n. 10, p. 1516-1531, 2017.

APÊNDICE A – ARTIGO PUBLICADO NA REVISTA MATERIALS SCIENCE AND ENGINEERING: C (FATOR DE IMPACTO: 7.32)



is an essential characteristic of any material with potential for use in biological applications [20,21]. The biocompatibility of PPY, which has been demonstrated in both *in vitro* and *in vivo* models [22], has allowed its use as a convenient substrate for the immobilization of biomolecules [23]. Due to their significant inhibitory activity on many species of bacteria [24,25], silver nanoparticles (AgNPs) alone exhibit a high antibacterial activity that has been exploited in medical applications and the development of commercial products [26]. However, it is well known that when used in their pristine form these particles can induce cell membrane damage and/or cause oxidative stress [27].

PPY-AgNPs composites have been used in the stabilization of oil-in-water emulsions [28] and as efficient nanocatalysts for the alkylation of amines [29]. It has been shown that the addition of AgNPs to a polymeric matrix can affect their interaction with cells and tissues [21]. Hybrid nanomaterials (such as polymer/metal nanoparticles) have been used in coatings with reduced toxicity and low level of particle aggregation [15,30], as well as antimicrobial agents [31,32] and platforms for cancer treatment [33]. Scarce information is available on the cytotoxic potential of hybrid composites based on PPY and AgNPs, and the evaluation of possible effects of AgNPs/PPY composites on mammalian cell systems remains an open field of investigation, with multiple avenues for multifaceted biomedical applications.

In this work, we report a novel synthesis of AgNPs/PPY composite by a single step *in situ* chemical oxidation of pyrrole monomers by silver cations. A 2³-factorial design was applied for the optimization of the physicochemical properties (maximum fluorescence emission, mean zeta potential, and mean particle size) of the AgNPs/PPY. UV-Visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), and transmission electron microscopy (TEM) were used to characterize the composite obtained using the optimal experimental conditions. We assayed the biocompatibility/cytotoxicity of the AgNPs/PPY composite on fibroblast, macrophages, Vero cells, and Human Cervix Epithelioid Carcinoma (HeLa) cells and examined its possible hemolytic activity toward ABO blood groups. The ultrastructural changes induced in these cells after exposure to the AgNPs/PPY composite was also investigated by Scanning Electron Microscopy.

2. Materials and methods

2.1. Chemical

Ethanol PA (C₂H₅OH - Química Moderna, Brazil) was used as a solvent, and silver nitrate (AgNO₃ 99% - Sigma-Aldrich, USA) as a metal precursor. The pyrrole monomer 95% (C₄H₅N) and (3-Mercaptopropyl)trimethoxysilane 95% ((HS(CH₂)₃Si(OCH₃)₃) - MPTS) were obtained from Sigma-Aldrich (USA). All reagents were used without further purification, except pyrrole, which was distilled under vacuum before use. Deionized water, was obtained by Millipore Synergy[®] Water Purification system (Merck, USA).

2.2. Synthesis of AgNPs/PPY composite

The synthesis of the AgNPs/PPY composite was carried out according to Santos et al. [34], with slight modifications. The reaction was performed in a round-bottom flask containing 10 mL of ethanol, followed by the addition of pyrrole monomer (3.0×10^{-2} mol/L), AgNO₃ solution (0.1×10^{-2} mol/L), and MPTS (2.7×10^{-2} mol/L). The resulting colloidal solution was maintained under vigorous stirring at 1000 rpm for 48 h, at room temperature. The dark AgNPs/PPY precipitate obtained was centrifuged at 10,000 $\times$ g and washed successively with methanol and ultrapure Milli-Q water, and then dried in an oven at 70 °C for 24 h.

2.3. Fluorescence emission screening of AgNPs/PPY composite

We investigated the fluorescence spectrum of the AgNPs/PPY composite in the (360–590) nm emission range, at room temperature

(23 °C), using a FluoroLog-3 spectrofluorimeter (Horiba, USA), under excitation in the (290–400) nm range. The excitation and emission slits had an opening of 2 nm, and 5 nm step sweeps were performed, using quartz cuvettes with a 1 cm optical path. The Spectra Lux program [35] was used to design a color coordinate graph in the RGB spectrum.

2.4. Optimization of the experimental condition of AgNPs/PPY composite by factorial design

A factorial experimental design method [36,37] was used to identify the main variables which have a key influence on the maximum fluorescence emission intensity (I_{max}), mean zeta potential (MZP), and mean particle size distribution (Z-Ave) of the AgNPs/PPY composite. The following variables were considered: (A) concentration of PPY monomers, (B) concentration of AgNPs, and (C) concentration of MPTS. A factorial design was implemented using two selected levels lower (–) and upper (+) for each variable (Table 1). By combining three-factor variables with the two levels for each one, 8 possible experiments (2³) were performed in duplicate, resulting in a total of 16 experiments (Table S1). The significance of the effects of each variable was evaluated by the analysis of variance (ANOVA) using the statistical software: Statistica 10 (Statsoft, USA). A p-value < 0.05 was used to indicate which variables were statistically significant, within a 95% confidence interval of Student's t distribution [38]. The statistical data were compared with Pareto chart analysis.

2.4.1. Z-average size and MZP measurements

The Z-Ave size and MZP of the sixteen AgNPs/PPY formulations were analyzed using a Zetasizer NANO-ZS90 (Malvern Instruments, UK). The Z-Ave were obtained by Dynamic Light Scattering (DLS) analysis, in which 10 μ L of the AgNPs/PPY composite were previously dispersed in 1 mL of ethanol P.A.. A polystyrene cuvette with a 1 cm optical path was filled, capped, and checked for the absence of any bubbles. The measurements were performed with a laser ($\lambda = 633$ nm) under a spreading angle of 90° and a temperature of 25 °C. The zeta potential was assayed from the electrophoretic mobility of each one of the sixteen runs of the complete factorial design. The mean zeta potential was calculated from the average of three values of the electrophoretic mobility of each one of the sixteen AgNPs/PPY composite formulations. All measurements were performed in a polystyrene cuvette with a 1 cm optical path, using an electrode for aqueous systems.

2.5. Characterisation methods

2.5.1. UV-visible and FTIR spectroscopies

The absorption UV-Visible spectra of dilute AgNPs/PPY solutions (5:1000 v/v) were obtained in the 200–1000 nm range, by using a quartz cell with an optical length of 1.0 cm, in a UV-2600 spectrophotometer (Shimadzu, Japan). The FTIR spectra were recorded on an FTIR-ATR spectrophotometer 4600 (Jasco Corporation, Japan) equipped with an ATR attachment comprising a ZnSe crystal with a central incidence angle of 45°, a circular contact area of 4.0 mm² and operating at a resolution of 4 cm^{–1} in the 4000 cm^{–1} to 400 cm^{–1} range.

Table 1
Experimental factors and levels in the 2³-factorial design for the AgNPs/PPY composite synthesis optimization.

Factors	Levels (mol/L)	
	Lower (–)	Upper (+)
MPTS	1.6×10^{-2}	2.7×10^{-2}
Pyrrole	1.4×10^{-2}	3.0×10^{-2}
AgNO ₃	3.6×10^{-3}	0.1×10^{-2}

MPTS: (3-Mercaptopropyl)trimethoxysilane.

2.5.2. Transmission electron microscopy

The structural characterization of the AgNPs/PPy composite was carried out using an FEI Tecnai Spirit transmission electron microscope (FEI, USA) operating at 120 kV. For this, we dropped 10 μ L of diluted AgNPs/PPy solution on a 400-mesh carbon-coated copper grid that was allowed to dry completely, at room temperature, before obtaining the TEM images.

2.6. In vitro biocompatibility of AgNPs-PPy composite

2.6.1. Cell culture and cytotoxicity assay

For the biocompatibility studies, we used four mammalian cell lineages: J774A.1 macrophage (ATCC® TIB-67™), Vero (ATCC® CCL-81™), fibroblast (ATCC® PCS-201-012™), and HeLa (ATCC® CCL-2™). The Vero cells and macrophages (5×10^5 cells/well) were cultured in 96-well plates containing 100 μ L of an RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Sigma-Aldrich, USA). The fibroblasts and HeLa cells were cultivated at the same conditions for the macrophages and Vero cells, except by the use of a DMEM medium (Sigma-Aldrich, USA). After the adhesion, the cells were incubated in the appropriate culture medium in the absence (untreated control cells) or presence of different concentrations (31.2–500 μ g/mL) of AgNPs/PPy composite for 24 h. After the incubation time, the cells were washed and reincubated in a fresh RPMI culture medium without phenol red (Sigma-Aldrich, USA), containing 5 mg/mL of 3-(4,5-Dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, USA) and cultivated for an additional period of 3 h at 37 °C. Afterward, the cells were solubilized in 100 μ L of an acidified isopropanol solution (0.04 M HCl + absolute isopropanol), to dissolve the formazan dye. The absorbance was read in a microplate spectrophotometer (Bio Rad®, California, USA) at 540 nm. Each analysis was performed in quadruplicate, in two independent experiments.

2.6.2. Ultrastructural analysis

The scanning electron microscopy (SEM) analysis was performed to investigate the effects of AgNPs/PPy on the morphology of HeLa and J774A.1 macrophage cells. For this, the HeLa and macrophages cells were treated with the AgNPs/PPy composite solutions at CC_{50} or $2 \times CC_{50}$ concentration, for 24 h. Untreated cells were used as a negative control. After incubation time, both the control and treated cells were washed with PBS and fixed for 2 h at room temperature in a solution containing 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 M of cacodylate buffer at pH 7.2. After washing in the same buffer, the cells were post-fixed for 1 h with 1% osmium tetroxide/0.05% potassium ferricyanide/5 mM $CaCl_2$ diluted in 0.1 M cacodylate buffer solution at pH 7.2. The samples were dehydrated in an increasing gradient series of ethanol, drying by critical point, covered with a 20 nm thick gold layer, and analyzed in a JEOL T-200 scanning electron microscope (JEOL, Japan).

2.6.3. Hemolytic assay

The hemolytic activity of AgNPs/PPy composite was investigated on human erythrocytes (A, B, O, and AB), according to the protocol established by Yang et al. [39]. Briefly, erythrocytes of each blood type were serially diluted in saline (NaCl 0.9%) and incubated in a 96-well microplate, in the presence of AgNPs/PPy solution at different concentrations (31.2–500 μ g/mL), for 30 min at 37 °C. After incubation, the microplates were centrifuged at 1500 $\times$ g for 4 min, and the hemolysis activity was quantified in a UV-Vis spectrophotometer at 412 nm. Erythrocytes incubated in 100 μ L of saline solution (NaCl 0.9%) or treated with 100 μ L of Triton X-100 (0.1%) were used as a negative and positive control of hemolysis, respectively. The hemolysis rate was determined as

$$\% \text{ hemolysis} = \frac{A_t - A_N}{A_P - A_N} \times 100,$$

where A_t , A_P , and A_N are the absorbances of the tested sample, the positive (100% of lysis), and the negative control, respectively. Each analysis was carried out in three independent experiments in triplicate.

2.7. Ethical considerations

The present study was carried out following the ethical principles adopted by the Brazilian Law 11.794/2008 and National Research Ethics Committee (CEP/CONEP), under the number CAAE 06959119.8.0009.5048.

2.8. Statistical analysis

The statistical analyses were performed using the SPSS Statistics for Windows (Version 18.0, IBM Corp. USA). The 50% cytotoxic concentration (CC_{50}) was calculated by linear regression. All data are presented as the average value $\pm$ standard deviations. A value of $p < 0.05$ was considered as statistically significant by one-way ANOVA test, and Dunnett's Post-test in GraphPadPrism 5.0 (Graphpad, California, USA).

3. Results and discussion

3.1. Synthesis of the AgNPs/PPy composite

In this work, the AgNPs/PPy composite was obtained efficiently and economically through a one-pot reaction. A schematic diagram and the mechanism proposed for the colloidal formation of AgNPs/PPy are depicted in Fig. 1, according to Oliveira et al. [40]. Initially, the silver precursor ($AgNO_3$) dissociates into silver cations (Ag^+) and nitrate anions (NO_3^-) (Step a). Consequently, the Ag^+ cation acts as an oxidizing agent for the *in situ* chemical polymerization of the pyrrole monomers, resulting in the simultaneous formation of pyrrole radical cation and metallic silver (Ag^0) (Step b). The PPy molecules are absorbed by hydrogen bonding and electrostatic interaction onto the mercaptocarboxylic acid, capping the AgNPs [41,42]; the silver nanoparticles are aggregated inside the PPy chains (Step c) [28,40,43,44]. The MPTS is widely used to prevent the agglomeration of the formed nanoparticles, due to its chemical affinity – which is associated with the presence of thiol groups – toward different noble metals [45,46].

3.2. Fluorescence measurements and optimization of the experimental condition of AgNPs/PPy composite

Highly fluorescent nanoparticles, as conductive polymers, have attracted scientific and industrial interest due to their remarkable optical/fluorescence properties, which can be exploited in various applications such as high-throughput screening, ultrasensitive assays, live-cell imaging, and photodynamic therapy [47]. Previous work conducted by our group demonstrated that the interaction between the metal particles and the polymeric chains in the AuNPs/Polyaniline composite results in remarkable fluorescence emission in the visible region [34]. To investigate the photoluminescence of the as prepared AgNPs/PPy, an excitation and emission spectrum screening was performed (Fig. 2A–B). The maximum fluorescence emission intensity (I_{max}) was observed at ~ 425 nm (Fig. 2B), for a $\lambda_{exc} = 350$ nm (Fig. 2A). This corresponds to the blue region in the visible spectrum, as confirmed by the corresponding color coordinate graph (Fig. 2C). Dong et al. [47] have reported a fluorescence emission peak around 440 nm for PPy nanospheres excited at 360 nm. The interaction of the AgNPs with PPy chains can account for the blue-shift observed in our case. Ramanavicius et al. [48] showed that quantum dots covered with PPy layer presented enhanced photoluminescence and resistance to the photobleaching. It has been reported

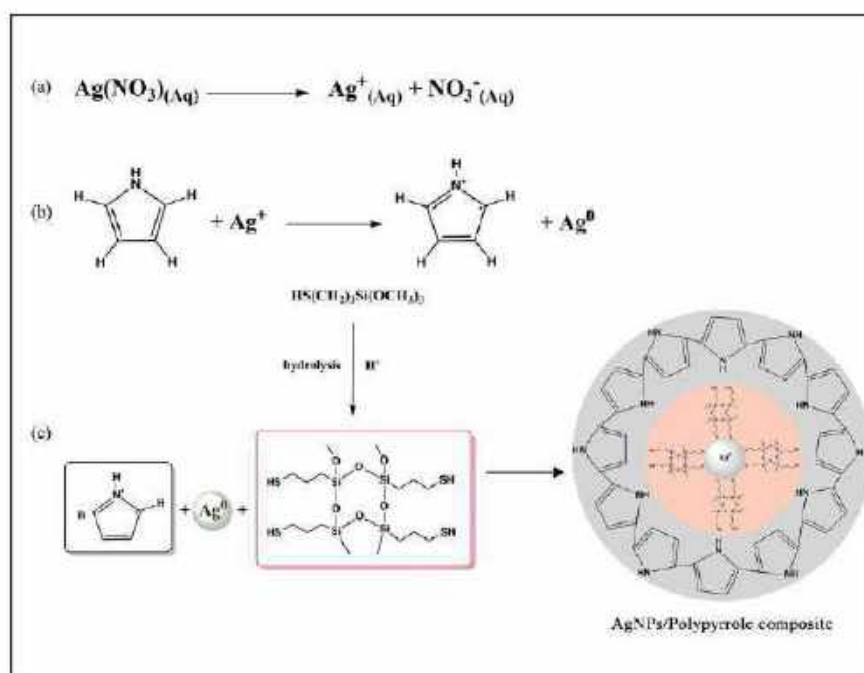


Fig. 1. Mechanism proposed for the of AgNPs/PPy composite formation.

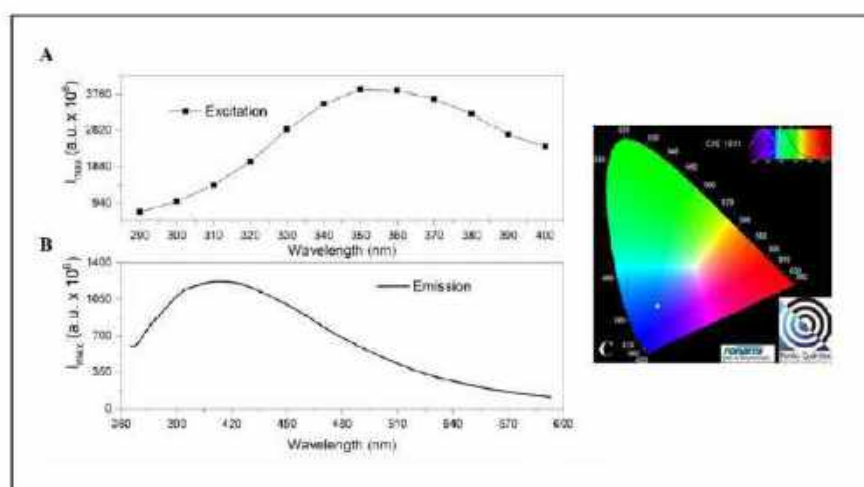


Fig. 2. Fluorescence intensity of the AgNPs/PPy composite at excitation wavelengths at 290–400 nm excitation wavelengths range (A), fluorescence emission spectrum ($\lambda_{\text{exc}} = 350 \text{ nm}$) (B), and color coordinate in the RGB spectrum (C).

that besides playing an important role in modulating the fluorescence of dyes, noble metals as gold and silver can become fluorescent *per se*, when their size is sharply decreased to the subnanometer range and/or they

are stabilized with appropriate ligands [49]. The fluorescence emission of the AgNPs/PPy composite may result from the interaction between the pyrrole monomers and the AgNPs formed during the AgNPs/PPy

synthesis process [34,50].

Because the existence of fluorescence expands the diversity of biological applications of the AgNPs/PPy composite, we further investigated the influence of the concentrations of Ag, pyrrole monomers, and MPTS (Independent variables) on the maximum emission fluorescence intensity (I_{max} , under 350 nm excitation), mean zeta potential (MZP), and the mean particle size distribution (Z-Ave) using the 2^3 -factorial design. This approach also allowed us to choose the optimal conditions for the synthesis of AgNPs/PPy that would better fit the physicochemical parameters evaluated. A matrix consisting of the selected variables (concentration of pyrrole, AgNPs, and MPTS) together with the corresponding responses (I_{max} , Z-ave, and MZP) is presented in Table 2.

The Pareto chart analysis showed which of three variables analyzed (concentration of MPTS, AgNO₃, and pyrrole) or its interactions should be statistically significant based on the variation between the two levels chosen. The red line at 0.05 indicated the statistical significance (Fig. 3). Our results demonstrated that the I_{max} is increased when the AgNPs/PPy composite was synthesized with the highest concentration of pyrrole and AgNO₃ (Fig. 3A), as indicated by the positive value of the standardized effect (AgNO₃ = 7.73; pyrrole = 3.51; Ag and Pyrrole interaction = 2.77). The positive interactions between these variables were confirmed by the analysis of variance (ANOVA) (Table S2).

The MZP, which depends on the surface charge, plays a key role in the stability of nanoparticles in suspension and is an important parameter for the synthesis and functionality of solid-state and soft matter synthetic colloids [51]. The analysis of the MZP measurements showed negative absolute values, for all 16 samples tested indicating a able colloidal dispersion of AgNPs/PPy composite (Table 2). As shown in the Pareto chart (Fig. 3B), the concentration of MPTS, as expected, was the variable that presented the most significant influence on MZP, as also demonstrated by the ANOVA analysis (Table S2). When MPTS, used in its lower level (-), was combined with the upper levels (+) of AgNO₃ and pyrrole there is a reduction in the average value of the main zeta potential (run 7 and 8; and run 15 and 16, respectively). When the amount of PPy was increased, the MZP was more negative (run 7 and 8; and run 15 and 16, respectively). Wen et al. [52] have found that the zeta potential of PPy/Ag composites becomes more negative when the relative amount of PPy is increased, as a result of a stronger interaction between the PPy chains and AgNPs.

The influence of reagent concentrations on Z-Ave was also analyzed. The Z-ave measurements of the samples (1–16 runs) showed that the size of the composite is influenced by reagent concentration, varying from 971.30 nm for MPTS (+), pyrrole (-), and AgNO₃ (+) to 942.70 nm for

MPTS (+), pyrrole (+) and AgNO₃ (-) (run 14 and 16). The Pareto chart demonstrated that pyrrole concentration has the highest standardized effect on this parameter (3.42) at a 95% confidence level (Fig. 3C). This result indicates that only the pyrrole concentration was statistically significant and the best response for the dispersion of the average particle size occurred when this variable was at its upper level (+). The positive effect showed on the Pareto chart also corroborates with the main effect of pyrrole concentration (statistical test - Table S2) that was observed for I_{max} .

Taking into account the observed influence of the concentration of composite constituents on the I_{max} , MZP, and Z-ave, the optimal condition for the synthesis of colloidal formulation of AgNPs/PPy was those where MPTS (-), AgNO₃ (+), and pyrrole (+). In this regard, this optimized formulation was used for the subsequent characterizations and the investigation of its biocompatibility and antitumoral activity.

3.3. Spectroscopic and structural characterization of the Ag/polypyrrole composite

The absorption spectrum of the AgNPs/PPy composite is shown in Fig. 4. It has been verified that the predicted optimal point of the AgNPs/PPy has two absorption bands at 230 nm and 280 nm, corresponding to the $\pi-\pi^*$ transitions of the benzenoid rings and the $\pi-\pi^*$ transitions of the pyrrole rings, respectively [47]. The shoulder at 470 nm (Fig. 4, inset) is associated with the overlapping of the $\pi-\pi^*$ transitions of the PPy and the presence of plasmons resonance surface of metallic nanoparticles [53]. Although the AgNPs commonly exhibit plasmon resonance bands in the (410–440) nm region [54], a band shift should be expected upon their incorporation into the polymeric chains [55,56].

The FTIR spectrum of the synthesized AgNPs/PPy composite confirmed the successful polymerization of pyrrole and the incorporation of the AgNPs (Fig. 5), even though some peaks become slightly displaced due to the interaction of the metallic nanoparticles with the PPy chains [42,57–59]. Characteristic peaks of PPy were observed in 1329 cm⁻¹, 1044 cm⁻¹, 679 cm⁻¹, and 601 cm⁻¹, which can be attributed respectively to the C=C stretch in the ring, C–H twist of the ring outside the plane, stretching of C–C, and deformation of C–C–N–H of the ring outside the plane [60]. The peak attributed to the pyrrole oxidation appears near 1379 cm⁻¹ [61]. The peak at 1660 cm⁻¹ is characteristic of the C=C bond stretch or can be assigned to the O=C–NH₂ groups. The peak around 1096 cm⁻¹ can be assigned to the vibrations of the pyrrole ring [42,44,62].

The structural analysis of the AgNPs/PPy composite by TEM revealed the presence of both monodisperse and aggregated regions. In the monodisperse structures, the electron-dense core, which corresponds to single spherical silver nanoparticles with sizes smaller than 100 nm, is surrounded by an amorphous electron lucent PPy matrix (Fig. 5A and B). On the other hand, the aggregates corresponded to a large polymeric matrix containing various inner silver nanoparticles, with sizes ranging from 200 nm to 500 nm (Fig. 5C and D). These agglomerate structures could have been formed by the concomitant reduction of silver cations and the growth of the PPy chains, which explains the higher mean particle size values obtained by DLS (Section 3.2).

3.4. Cytotoxic assay on mammalian cells

The biocompatibility of a given material can be assessed by examining the subsequent viability of selected cells exposed to it [26,63]. In this work, the cytotoxicity of as-prepared AgNPs/PPy composite was examined by performing MTT assays on fibroblasts, macrophages, and Vero cells at different concentrations of the composite. While fibroblasts and Vero Cells are frequently used for the evaluation of the cytotoxicity of biomaterials [29,64], macrophages are immunocompetent cells that first interact with foreign particles, efficiently phagocytizing them [65]. Macrophages also participate in the immune response to cancer cells [66]. Human cervix epitheloid carcinoma (HeLa) cells have been

Table 2

Matrix of interactions and results of the complete factorial design of the synthesis of AgNPs/PPy composite. Response: higher emission intensity (I_{max}) recorded at $\lambda_{exc} = 350$ nm, mean zeta potential (MZP), and the mean particle size (Z-Ave).

Run	Variables			Responses		
	MPTS	Pyrrole	AgNO ₃	I_{max} (a.u.)	MZP (mV)	Z-Ave (nm)
1	–	–	–	4.62×10^3	–25.7	843.20
2	–	–	+	3.59×10^3	–38.9	704.10
3	–	+	–	6.42×10^3	–19.2	758.70
4	–	+	+	7.72×10^3	–21.6	823.70
5	+	–	–	3.92×10^3	–32.3	950.00
6	+	–	+	5.64×10^3	–44.1	896.20
7	+	+	–	1.14×10^4	–35.1	821.30
8	+	+	+	1.12×10^4	–43.9	867.20
9	–	–	–	5.97×10^3	–30.9	892.60
10	–	–	+	4.99×10^3	–36.4	843.50
11	–	+	–	8.58×10^3	–25.9	864.10
12	–	+	+	8.61×10^3	–36.4	795.70
13	+	–	–	6.48×10^3	–32.6	885.70
14	+	–	+	5.73×10^3	–45.0	971.30
15	+	+	–	1.23×10^4	–41.1	969.50
16	+	+	+	1.08×10^4	–45.4	942.70

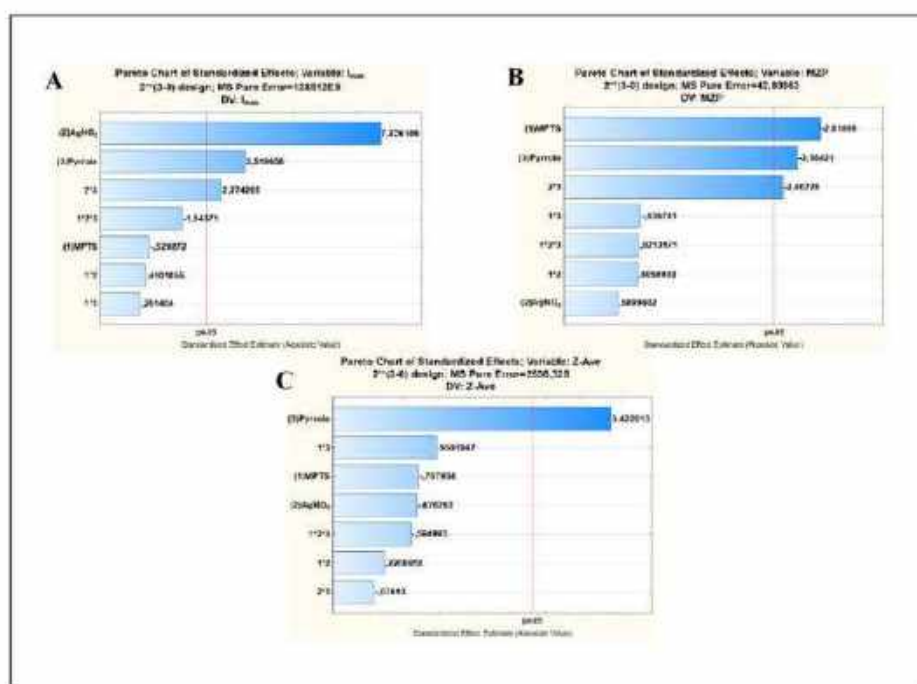


Fig. 3. Pareto chart of the effect of MPTS, AgNO₃, and pyridine concentration and on lmax (A), MZP (B), and Z-Ave (C) of AgNPs/PPy composite. The vertical red line in the Pareto chart indicates the significant effects at a 95% confidence level. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

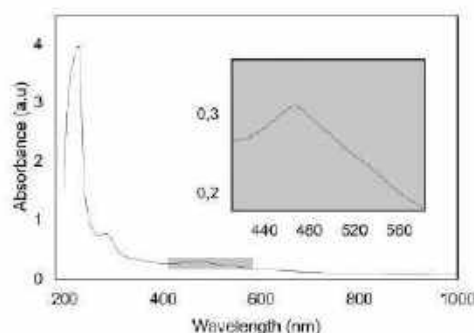


Fig. 4. UV-Visible spectrum of the AgNPs/PPy composite.

efficiently used *asa* model to evaluate the antitumor activity and tumor targeting of hybrid nanomaterials [67]. As a manner to assess the possible role of the AgNPs/PPy composite on cancer therapy, we evaluated its effect on HeLa cells.

Our results indicated that the AgNPs/PPy composite did not induce significant cytotoxic activity on macrophages and fibroblast and Vero cells (Fig. 7A–C). The corresponding CC₅₀ could not be estimated by regression analysis, and so it was considered greater than the highest concentration tested (500 µg/mL) in these cells (Table 3). Our results are consistent with the good biocompatibility already reported in the

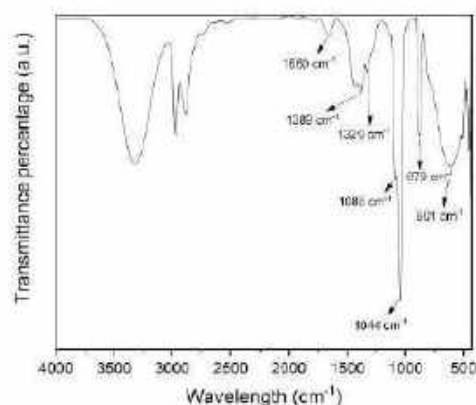


Fig. 5. FTIR spectrum of the AgNPs/PPy composite.

literature for PPy-based molecules [68–70]. It has been demonstrated that PPy was not toxic to mouse marrow-derived stem cells and sustain the attachment and proliferation of these cells on the PPy-modified surfaces. It has been also shown that the presence of amine groups and positive charges in the PPy structures allows the adhesion and proliferation of fibroblasts and other adherent cells [70–72]. Our results

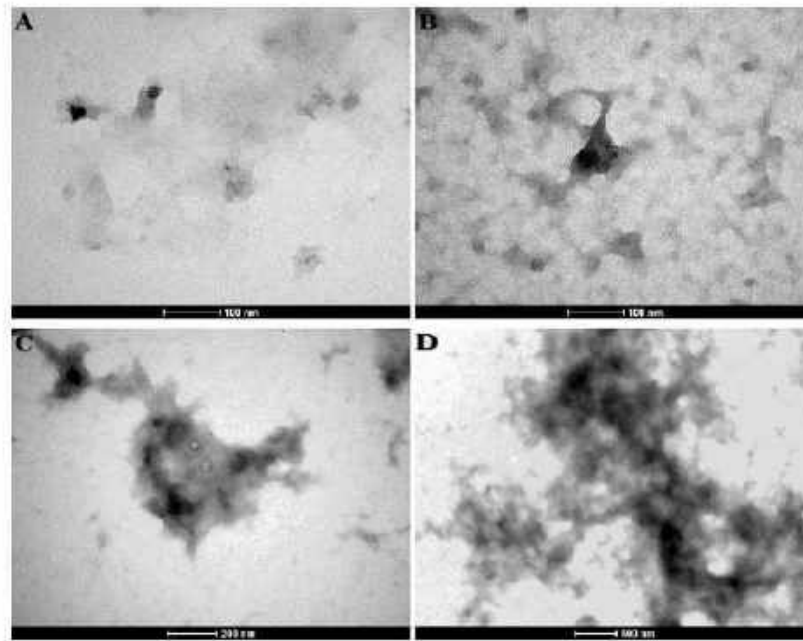


Fig. 6. TEM of the AgNPs/PPy composite in monodisperse shapes (A and B) and its agglomerated morphology (C and D).

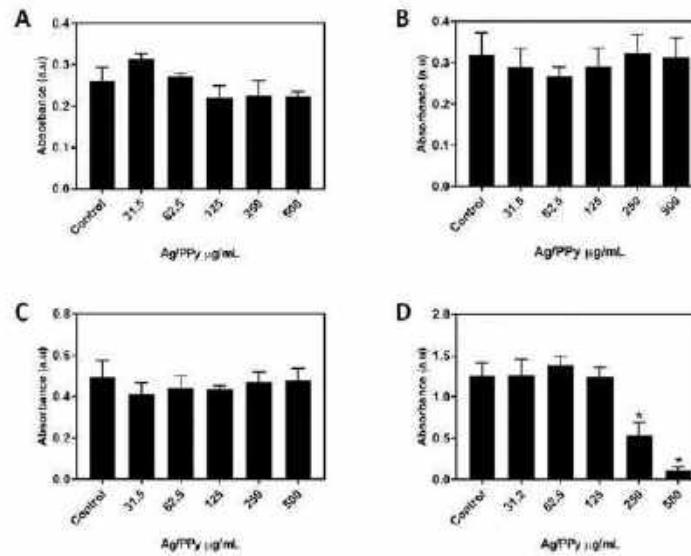


Fig. 7. Effect of the AgNPs/PPy composite on mammalian cells: (A) fibroblasts, (B) Vero, (C) J774A.1, and (D) HeLa. Values represent the mean $\pm$ standard deviation of three independent experiments in triplicate. *Significant differences at $p < 0.05$ compared to untreated control.

Table 3
The effect of AgNPs/PPy on the viability of mammalian cells.

Cell line	CC ₅₀ (μg/mL)
Fibroblast	>500
Vero	>500
Macrophage (J774A.1)	>500
HeLa	210.06

CC₅₀ – concentration (μg/mL) able to decrease the cell viability by 50%.

indicated that while the viability of fibroblasts cultivated in presence of the lowest concentration of AgNPs/PPy slightly increases (Fig. 7A), a significant decrease in the viability of HeLa cells treated with 250 and 500 μg/mL of the composite occurs. For the highest concentration tested, the inhibition of cell viability was close to 90%. The estimated CC₅₀ for HeLa cells after 24 h of incubation was 210 μg/mL (Table 3). The action of PPy-based materials on the viability of cancer cells has already been pointed out [73]. However, most of the previous works have been limited to the use of such materials as active agents for photothermal therapy or as biosensors for cancer diagnosis [7,18,74,75]. To the best of our knowledge, the present results correspond to the first report of the antitumoral activity of AgNPs/PPy composite *per se*.

3.5. Effect of AgNPs/PPy composite on the ultrastructure of mammalian cells

We used scanning electron microscopy to investigate the effects of the AgNPs/PPy composite on the morphology of J774A.1 macrophages and HeLa cells. To establish a baseline for comparison, in Fig. 8A and B we present the characteristic morphology of untreated J774A.1 macrophages and HeLa cells. The control macrophages, which present round to flattened shapes, were found to firmly attach to the substrate. Prominent filopodia and lamellipodia projections are commonly observed in these cells (Fig. 8A). The HeLa cells presented flattened and/or polygonal morphology. The cell surface of these cells is decorated with numerous microvilli (Fig. 8B) [76–78]. The surface topography of both types of cells indicates that they are extraordinarily active [79]. No damage to the plasma membrane was observed when the J774A.1 macrophage was treated with CC₅₀ of the AgNPs/PPy composite (Fig. 8C). However, under this treatment condition, these cells became more flattened and with longer pseudopodia compared to non-treated cells (Fig. 8C). This phenotype has been usually associated with macrophage activation [80,81]. The treatment of macrophages with LPS, which is a standard activator of macrophages toward classical M1 activation profile, quickly induces the formation of pseudopods, filopodia-like projection, and the spreading of these cells on the substrate, in a similar way to those observed in our study [82]. Similarly,

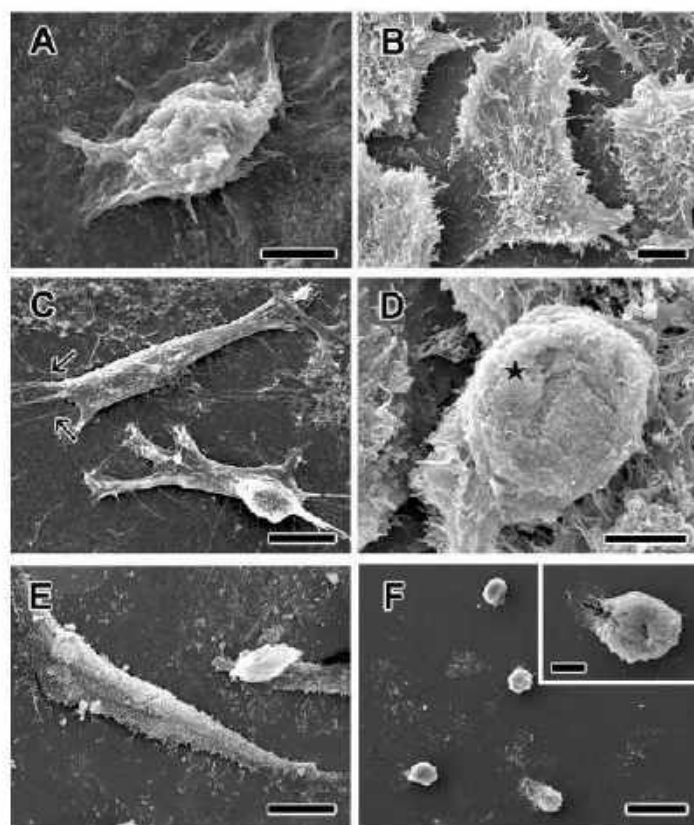


Fig. 8. Representative micrographs of the effects of AgNPs/PPy on the morphology of macrophages (left column) and HeLa cells (right column). (A) Untreated macrophages presenting a round shape and evident filopodia and lamellipodia tightly attached to the substrate with a clear emission of filopodia and lamellipodia. (B) Untreated HeLa cells presenting flattened morphology. The cell surface showed characteristic microvilli extending from the cell surface. (C–D) Cells treated with CC₅₀ of AgNPs/PPy. (C) treated macrophages showing spread and flattened morphology with long filopodia projections (black arrow). (D) Detail of a HeLa cell showing a rounding of the cell body and loss of microvilli (black star). (E–F) Macrophages (E) and HeLa cells (F) were treated with $2 \times \text{CC}_{50}$ of AgNPs/PPy. (F). Detail of HeLa cell culture showing scant adhered cells, all of them with a round morphology. Note the presence of membrane perforations (black arrow) and loss of microvilli structures (inset). Bars: A, B and D = 2 μm; C, E = 5 μm; F = 10 μm; inset = 1 μm.

Gniadek et al. [93] demonstrated that THP-1 monocytes in contact with PPY-coated materials, including AgNPs/PPY, showed numerous funnel-like lamellipodia characteristic of macrophage activation. Classically activated M1-polarized macrophages have an important role in re-educating the immune system for the elimination of cancer. Thus, the AgNPs/PPY composite may induce the polarization of macrophages toward the M1 phenotype, making them more competent to attack and phagocytose tumor cells [93,94].

The treatment of HeLa cells with CC₅₀ of AgNPs/PPY composites induced the loss of microvilli, whereas their plasma membrane assumes a corrugated appearance (Fig. 8D). These morphological changes are indicative of a loss of plasma membrane function. Only at the higher concentration tested, the AgNPs/PPY composite induced some damage to the macrophage membrane (Fig. 8E), and, even so, to a lesser extent than the damage caused to the HeLa cells. In all concentrations tested, the morphology of the HeLa cells assumed a more globular aspect, with damage to the plasma membrane being observed even at the lowest AgNPs/PPY concentration tested (Fig. 8D and F). Hence, our results indicate that the AgNPs/PPY composite selectively affects the HeLa cells relative to the treated fibroblast, Vero cells, and macrophages.

It is usually assumed that the antitumor activity of AgNPs is mainly due to the possible release of metallic silver (Ag^0) and silver ions (Ag^+), which can trigger increased ROS production leading to oxidative stress, DNA, mitochondrial and membrane damage, genotoxicity, resulting in cell death by necrosis or apoptosis [85]. Consistently, our morphological results showed that the treatment of HeLa cells with the composite caused damage to the plasma membrane and the presence of cellular debris, characteristic of necrosis. It has been demonstrated that reactive oxygen species (ROS) have a paradoxical role in cancer. ROS can promote protumorigenic signaling, involving cancer cell proliferation, survival, and adaptation to hypoxia [86]. On the other hand, ROS can induce antitumorigenic signaling and trigger oxidative stress-induced cancer cell death [97,98]. The high metabolic activity in cancer cells, needed for cell transformation and tumorigenesis, increases ROS production by these cells. Concurrently, to maintain ROS homeostasis, tumor cells altered their antioxidant capacity increasing their susceptibility to ROS-induced oxidative stress as compared to normal cells. Our results indicate that the combination of AgNPs with PPY chains has a noticeable effect on HeLa cells, without causing substantial damage to normal mammalian cells (macrophages, fibroblasts, and Vero cells). These results suggest that the presence of the PPY chains reduced the cytotoxic potential of AgNPs on non-tumoral cell lines. Despite the good biocompatibility of PPY to mammalian cells have been already reported in the literature, we cannot rule out that this CP also contributes, at least in part, to the cytotoxicity of AgNPs/PPY toward HeLa cells. Valdivia et al. [72] showed that PPYNPs presented considered cytotoxic effects to the Jurkat, M122A tumoral lineages whereas the cytotoxic effect of these nanoparticles on primary mouse embryonic fibroblast was observed only at higher concentrations.

3.6. Hemolytic assay

The hemolytic activity is an important parameter to be considered when assessing the biocompatibility of a given material [99]. The hemolysis corresponds to the breakdown or destruction of the plasma membrane of Red Blood Cells (RBCs), with the subsequent release of hemoglobin [90]. A significant RBC rupture in response to the exposure to a material is an adverse effect that could lead to important pathological conditions, limiting its systematic use in medical devices and applications [91]. We used RBCs from the A, B, AB, and O human blood groups to investigate the hemolytic potential of the AgNPs/PPY composites (Fig. 9). The hemolytic activity of AgNPs/PPY composites was less than 20%, for all blood groups tested, at concentrations up to 125 $\mu\text{g}/\text{mL}$, which is a threshold value to consider a biomaterial as non-hemocompatible [62,92,93].

It is important to note that the composition of the human erythrocyte

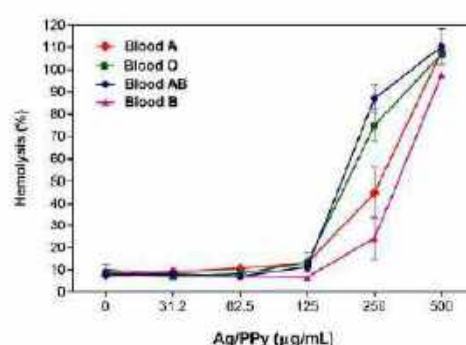


Fig. 9. Hemolytic percentage of AgNPs/PPY in different human erythrocyte groups.

plasma membrane varies according to the blood group [94], leading to differences in the susceptibility of these cells to biomaterials [95,96]. Membrane proteins contribute considerably to the total surface charge of these cells. Particularly, the intermolecular interactions of composite materials with the surface of the different blood groups erythrocytes can modify the zeta potential of these cells [97–99]. It is worth mentioning that isolated silver nanoparticles can induce the rupture of the erythrocyte membrane [15,91,95]. The cytotoxic and hemolytic effects of AgNPs or silver-based nanocomposites on mammalian cells have been reported by various groups [91,100,101]. The hemolytic activity of isolated AgNPs seems to depend on their surface/volume ratio, with nanoparticles presenting a higher hemolytic activity than micron-size particles. On the other hand, as a hemocompatible material [22], PPY can protect the erythrocytes from the deleterious effects of Ag nanoparticles.

4. Conclusions

We presented an easy method of preparing AgNPs/PPY composite which emits a fluorescent signal in the blue region of the visible spectrum, characterizing them by UV–Visible and FTIR spectroscopies, and TEM analyses. The impact of the concentration AgNPs, pyrene, and MPIS on the maximum fluorescence intensity, zeta potential, and Z-Ave size was demonstrated by factorial design methodology and validated by statistical analysis (Pareto charts and ANOVA). This approach allowed us to optimize the experimental condition for the synthesis of AgNPs/PPY composite. We have shown that the optimized AgNPs/PPY composite exhibits low cytotoxicity toward non-tumoral cell lineages but has highly selective action against HeLa cells, in which severe membrane damage is induced, as observed by SEM. The AgNPs/PPY composite also exhibited a low hemolytic activity, when used at concentrations below 125 $\mu\text{g}/\text{mL}$. Overall, these results confirm the good biocompatibility and promising potential of the AgNPs/PPY composites as cytotoxic agents against HeLa cells without induces damage to healthy cells. Additionally, the fluorescence of these composites opens up the possibility of their use as biomarkers for diagnosis and biosensing. We believe that further studies are necessary to elucidate the mechanism of action of the AgNPs/PPY composite on HeLa cells, as well as on other tumoral cells both *in vitro* and *in vivo*.

CRediT authorship contribution statement

Elton Marlon de Araújo Lima: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. Vanderlan Nogueira Holanda: Methodology, Formal analysis, Data curation. Gabriel

Plautz Ratkovski: Methodology, Formal analysis, Data curation, Wellson Vicente da Silva: Investigation, Resources. Pedro Henrique do Nascimento: Investigation, Resources. Regina Célia Bressan Queiroz de Figueiredo: Conceptualization, Writing – original draft, Writing – review & editing, Supervision. Celso Pinto de Melo: Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

On behalf of all authors, I hereby certify that there is no conflict of interests in the publication of the manuscript "A new biocompatible silver/polypyrrole composite with in vitro antitumor activity", by Elton Marlon de Araújo Lima, Vanderlân Nogueira Holanda, Gabriela Plautz Ratkovski, Wellson Vicente da Silva, Pedro Henrique do Nascimento, Regina Célia Bressan Queiroz de Figueiredo, and Celso Pinto de Melo, as no author has any financial/personal interest or belief, dual commitments, competing interests, or competing loyalties that could the corresponding work.

Acknowledgments

This work was supported by Brazilian agencies FACEPE (Fundo de Amparo à Ciência e Tecnologia de Pernambuco, Brazil - IBPG-1510-3.03/16), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). The authors would like to thank Professor C. A. S. Andrade (Departamento de Bioquímica - Universidade Federal de Pernambuco) for the support on synthesis of composite, Professor C. F. Pereira (Departamento de Química Fundamental - Universidade Federal de Pernambuco) for the support on factorial analyses, and the Instituto Aggeu Magalhães for the use of the electron microscopy facility.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.msec.2021.112314>.

References

- [1] N.K. Guimard, N. Gouaux, C.E. Schmidt, *Prog. Polym. Sci.* 32 (2007) 875–921, <https://doi.org/10.1016/j.proppolym.2007.05.012>.
- [2] H. Shirakawa, E.J. Louis, A.O. MacDiarmid, C.K. Chiang, A.J. Heeger, *J. Chem. Soc. Chem. Commun.* (1977) 578–580, <https://doi.org/10.1039/J19770000578>.
- [3] B. Liu, *Energy Biomater.* 19 (2018) 1783–1803, <https://doi.org/10.1021/acs.energymater.8b00575>.
- [4] C.K. Chiang, C.R. Fincher, Y.W. Park, A.J. Heeger, H. Shirakawa, E.J. Louis, S. C. Gau, A.O. MacDiarmid, *Phys. Rev. Lett.* 39 (1977) 1096–1101, <https://doi.org/10.1103/PhysRevLett.39.1096>.
- [5] M. Delvaux, J. Duerksen, P.-Y. Stancu, R. Legras, S. Dumortier-Champagne, *Synth. Met.* 113 (2000) 225–230, [https://doi.org/10.1016/S0927-7759\(99\)00326-3](https://doi.org/10.1016/S0927-7759(99)00326-3).
- [6] A.G. MacDonald, R.J. Marmorek, R.B. Kacer, L. Porter, R. Perzig, A.J. Heeger, D. R. Rosolowsky, R.J. Gillespie, P. Day, *Philos. Trans. R. Soc. Lond. A Math. Phys. Sci.* 314 (1968) 3–18, <https://doi.org/10.1098/rsta.1968.0004>.
- [7] Z. Jia, X. Yue, Q. Ren, S. Dai, *Adv. Mater.* 25 (2013) 777–782, <https://doi.org/10.1002/adma.201202211>.
- [8] E. Balint, N.J. Casady, S.H. Cortwell, *Acta Biomater.* 10 (2014) 2341–2353, <https://doi.org/10.1016/j.actbio.2014.02.015>.
- [9] B.S. Dolshayin, K.R. Reddy, A. Mishra, N.D. Shrivastava, S.J. Mulede, S. Banu, S. Narayana, A.V. Raghun, *Microchem. J.* 147 (2019) 7–21, <https://doi.org/10.1016/j.microc.2019.02.061>.
- [10] S. Li, X. Shan, Q. He, X. Cao, *Nanoscale* 11 (2019) 19351–19365, <https://doi.org/10.1039/C9NR05460A>.
- [11] A.P. O'Mallone, S.E. Dale, J.V. Macpherson, F.R. Urrutia, *Chem. Commun.* (2004) 1606–1607, <https://doi.org/10.1039/B404030F>.
- [12] D. Upadhyay, B.J. Boyd, S. Garg, J. Travas-Sejdic, D. Sankila, *Biomaterials* 111 (2016) 144–162, <https://doi.org/10.1016/j.biomaterials.2016.06.021>.
- [13] M. Qiu, Z. Song, Y. Wu, B. Guo, X. Pan, X. Luo, *Biosens. Bioelectron.* 79 (2016) 296–304, <https://doi.org/10.1016/j.bios.2015.12.011>.
- [14] E.J. da Silva, R.C. Maril, J.C. Medeiros, A.F. Chaves-Cunha, J. J. Almeida-Egipson, C. Pinto de Melo, *Arch. Biochem. Biophys.* 575 (2019) 22–35, <https://doi.org/10.1016/j.ab.2019.03.003>.
- [15] L. Han, Y. Zhang, Y. Zhang, Y. Shi, X. W. Chen, J.-H. Wang, *Talanta* 171 (2017) 33–38, <https://doi.org/10.1016/j.talanta.2017.04.055>.
- [16] A. Sajjalal, *Chem. Pap.* 67 (2013) 819–825, <https://doi.org/10.2478/s11695-012-0304-6>.
- [17] T.Y. Tekkozoglu, T. Segner, M. Ali, A. Koca, M.K. Gencer, *Biosens. Bioelectron.* 87 (2017) 81–88, <https://doi.org/10.1016/j.bios.2016.08.020>.
- [18] S. Ye, S. Chen, H. Wang, Y. Lu, *Phys. Chem. Chem. Phys.* 13 (2011) 4668–4673, <https://doi.org/10.1039/C1CP201134A>.
- [19] F. Zou, F.N. Solak, G. Okada, D.F. Williams, *Nanoscale* 14 (2010) 2768–2775, <https://doi.org/10.1039/T9NM140140>.
- [20] D. Humpalová, V. Kozubeková, J. Pávek, J. Štekl, P. Bobek, Z. Čapková, E. A. Rudzinskiewicz, I. Jurek, M. Lachovský, *Mater. Sci. Eng. C* 91 (2018) 303–310, <https://doi.org/10.1016/j.msec.2018.07.037>.
- [21] M. Ziabka, M. Dziadek, B. Mentasch, *Polymers* 10 (2018) 1257, <https://doi.org/10.3390/polym1011257>.
- [22] X. Wang, X. Gu, C. Yuan, S. Chen, P. Zhang, T. Zhang, J. Yan, F. Chen, G. Chen, *J. Biomed. Mater. Res. A* 88A (2004) 411–422, <https://doi.org/10.1002/jbm.b.20076>.
- [23] A. Romanavicius, A. Piskunovskis, H. Ocilins, A. Romanaviciene, *Bioelectrochemistry* 79 (2010) 11–18, <https://doi.org/10.1016/j.bioelect.2009.09.013>.
- [24] U.T. Khatone, G.V.S.N. Rao, M.R. Mohan, A. Romanaviciene, A. Romanavicius, *J. Environ. Chem. Eng.* 6 (2018) 5637–5644, <https://doi.org/10.1016/j.jece.2018.08.009>.
- [25] U.T. Khatone, G.V.S. Nagarajan Rao, M.R. Mohan, A. Romanaviciene, A. Romanavicius, *Vacuum* 146 (2017) 259–265, <https://doi.org/10.1016/j.vacuum.2017.10.061>.
- [26] L. Paulock, S. Herrmann, M. Bohalek, G. Szalay, V. Ali, P. Schneider, K.S. Lips, *Acta Biomater.* 10 (2014) 459–469, <https://doi.org/10.1016/j.actbio.2013.08.037>.
- [27] M.J. Piao, K.A. Karg, L.K. Lee, H.S. Elm, S. Kim, J.Y. Choi, J. Choi, J.W. Hyun, *Techol. Lett.* 201 (2017) 95–100, <https://doi.org/10.1016/j.tlet.2016.12.010>.
- [28] S. Fujii, A. Akita, K. Akamatsu, H. Nishizawa, Y. Nakamura, *J. Mater. Chem.* 17 (2007) 3777–3779, <https://doi.org/10.1039/B709415B>.
- [29] U. Maril, S. K. Kozubek, A. Kozubek, S.M. Islam, J. Colloid Interface Sci. 467 (2016) 291–299, <https://doi.org/10.1016/j.jcis.2016.01.017>.
- [30] F.J. Rodriguez, S. Oufeyre, J.G. Hauser, J.L. Brown, N. Botin, *Environ. Sci. Technol.* 34 (2000) 3018–3023, <https://doi.org/10.1021/es99040a>.
- [31] E. Fico, B. Bala, P. Dandapani, S. Marchionni, M. Ando, *Electrochim. Acta* 90 (2012) 1257–1263, <https://doi.org/10.1016/j.electacta.2012.07.036>.
- [32] M. Hanczka, P. Hanczka, V. Kozubek, W. Czapkiewicz, L. Marcinowski, P. Bobek, M. Trichová, J. Štekl, *Appl. Surf. Sci.* 396 (2017) 169–176, <https://doi.org/10.1016/j.apusc.2017.11.014>.
- [33] K. Tan, J. Wang, K. Pang, L. Lin, Q. Sun, Q. You, F. Tan, S. Li, *ACS Appl. Mater. Interfaces* 8 (2016) 34991–34995, <https://doi.org/10.1021/acsami.6b12852>.
- [34] R.F.A. Freitas, C.A.S. Andrade, G.F. dos Santos, G.P. de Melo, J. Neurosci. Res. 13 (2013) 1433, <https://doi.org/10.1007/s12031-012-1468-1>.
- [35] P. Santa-Cruz, F. Tellez, *Prote Quantitaz Nucleotidomorf. TERNABR*, 2003.
- [36] J.P. Oliveira, A.R. Prado, W.J. Kajlich, M.R.N. Ribeiro, M.J. Pereira, B.V. Nogueira, M.C.C. G. Amaral, *Arch. J. Chem.* 15 (2020) 216–226, <https://doi.org/10.1016/j.archsc.2020.100393>.
- [37] A.L. de Paula Neves, C.C. Milski, L. Müller, H.O. Ruela, S.C. Kalman, H. E. Sulkow, *Colloid Polym. Sci.* 292 (2014) 34–39, <https://doi.org/10.1007/s00382-013-2058-8>.
- [38] L. Wilkinson, *Am. Stat.* 60 (2006) 332–334, <https://doi.org/10.1198/08931640600152242>.
- [39] Z.-D. Yang, H.-X. Sun, W.-H. Fang, *Vaccine* 23 (2005) 5196–5203, <https://doi.org/10.1016/j.vaccine.2005.06.014>.
- [40] L.V.P. Oliveira, P.F. Camilo, *Synth. Met.* 247 (2019) 219–227, <https://doi.org/10.1016/j.synmet.2019.12.001>.
- [41] W. Zhou, *Microchim. Rapid Commun.* 21 (2000) 745–752, [https://doi.org/10.1002/1522-2925\(200003/04\)21:03<745::AID-MR745>3.0.CO;2-3](https://doi.org/10.1002/1522-2925(200003/04)21:03<745::AID-MR745>3.0.CO;2-3).
- [42] S. Jiang, S. Xie, L. Yu, B. Zhao, *Mater. Lett.* 61 (2007) 4529–4534, <https://doi.org/10.1016/j.matlet.2007.02.045>.
- [43] Y. Tan, K. Ghosh, *Synth. Met.* 175 (2013) 183–191, <https://doi.org/10.1016/j.synmet.2013.01.014>.
- [44] A. Xu, X. Zhang, M. Sun, Y. Xia, P. Wu, *Mater. Des.* 104 (2016) 192–202, <https://doi.org/10.1016/j.matdes.2016.05.029>.
- [45] S.A.H. Jalili, A.R. Allahfar, S.S. Sarmadani, J. Aghaei Tamati, J. Taiwan Inst. Chem. Eng. 66 (2016) 357–362, <https://doi.org/10.1016/j.jtice.2016.05.011>.
- [46] J.D. Kim, H. Yun, G.C. Han, C.W. Lee, H.C. Choi, *Appl. Surf. Sci.* 283 (2015) 227–235, <https://doi.org/10.1016/j.apsusc.2015.05.066>.
- [47] B. Dong, M. Yang, S. Ge, Y. Cao, B. Li, Y. Lu, *RSC Adv.* 6 (2016) 23737–23745, <https://doi.org/10.1039/C6RA01465B>.
- [48] A. Romanavicius, V. Kozubeková, A. Romanaviciene, R. Romaniuk, J. Narosci, *Nanoscale* 9 (2009) 1909–1915, <https://doi.org/10.1039/b9nr00035i>.
- [49] E. Jia, P. Wang, L. Yang, X. Zhou, W. Chen, Y. Liu, *J. Mater. Chem. C* 3 (2015) 3522–3529, <https://doi.org/10.1039/C4TC02330C>.
- [50] D. Munoz-Rojas, J. Oro-Sola, O. Ayyad, P. Gomez-Romero, *J. Mater. Chem.* 21 (2011) 2078–2086, <https://doi.org/10.1039/C0JM001440>.
- [51] M.R. Romanova, J.N. Pedersen, E. Madsen, *Nat. Commun.* 11 (2020) 2307, <https://doi.org/10.1038/s41467-020-15089-2>.
- [52] J. Wen, Y. Tian, Z. Mai, W. Wu, Y. Tian, *RSC Adv.* 7 (2017) 53219–53225, <https://doi.org/10.1039/C7RA04725B>.

- [53] Y.-C. Liu, T.C. Chung, *J. Phys. Chem. B* 107 (2003) 12303–12306, <https://doi.org/10.1021/jp035686v>.
- [54] S.C. Shi, M.O. Wolf, *Chem. Commun.* (2005) 3375–3384, <https://doi.org/10.1039/B501443D>.
- [55] J.C. Gustafson, B. Liedberg, O. Inganäs, *Solid State Ionics* 65 (1994) 145–152, [https://doi.org/10.1016/0167-2738\(94\)90403-0](https://doi.org/10.1016/0167-2738(94)90403-0).
- [56] J. Xu, J. Hu, B. Quan, Z. Wei, *Macromol. Rapid Commun.* 30 (2009) 936–940, <https://doi.org/10.1002/mrac.200900764>.
- [57] P. Dullius, D. Marchese, D. Vekovic, N. Boukacem, S. Nejovali, C. Timpello, D. Penttilä, *Polymer* 48 (2007) 2067–2073, <https://doi.org/10.1016/j.polymer.2007.01.050>.
- [58] H.V.R. Dias, M. Flacchini, R.M.O. Rulapaka, *Polymer* 47 (2006) 7349–7354, <https://doi.org/10.1016/j.polymer.2006.09.023>.
- [59] S. Ylag, G. Zhao, *Mater. Lett.* 61 (2007) 2040–2044, <https://doi.org/10.1016/j.matlet.2006.01.011>.
- [60] R. Kozin, D. Rukhovich, S.A. Egorov, L.E. Davidova, L.A. Galkin, *J. Chem. Phys.* 120 (2004) 3104–3109, <https://doi.org/10.1063/1.1666020>.
- [61] X. Peng, H. Huang, Q. Ye, J.-J. Zuo, W. Han, *J. Phys. Chem. C* 111 (2007) 8463–8468, <https://doi.org/10.1021/jp071110e>.
- [62] K.H. Kato, S.R. Dandekar, P. Khanna, G. Jain, *J. Nanosci. Nanotechnol.* 11 (2011) 7863–7869, <https://doi.org/10.1166/jnn.2011.4700>.
- [63] S. Kim, W.-K. Oh, Y.S. Jeong, J.-Y. Hong, S.-R. Cho, J.-S. Hahn, *J. Inorg. Biochem.* 102 (2011) 2342–2350, <https://doi.org/10.1016/j.jinorgbio.2010.11.016>.
- [64] N. Basu, A. Ghosh, A. Boroi, B. Basu, *J. Biomater. Appl.* 33 (2019) 1055–1052, <https://doi.org/10.1177/0885262219821340>.
- [65] J.E. Burchin, R.A. Gemeinhart, Activation of macrophages in response to biomaterials, in: M. Kloe (Ed.), *Macrophages: Origin, Function and Intervention*, Springer International Publishing, Cham, 2017, pp. 317–381, https://doi.org/10.1007/978-3-319-54050-6_13.
- [66] A. Nardin, J.-P. Abadie, *Front. Biosci.* 13 (2008) 494–505, <https://doi.org/10.2741/2944>.
- [67] C. Du, A. Wang, J. Fei, J. Song, J. Li, *J. Mater. Chem. B* 3 (2015) 4533–4545, <https://doi.org/10.1039/C5TB00560D>.
- [68] M. Minakata, C. Ungureanu, R. Ioa, C. Pirvu, *Appl. Surf. Sci.* 275 (2013) 401–410, <https://doi.org/10.1016/j.apsusc.2012.03.102>.
- [69] A. Romanovicene, A. Romanic, S. Trifunovic, A. Romanovicene, *J. Pharm. Pharmacol.* 59 (2007) 311–315, <https://doi.org/10.1211/jpp.59.3.0017>.
- [70] A. Valdivia, V. Romanic, L. Mikolajczyk, V. Knera, G. Romanovicene, O. Bialkiewicz, A. Romanovicene, A. Romanovicene, *Colloids Surf. A Physicochem. Eng. Asp.* 442 (2014) 132–150, <https://doi.org/10.1016/j.colsurfa.2014.06.003>.
- [71] J.-Y. Lee, G.L. Schmidt, *J. Biomed. Mater. Res. A* 103 (2015) 2126–2132, <https://doi.org/10.1002/jbm.b.33344>.
- [72] A. Valdivia, V. Romanic, J. Veronovic, G. Romanovicene, O. Bialkiewicz, A. Romanovicene, A. Romanovicene, *J. Inorg. Mater.* 250–251 (2013) 167–174, <https://doi.org/10.1016/j.jinorgmat.2012.04.004>.
- [73] Y. Chen, B. Xiang, L. Wang, M. Wang, G. Wang, B. Jia, K. Zhang, B. Yang, *ChemPharmChem* 15 (2018) 1127–1134, <https://doi.org/10.1002/cphc.201800090>.
- [74] C.-X. Qian, Y.-A. Chen, P.-J. Feng, X.-Z. Xiao, M. Dong, J.-C. Yu, Q.-Y. Hu, Q.-D. Shao, Z. Gu, *Acta Pharmacol. Sin.* 38 (2017) 764–771, <https://doi.org/10.1038/npp.2017.12>.
- [75] B. Tian, C. Wang, S. Zhang, L. Peng, Z. Liu, *ACS Nano* 5 (2011) 7000–7009, <https://doi.org/10.1021/nn101550a>.
- [76] S. Hinkel, M. Rohde, M. von Neuhoff, M. Nimz, I. Steinmetz, *Infect. Immun.* 71 (2003) 2970, <https://doi.org/10.1128/IAI.71.5.2970-2975.2003>.
- [77] A. Lam, M. Hermit, M. Debnio, V. Heinrich, *Biophys. J.* 95 (2009) 240–254, <https://doi.org/10.1524/biophys.1.09.139154>.
- [78] H.R. Percy, D.G. Hoffman, H.M. McConnell, *J. Cell Biol.* 30 (1981) 228–229, <https://doi.org/10.1083/jcb.30.2.228>.
- [79] E.R. Perera, V. Zengin, *C. Weiss, Cancer Res.* 34 (1974) 1305.
- [80] J. Hardie, J.A. MacKinnon, S. Ho, E.M. Rizzo, M.E. Parker, *Pharmacol. Res.* 148 (2019) 104452, <https://doi.org/10.1016/j.phrs.2019.104452>.
- [81] H.M. Rocco, P.M. Rocco, M.R. Almeida, N. Godegnard, A. M. Ghassemlahani, *Sci. Rep.* 7 (2017) 3521, <https://doi.org/10.1038/s41598-017-03704-z>.
- [82] S.-R. Kim, D.-Y. Kim, K.-J. Park, H.-S. Park, Y.-S. Cho, H.-J. Lee, W.-S. Lee, C. H. Ryu, Y.L. Hu, D.H. Lee, *Sci. Based Complement. Alternat. Med.* 2011 (2011), <https://doi.org/10.1155/2011/240588>.
- [83] M. Gzdeck, A. Wichowski, M. Aros-Bielska, P. Oskowski, M. Rzyzewska, M. Dantes, *Synth. Met.* 266 (2020) 116490, <https://doi.org/10.1016/j.synmet.2020.116490>.
- [84] R.G.D. Andrade, B. Reis, B. Cortez, S.A.C. Lima, S. Reis, *Polymer* 13 (2021) 88, <https://doi.org/10.1016/j.polymer.2021.08.005>.
- [85] L.A.R. Pereira, F. Garcia-Perez, A. Rialta, N. Durán, W.L. Figueira, M.R. de Jesus, *Eur. J. Pharm. Biopharm.* 151 (2020) 162–170, <https://doi.org/10.1016/j.ejpb.2020.04.012>.
- [86] M.C. Szarek, G. Wei, E.S. McLennan, D.M. Patterson, A. Wei, M.S. Sepúlveda, *Nanomedicine* 6 (2011) 879–888, <https://doi.org/10.2217/nmm.11.26>.
- [87] J. Wang, J. Yi, *Cancer Biol. Ther.* 7 (2008) 1075–1084, <https://doi.org/10.4161/cbt.7.12.7897>.
- [88] C.R. Beczak, S.S. Ghoshal, *Annu. Rev. Cancer Biol.* 1 (2017) 79–94, <https://doi.org/10.1146/annurev-cancerbio.041615-005868>.
- [89] Q. Gou, J. Huang, X. Zhang, *Small* 14 (2018) 1803101, <https://doi.org/10.1002/smll.201803101>.
- [90] S. Han, L. Han, G. Roldan, J. Blum, W. van Oosterhout, *Mater. Sci. Eng. C* 29 (2009) 1650–1654, <https://doi.org/10.1016/j.msec.2009.01.002>.
- [91] J. Upadhyay, A. Kumar, B. Gogoi, A.K. Bhattacharya, *Mater. Sci. Eng. C* 54 (2015) 6–18, <https://doi.org/10.1016/j.msec.2015.04.027>.
- [92] E.C. Barick, P.A. Hassan, *J. Colloid Interface Sci.* 369 (2012) 90–102, <https://doi.org/10.1016/j.jcis.2011.12.038>.
- [93] D. Xiong, Y. Deng, N. Wang, Y. Yang, *Appl. Surf. Sci.* 290 (2014) 55–61, <https://doi.org/10.1016/j.apsusc.2014.02.085>.
- [94] B. Hoon, *J. Med. Imaging* 55 (2008) 174–182, <https://doi.org/10.3152/jmi.55.174>.
- [95] J. Choi, V. Briga, V.M. Hitchens, P.L. Goring, P.A. McManus, *Toxicol. Sci.* 123 (2011) 130–142, <https://doi.org/10.1093/toxsci/kfr195>.
- [96] L. Lavigne, M.R. Infante, M.A. Reyes, A. Piacca, M.R. Vinardelli, M. Mitjana, M. A. Manresa, L. Perez, *Soft Matter* 9 (2013) 306–319, <https://doi.org/10.1039/C2SM26870A>.
- [97] W.-S. Cho, R. Duffin, F. Thielheer, M. Bradley, L.L. Magoon, W. McNeen, C. A. Poland, G.L. Tran, B. Donaldson, *Toxicol. Sci.* 128 (2012) 469–477, <https://doi.org/10.1093/toxsci/kfr195>.
- [98] C. McQuinn, R. Duffin, R. Brown, M.L. Mills, L.L. Magoon, W. McNeen, S. Johnston, S.L. Lo, L. Tran, R. Li, X. Wang, D.B. Newby, K. Donaldson, *Toxicol. Sci.* 119 (2010) 359–364, <https://doi.org/10.1093/toxsci/kfp149>.
- [99] F. Tokumura, G.R. Otonari, C. Amaratunga, R.M. Fakhour, *Exp. Pathol.* 131 (2012) 245–251, <https://doi.org/10.1016/j.exppath.2012.09.005>.
- [100] T.J. Jayaram, C.P. Newby, T. Rajaratnam, *J. Int. Eng. Chem.* 39 (2020) 268–300, <https://doi.org/10.1016/j.jiec.2020.05.025>.
- [101] Z. Lu, X. Zhang, Z. Li, Z. Wu, J. Song, C. Li, *Polym. Chem.* 6 (2015) 772–778, <https://doi.org/10.1039/C5PY00401B>.

APÊNDICE B – ARTIGO PUBLICADO NA REVISTA JOURNAL OF APPLIED MICROBIOLOGY (FATOR DE IMPACTO: 3.77)

Received: 13 January 2022 | Revised: 11 March 2022 | Accepted: 21 March 2022
DOI: 10.1111/jam.15542

ORIGINAL ARTICLE



Activity of poly(methacrylic acid)-silver nanoparticles on fluconazole-resistant *Candida albicans* strains: Synergistic and cytotoxic effects

Cecília Maria Cruz Falcão¹ | Audrey Andrade^{2,3} | Vanderlan Nogueira Holanda⁴ | Regina Celia Bressan Queiroz de Figueiredo⁴ | Eulália Azevedo Ximenes⁵ | Anderson Stevens Leonidas Gomes^{1,2}

¹Postgraduate Program in Dentistry, Federal University of Pernambuco, Recife, Brazil

²Department of Physics, Federal University of Pernambuco, Recife, Brazil

³Laboratory of Microscope and Microanalysis, Strategic Technologies Center of Northeast, Recife, Brazil

⁴Department of Microbiology, Aggeu Magalhães Research Centre, Recife, Brazil

⁵Department of Antibiotics, Federal University of Pernambuco, Recife, Brazil

Correspondence

Cecília Maria Cruz Falcão, Postgraduate Program in Dentistry, Federal University of Pernambuco, 50670-901, Recife, Prince Edward Island, Brazil. Email: ceciliadonto@gmail.com

Funding information

Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: PQ-1A/307239/2015-3; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Grant/Award Number: 001CAPES-UFPE; Instituto Nacional de Física, Grant/Award Number: INCT 465763/2014-6

Abstract

Aims: To synthesize and evaluate the antifungal activity of poly(methacrylic acid)-silver nanoparticles (PMAA-AgNPs) against nine *Candida albicans* isolated from clinical specimens.

Methods and Results: The effects of PMAA-AgNPs-fluconazole combination was analysed by checkerboard methodology. The synergistic potential of PMAA-AgNPs-fluconazole was determined by the fractional inhibitory concentration index (FICI). The inhibition of germ tube formation and the determination of PMAA-AgNPs cytotoxicity were also performed. All *C. albicans* strains were susceptible to PMAA-AgNPs and resistant to fluconazole. PMAA-AgNPs at subinhibitory concentrations restored the susceptibility of resistant *C. albicans* to fluconazole, whose FICI ranged from 0.3 to 0.5. The synergistic interaction of the combination was observed in eight of nine strains. The PMAA-AgNPs-fluconazole combination was also able to inhibit the germ tube formation. PMAA-AgNPs showed a dose-dependent decrease in viability for cells tested, with 50% cytotoxic concentration (CC₅₀) values of 6.5, 4.9 and 6.8 µg ml⁻¹ for macrophages, fibroblasts and Vero cells, respectively.

Conclusions: This study demonstrated that, in general, PMAA-AgNPs acts synergistically in combination with fluconazole, inhibiting fluconazole-resistant *C. albicans* strains. PMAA-AgNPs-fluconazole combination was also able to inhibit germ tube formation, an important virulence factor. Inhibitory effect of PMAA-AgNPs alone or in combination was higher in *C. albicans* than in mammalian cells.

Significance and Impact of Study: This study shows the potential of PMAA-AgNPs combined with fluconazole to inhibit fluconazole-resistant *C. albicans* strains.

KEYWORDS

Candida albicans, fluconazole, germ tube, silver nanoparticles, synergism

INTRODUCTION

Candidiasis is an opportunistic pathology ranging from superficial to systemic and life-threatening infection, being *Candida albicans* the most prevalent species (Arya & Rafiq, 2020). Usually, infections caused by *Candida* are treated by different classes of antifungals including azoles, polyenes and echinocandins (Pappas et al., 2016). Besides the emergence of resistance, the limited spectrum of available antifungals constitutes a major barrier to the effective treatment of systemic and local fungal infections (Nguyen et al., 2020).

The development of resistance to the currently used antifungals has been an important health concern. In this regard, the search of novel antifungal alternatives to traditional antibiotics, aiming to overcome this limitation, represents an urgent need in the field. (Pimental et al., 2022; Nadhe et al., 2019).

Metal nanoparticles have been demonstrated to have not only antimicrobial effects, but also advantages such as: lower toxicity, hydrophilicity, biocompatibility and high stability. These nanoparticles alone or in combination with currently antifungal agents can be used to circumvent the fungal resistance, as well as the complications associated with prolonged use of antifungals (Mussin et al., 2019). Silver nanoparticles (AgNPs) are among the most studied nanomaterials for biomedical applications due to their potent antimicrobial properties, good chemical stability and biocompatibility (Singh et al., 2019). The activity of these nanoparticles against *C. albicans* has been already demonstrated (Hamida et al., 2021; Hampe et al., 2017; Lee et al., 2019; Longhi et al., 2016). However, the search of novel AgNPs with more suitable physicochemical properties and lower cytotoxicity with antifungal activity is still an urgent need.

Although nanoparticles are considered as a promising tool in the areas of science and technology, their cytotoxicity potential has been a matter of concern (Punjabi et al., 2018). Development of AgNPs with antifungal activity, environmentally harmless and cheap procedures in AgNP production has been sought. Thus, it is necessary to develop not only an innovative, cost-effectively and eco-friendly method to produce AgNPs, but also to evaluate its antimicrobial activity and biosafety to prove their therapeutic potential (Różalska et al., 2018).

In a previous study, a solution of silver nitrate and biocompatible poly(methacrylic acid) (PMAA) polymer were used as starting point to prepare spherical AgNPs (PMAA-AgNPs), with diameter smaller than 40 nm (Carvalho et al., 2018). To the best of our knowledge, studies on the effect of PMAA-AgNPs combined with antifungal agents against *C. albicans* have not yet been published. In this regard, this study aims to evaluate the in vitro effects of the

combination of PMAA-AgNPs with fluconazole against *C. albicans* strains isolated from different clinical samples, the ability of these nanoparticles to inhibit the germ tube formation as well the determination of its cytotoxicity potential on mammalian cells.

MATERIALS AND METHODS

Synthesis and characterization of PMAA-AgNPs

Poly(methacrylic acid)-silver nanoparticles (PMAA-AgNPs) were synthesized according to the methodology described by Carvalho et al. (2018). Aqueous solution of silver nitrate (AgNO_3 , 50 ml, 5 mmol L^{-1}) was mixed with PMAA (sodium salt, 50 ml, 0.5 mmol L^{-1}) and magnetically stirred at 25°C for 5 min. This solution was exposed to UV light (8 W, central wavelength 370 nm) for 6 h for silver reduction and stored in amber vials (Spadaro et al., 2009). Then, size and morphology of synthesized PMAA-AgNPs was analysed. UV-Visible transmission spectroscopy (Cary 50 UV-Vis spectrometer; Varian/Agilent) was used to verify the conversion of silver cations to AgNPs. Nanostructure of samples were characterized by transmission electron microscopy (JEOL; JEM-2100) operating at 200 kV and energy dispersive X-ray fluorescent spectrometer module of the Nuclear and Energy Research Institute (SP, Brazil). The zeta potential measurements were performed using Zetasizer Nano from the laboratory of Non-Conventional Polymers, Department of Physics—Federal University of Pernambuco.

Candida albicans strains and inoculum standardization

Candida albicans strains ($n = 9$) were obtained from stock cultures maintained in the Laboratory of Physiology and Biochemistry of Microorganisms (LFBM), Department of Antibiotics—Federal University of Pernambuco. These strains were isolated from urine (LFBM Ca01, LFBM Ca02, LFBM Ca03 and LFBM Ca04), oral cavity (LFBM Ca05 and LFBM Ca06) and blood (LFBM Ca07, LFBM Ca14 and LFBM Ca1902). *C. albicans* ATCC[®] 10231 was used as standard strain.

Candida albicans cultures were reactivated in Sabouraud Dextrose Broth (DIFCO), seeded onto Sabouraud Dextrose Agar (DIFCO) and incubated at 35°C for 24 h. For standardization of the inoculum, single colonies were selected and yeast suspension was prepared in saline solution (0.85%). Turbidity of these suspensions was adjusted to 90% transmittance at 530 nm which is

equivalent to 10^6 colony forming units per ml (cfu ml^{-1}). Then, two dilutions were performed to obtain a final inoculum (10^3 cfu ml^{-1}). First, *C. albicans* culture were diluted in saline solution (1:100) following by a second dilution (1:10) in Roswell Park Memorial Institute Medium (RPMI-1640; Gibco) buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS; Sigma-Aldrich) (CLSI, 2017b). *Candida albicans* growth in the culture was confirmed by direct cell counting.

Antifungal agents

Amphotericin B, nystatin and fluconazole (Sigma-Aldrich) were chosen as reference drugs, based on Guideline of Infectious Diseases Society of America (Pappas et al., 2016). Resistance was defined for each case, considering minimal inhibitory concentration (MIC) values required to inhibit the growth of 90% of organisms: amphotericin B MIC $>1.0 \mu\text{g ml}^{-1}$, fluconazole MIC $\geq 8.0 \mu\text{g ml}^{-1}$. For nystatin, MIC between 8.0 and $32.0 \mu\text{g ml}^{-1}$ was defined as dose-dependent susceptibility (CLSI, 2017a, 2017b). Amphotericin B, nystatin and fluconazole were solubilized in dimethyl sulfoxide/tween 80/water (1.0/0.5/8.5) while AgNPs were solubilized in water. *Candida albicans* cultivated in medium containing dimethyl sulfoxide/tween 80/water was used as negative control to exclude the possibility of toxic effects of this diluent on the pathogen.

Antifungal activity

Susceptibility of *C. albicans* to antibiotics was carried out using broth microdilution method, in accordance with CLSI M27-A4 guideline with slight modifications. Stock solution of PMAA-AgNPs, amphotericin B, nystatin and fluconazole were serially diluted in RPMI-1640 buffered with MOPS to obtain concentrations that ranged from 64.0 to $0.5 \mu\text{g ml}^{-1}$, except to fluconazole, whose concentration ranged from 1024 to $8.0 \mu\text{g ml}^{-1}$. Aliquots ($100 \mu\text{l}$) of drugs solutions were distributed in sterile 96-well microplates and then a standardized suspension of *C. albicans* strains ($100 \mu\text{l}$) was added to wells. These plates were incubated at 35°C for 24 h. Minimal inhibitory concentration was reported as the lowest concentration of compounds that inhibited *C. albicans* growth (CLSI, 2017b).

To determine the minimal fungicidal concentration (MFC), defined as the lowest concentration that killed more than 99.9% of the initial inoculum, aliquots ($5 \mu\text{l}$) of each well, where no growth was detected, were seeded onto Sabouraud Dextrose Agar. These plates were

incubated under the same conditions and the number of colonies was determined.

Checkerboard assay

Checkerboard assay was performed according to the methodology described by Odds (2003). Appropriate dilutions of PMAA-AgNPs (4.0 – $0.031 \mu\text{g ml}^{-1}$) and fluconazole (1024 – $0.5 \mu\text{g ml}^{-1}$) were prepared into RPMI-1640. From these dilutions, $50 \mu\text{l}$ were added into 96-well microplates to obtain a final concentration corresponding to 1/8 and 1/12 of MIC value for PMAA-AgNPs and fluconazole, respectively. Then, $100 \mu\text{l}$ of cell suspension was added to each well (10^3 cfu ml^{-1}) and microplates were incubated at 35°C for 24 h.

Fractional inhibitory concentration index (FICI) was determined as: (MIC of the PMAA-AgNPs in combination with fluconazole/MIC of the PMAA-AgNPs) + (MIC of fluconazole in combination with PMAA-AgNPs/MIC of fluconazole).

The effect of the combination was defined as synergistic if FICI was ≤ 0.5 , additive when the FICI was >0.5 to ≤ 1.0 and antagonistic when >1.0 (Odds, 2003).

Germ tube formation assay

LFBM Ca06 strain was randomly chosen for the germ tube formation tests. This strain was cultivated on Sabouraud Dextrose Broth at 35°C for 24 h. Cultures were diluted in the same broth to obtain yeast standard suspensions (10^3 cfu ml^{-1}). These suspensions were exposed to fluconazole, PMAA-AgNPs and their combination in subinhibitory concentrations. Aliquots of 1 ml of each *C. albicans* culture were added to 1 ml of fetal bovine serum (FBS; Gibco). The test tubes were incubated at 37°C for 3 h under gently shaking. Samples cultivated in free-drug medium supplemented with FBS were considered as positive control of germ tube formation. A drop of each *C. albicans* culture was placed on a slide and observed under light microscope at $400\times$ magnification (Olympus BX51) (Matare et al., 2017). Yeasts were considered to have germinated if the germ tube formation was at least twice the length of the cell.

Cytotoxicity assay

Cytotoxic effects of the PMAA-AgNPs on J774.A1 macrophages (ATCC TIB-67TM), Vero cells (ATCC CCL-81TM) and fibroblasts (ATCC PCS-201-018) were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-di-

phenyl tetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983). For each cell types, 10^5 cells ml^{-1} was seeded in 96-well plates containing RPMI-1640 for macrophages and Vero cells, or Dulbecco's Modified Eagle Medium for fibroblasts, supplemented with 10% inactivated FBS. Cultures were incubated at 37°C for 3 h in 5% CO_2 atmosphere. Nonadherent cells were removed by washing with phosphate buffered saline, pH 7.0. Remaining cells were allowed to grow for additional 48 h in RPMI-1640 in the absence or the presence of PMAA-AgNPs (100 μl), at concentrations ranging from 2.0 to 64.0 μgml^{-1} . Treated and untreated cells were washed and incubated at 37°C for 3 h in fresh culture media containing 0.5 mgml^{-1} of MTT (Sigma-Aldrich). After incubation, MTT was removed and the cells were treated with an (100 μl) acid isopropanol (0.04 N HCl in isopropanol) for formazan crystal solubilization derived from MTT reduction. Samples aliquots (100 μl) were transferred to 96-well microplates and analysed at Benchmark PlusTM Microplate Spectrophotometer (Bio-Rad[®]) at 540 nm.

Statistical analysis

All assays were performed in triplicate on two independent experiments and the results were expressed as an average of the assays. Cytotoxicity assay results were plotted as the percentage of viable cells normalized to untreated cells. The cytotoxic concentration 50% value (CC_{50}) was calculated by nonlinear regression analysis. Data were compared by one-way analysis of variance followed by the Tukey posttest using GraphPad Prism 9.1.1 (GraphPad Software). p -value <0.05 was considered statistically significant.

RESULTS

Synthesis and characterization of PMAA-AgNPs

Poly(methacrylic acid)-silver nanoparticles showed maximum intensity of the extinction spectrum in 420 nm (Figure 1), which is correlated to the plasmon absorption peak of spherical AgNPs (Maier, 2007). Monodispersed AgNPs, spherical and without significant agglomeration, were observed in transmission electron microscopy image (Figure 2a). Particle size distribution ranges from 10 to 40 nm with a mean size of 15 nm (Figure 2b). The presence of PMAA prevented the particles clustering while they remain in suspension. Poly(methacrylic acid)-silver nanoparticles presented a zeta potential of -41.83 mV .

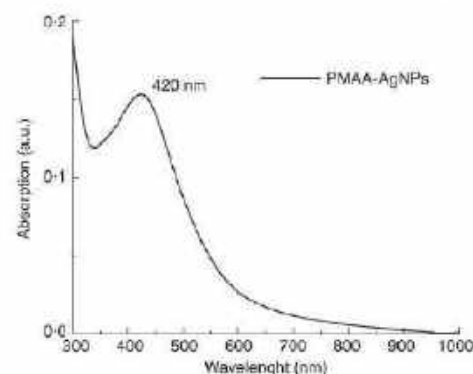


FIGURE 1 UV-visible absorption spectrum of PMAA-AgNPs. PMAA-AgNPs, poly(methacrylic acid)-silver nanoparticles

Antifungal activity

Minimal inhibitory concentration and MFC values of PMAA-AgNPs and antifungals against *C. albicans* are presented in Table 1. Poly(methacrylic acid)-silver nanoparticles showed activity against all *C. albicans* strains tested, including *C. albicans* ATCC 10231 ($\text{MIC } 4.0\text{--}8.0 \mu\text{gml}^{-1}$). Compared to nystatin and fluconazole, amphotericin B showed the strongest activity whose MIC values ranging from 1.0 to 2.0 μgml^{-1} . However, three strains (LFBM Ca01, LFBM Ca05 and LFBM Ca14) showed resistance to this antifungal ($\text{MIC } 2.0 \mu\text{gml}^{-1}$).

All *C. albicans* strains were susceptible to nystatin treatment in a dose-dependent manner. On the other hand, all strains tested showed to be resistant to fluconazole, exhibiting MIC values equal or greater than $1024 \mu\text{gml}^{-1}$. In this regard, fluconazole was selected for the study of the interaction with PMAA-AgNPs.

Checkerboard assay

Minimal inhibitory concentration and FICI values obtained by the combination of PMAA-AgNPs with fluconazole against *C. albicans* strains are presented in Table 2.

Subinhibitory concentrations of PMAA-AgNPs ($\leq 0.5 \times \text{MIC}$) enhanced the anti-*Candida* activity of fluconazole. Among all *C. albicans* strains tested, five (LFBM Ca02, LFBM Ca04, LFBM Ca05, LFBM Ca14, LFBM Ca1002) had their sensitivity to fluconazole restored (initial MIC $1024 \mu\text{gml}^{-1}$; final MIC $4.0\text{--}2.0 \mu\text{gml}^{-1}$). Synergistic interaction ($\text{FICI} \leq 0.5$) was detected in eight strains leading to a reduction of MIC values (PMAA-AgNPs $\geq 50\%$; fluconazole $\geq 96.8\%$). Isobole curve corroborates the synergistic association

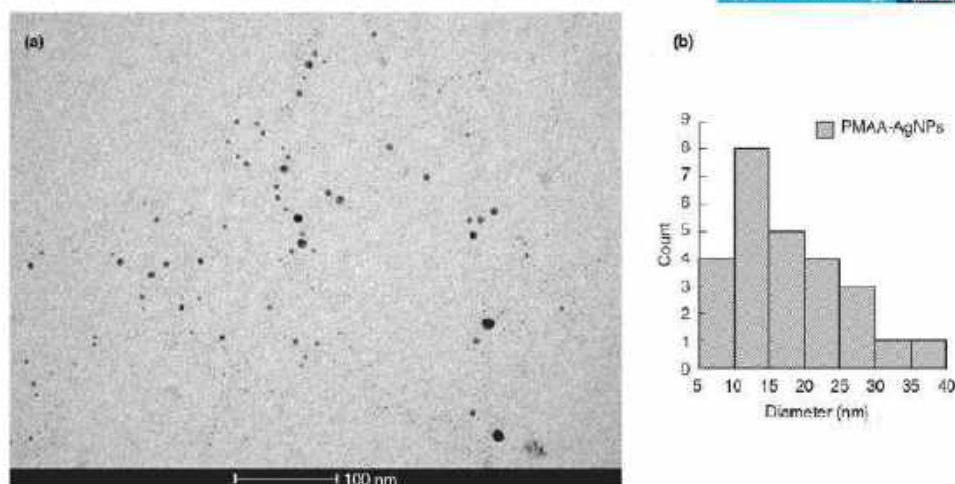


FIGURE 2 Transmission electron microscopy image of PMAA-AgNPs taken after synthesis (a); particle size distribution (b). PMAA-AgNPs, poly(methacrylic acid) silver nanoparticles.

TABLE 1 Minimal inhibitory and minimal fungicidal concentration (MIC/MFC) of PMAA-AgNPs and antifungals against *Candida albicans* strains

<i>Candida albicans</i> strains	MIC/MFC ($\mu\text{g ml}^{-1}$)			
	PMAA-AgNPs	Amphotericin B	Nystatin	Fluconazole
LFBM Ca01	4.0/8.0	2.0/2.0	16.0/32.0	>1024
LFBM Ca02	4.0/8.0	1.0/2.0	16.0/32.0	>1024
LFBM Ca03	4.0/8.0	1.0/2.0	16.0/32.0	>1024
LFBM Ca04	4.0/8.0	1.0/2.0	16.0/32.0	>1024
LFBM Ca05	4.0/8.0	2.0/2.0	16.0/32.0	>1024
LFBM Ca06	4.0/8.0	1.0/2.0	16.0/32.0	>1024
LFBM Ca07	8.0/16.0	1.0/2.0	16.0/32.0	>1024
LFBM Ca14	4.0/8.0	2.0/4.0	16.0/32.0	>1024
LFBM Ca1002	4.0/8.0	1.0/2.0	16.0/32.0	>1024

Abbreviations: LFBM Ca, Laboratory of Physiology and Biochemistry of Microorganisms *Candida albicans*; MFC, minimal fungicidal concentration; MIC, minimal inhibitory concentration; PMAA-AgNPs, poly(methacrylic acid) silver nanoparticles.

between PMAA-AgNPs and fluconazole (Figure 3). The greater synergistic interactions of PMAA-AgNPs + fluconazole was observed for LFBM Ca05 and LFBM Ca07 strains ($\text{FICI} = 0.3$). For these yeasts, the MIC value for fluconazole alone ($\geq 1024 \mu\text{g ml}^{-1}$) was lowered to 1/32–1/256 fold (combined MIC 32.0–4.0 $\mu\text{g ml}^{-1}$), respectively, when it was used in combination with PMAA-AgNPs. Although additive effect was observed against LFBM Ca01 strain ($\text{FICI} = 0.6$), PMAA-AgNPs tested at MIC = 2.0 $\mu\text{g ml}^{-1}$ was able to lower the MIC of fluconazole (individual MIC $\geq 1024 \mu\text{g ml}^{-1}$; combined MIC = 128.0 $\mu\text{g ml}^{-1}$).

Germ tube formation assay

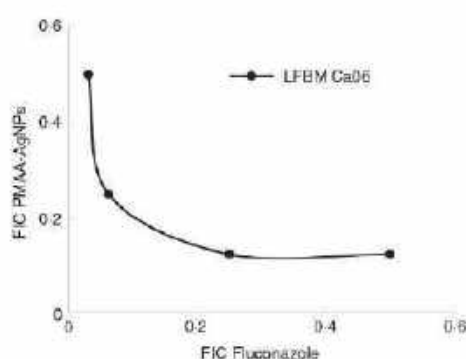
The treatment of *C. albicans* (LFBM Ca06 strain) sub-inhibitory concentrations of PMAA-AgNPs (2.0 or 1.0 $\mu\text{g ml}^{-1}$) or fluconazole (16.0 $\mu\text{g ml}^{-1}$) did not inhibit the germ tube formation (Figure 4a–c). However, when these cultures were exposed at PMAA-AgNPs-fluconazole combination at MIC of 2.0–16.0 or 1.0–16.0 $\mu\text{g ml}^{-1}$ for 3 h, an inhibition of germ tube formation could be observed (Figure 4d). It is noteworthy that germ tube inhibition occurred at concentration 1/64 $\times$ MIC for fluconazole when in combination with $\leq 1/2 \times$ MIC of PMAA-AgNPs.

TABLE 2 Combination testing of the PMAA-AgNPs with fluconazole against *Candida albicans* strains

<i>Candida albicans</i> strains	PMAA-AgNPs/FLC MIC ($\mu\text{g ml}^{-1}$)	PMAA-AgNPs/FLC Individual FIC	FICI	% MIC reduced
LFBM Ca01	2.0/128.0	0.5/0.125	0.6	50.0/87.5
LFBM Ca02	2.0/4.0	0.5/0.004	0.5	50.0/99.6
LFBM Ca03	2.0/16.0	0.5/0.02	0.5	50.0/98.4
LFBM Ca04	2.0/2.0	0.5/0.002	0.5	50.0/99.8
LFBM Ca05	1.0/4.0	0.25/0.004	0.3	75.0/99.6
LFBM Ca06	2.0/16.0	0.5/0.02	0.5	50.0/98.4
LFBM Ca07	2.0/32.0	0.25/0.03	0.3	75.0/96.8
LFBM Ca14	2.0/2.0	0.5/0.002	0.5	50.0/99.8
LFBM Ca1002	2.0/2.0	0.5/0.002	0.5	50.0/99.8

Note: % MIC Reduced = $(\text{MIC alone} - \text{MIC combined}) \times 100 / \text{MIC alone}$.

Abbreviations: FICI, fractional inhibitory concentration index; FLC, fluconazole; LFBM Ca, Laboratory of Physiology and Biochemistry of Microorganisms *Candida albicans*; MIC, minimal inhibitory concentration; PMAA-AgNPs, poly(methacrylic acid)-silver nanoparticles.

**FIGURE 3** Isobole curve revealing the synergistic effect of PMAA-AgNPs with fluconazole against *Candida albicans* LFBM Ca06. PMAA-AgNPs, poly(methacrylic acid)-silver nanoparticles.

Cytotoxicity assay

MTT assay showed a dose-dependent decrease in the viability for all the cell types tested, with CC_{50} values equal to $6.5 \mu\text{g ml}^{-1}$ (± 0.56), $4.9 \mu\text{g ml}^{-1}$ (± 0.25) and $6.8 \mu\text{g ml}^{-1}$ (± 0.36), for macrophages, fibroblasts and Vero Cells, respectively (Figure 5).

DISCUSSION

Silver nanoparticles are promising antimicrobial compounds applied in nanomedicine. Different methods of synthesis, as well the composition of the stabilizer used, produce nanoparticles with singular properties, which depend on the structure, size and shape of nanoparticle

(Akter et al., 2018; Nogueira et al., 2019). The surface characteristics of nanoparticles have an important role on their pharmacological cytotoxic activities (Das et al., 2017). For instance, both the size and the large surface contact area of these nanomaterials can influence its mechanism of action, allowing them to attach to the target cell membrane and easily penetrate, causing damage to cell structure (Hamida et al., 2021; Nogueira et al., 2019).

In this study, AgNPs were synthesized by a fast and easy to perform, nature-friendly method. For this, water is used as a solvent whereas ultra-violet radiation is employed as a catalytic agent of AgNPs, resulting in small size PMAA-AgNPs with great potential for technological development of novel nanocomposites (Carvalho et al., 2018). This method was first described by Spadaro et al. (2009) aimed to make the synthesis of nanoparticles simpler, minimizing the generation of final reaction by-products.

Because PMAA have been described as biocompatible, hydrophilic and cost-effective polymer, this compound was selected in our study as both: a reducer of the silver ions present in aqueous solution (from the precursor AgNO_3), in presence of UV radiation, and a stabilizer, which prevents the formation of clusters in the colloidal solution (Luo et al., 2010). In addition, chemical structure of PMAA presents negatively charged carboxylic groups ($-\text{COO}^-$), attached along the polymeric segments and acts as templates for incoming Ag^+ ions. PMAA offers the advantage of obtaining nanoparticles with more defined size and shape minimizing the chances of clustering and non-homogeneous distribution of AgNPs (Bajpai et al., 2013).

Consistently, zeta potential value of PMAA-AgNPs (-41.83 mV) corroborates a good dispersion and stability of these nanoparticles in solution (Shah et al., 2020), due to surface charge-based repulsion. Furthermore, negatively charged surface also facilitates the interaction between

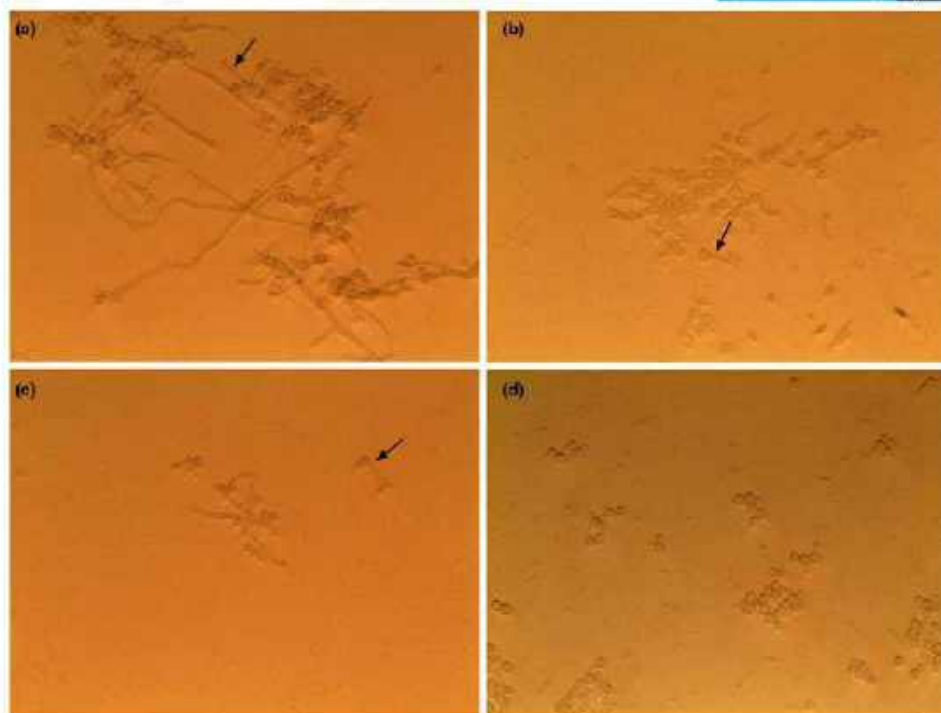


FIGURE 4 Germ tube formation by *Candida albicans* LFBM C06 in fetal bovine serum (a); exposed to fluconazole at $16.0 \mu\text{gml}^{-1}$ (b); PMAA-AgNPs at $2.0 \mu\text{gml}^{-1}$ (c) and germ tube inhibition by PMAA-AgNPs/fluconazole ($2.0 \mu\text{gml}^{-1}/16.0 \mu\text{gml}^{-1}$) combination (d). Arrows indicate germ tube formation. PMAA-AgNPs, poly(methacrylic acid)-silver nanoparticles

nanoparticle and target cell membranes (Nogueira et al., 2019).

An interesting finding of our study was the ability of PMAA-AgNPs to effectively act as an antifungal agent against fluconazole-resistant *C. albicans* strains at low concentrations ($4.0\text{--}8.0 \mu\text{gml}^{-1}$). It is already reported in the literature that AgNPs affect multiple cellular targets in *C. albicans*, altering the morphology and fluidity of its surface membrane, causing rupture of cellular microenvironment and interfering with the ergosterol content and fatty acid composition, especially oleic acid (Radhakrishnan, Dwivedi, et al., 2018; Radhakrishnan, Reddy Mudiam, et al., 2018). These effects could ultimately lead to the shrunk and collapse of yeast (Hamida et al., 2021; Khatoun et al., 2019).

The widespread use of azoles has led to the emergence of acquired resistance in fungal pathogens. Yeasts such as *C. albicans* can be resistant to fluconazole by at least three mechanisms: (1) change in the target enzyme to reduce drug-binding, (2) increase in the amount of target enzyme or (3) prevention from intracellular drug accumulation. A

usual resistance mechanism of *C. albicans* to antifungal is the active transport of drugs out of the cell by overexpression of efflux pumps, reducing the levels of azoles within the cell (Morschhäuser, 2016; Zavrel & White, 2015). Inhibiting and/or modulating the function of efflux pumps as well as the use of drug combinations has been an effective strategy to combat the multidrug resistance of pathogenic microorganisms (Hussain et al., 2019; Singh et al., 2018). Sun et al. (2016) showed that association of AgNPs with fluconazole could decrease expression levels of *CDR1* in *C. albicans*, the gene responsible for decoding of a major drug efflux pump. Thus, AgNPs may reverse resistance to fluconazole, further decreasing dose-dependent side effects and improving efficacy for the treatment.

Our results showed that PMAA-AgNPs combined with fluconazole in subinhibitory concentrations ($\leq 0.5 \times \text{MIC}$) was able to reverse fluconazole resistance in five *C. albicans* clinical strains. These results agree with Hampe et al. (2017). Silver nanoparticles combined with antifungal drugs may reduce the amount of drug and consequently,

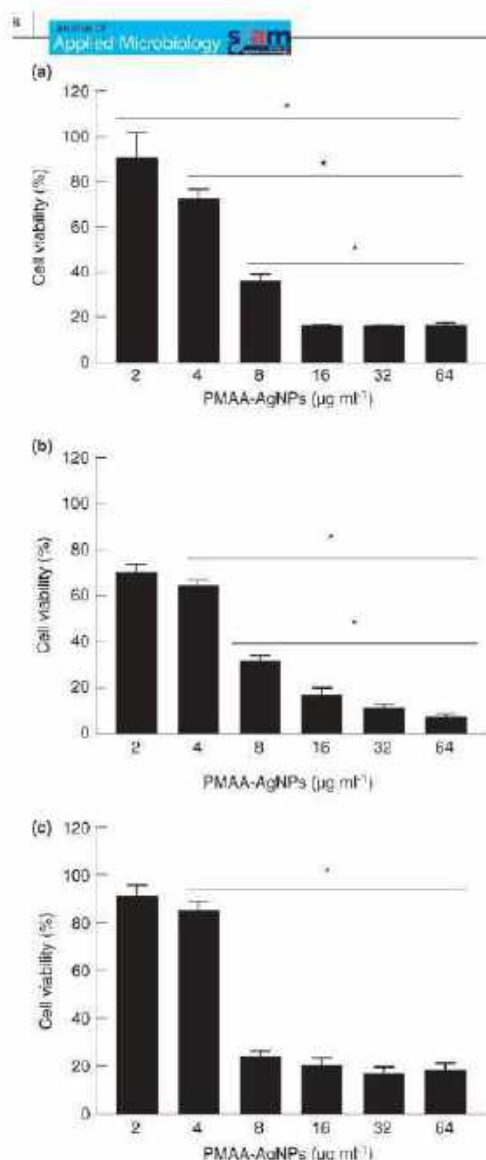


FIGURE 5 Effect of PMAA-AgNPs on viability of J774A.1 macrophages (a), fibroblasts (b) and Vero cells (c). Asterisks (*) denote a statistically significant difference compared to each other ($p \leq 0.05$). PMAA-AgNPs, poly(methacrylic acid)-silver nanoparticles

the cost of treatment and drug-related toxicity, thus providing a safer, more effective and economical alternative compared to the monotherapy (Ahmad et al., 2016).

Similar results were obtained by Longhi et al. (2016) whom evaluated the activity of AgNPs against

fluconazole-resistant *C. albicans* ($\text{MIC} = 4.35 \mu\text{g ml}^{-1}$). These authors showed a reduction of MIC values by 50% ($2.17 \mu\text{g ml}^{-1}$). However, no synergistic effect was found. On the other hand, the treatment with our PMAA-AgNPs caused a decrease of MIC by 75% and a synergistic effect in eight of nine strains when combined with fluconazole. Furthermore, compared to fluconazole alone, our results showed a significant susceptibility of *C. albicans* treated at lower concentration of AgNPs. Hussain et al. (2019) found that combined therapy of fluconazole with AgNPs against *C. albicans* induced drastic morphological depletion.

Germ tube formation is the first step in adhesion to host tissues playing important role in the mechanism of cell invasion by *C. albicans*. Therefore, morphological transition (yeast to hyphal) is pivotal for virulence and biofilm formation. Targeting virulence factors of *C. albicans* can be a useful strategy for the treatment of fungal infections (Halbange et al., 2019; Khatoon et al., 2019). In the present study, PMAA-AgNPs were able to inhibit the germ tube formation, at subinhibitory concentrations when combined with fluconazole.

According to Róžalska et al. (2018), the germ tube formation was also impaired in the presence of AgNPs, in both inhibitory and subinhibitory concentrations ($0.5 \times \text{MIC} = 3.125 \mu\text{g ml}^{-1}$). Halbange et al. (2019) suggested that AgNPs may affect signal transduction pathways in *C. albicans* by downregulating the expression of cell elongation gene (*ECE1*) and hyphal inducer gene (*TEC1*), which are important for yeast-hyphal transition. In this regard, the ability of AgNPs acting on multiple molecular and cellular targets can be crucial to overcoming drug resistance and pathogenicity of fungal cells (Radhakrishnan, Reddy Mudiam, et al., 2018).

The most important parameter to determine the therapeutic application of a given compound is its putative toxicity (Punjabi et al., 2018). Although AgNPs provide many benefits, little is known about their overall toxicity due to the absence of precise comparative analysis among AgNPs with different shapes, sizes and capping agents. Silver nanoparticles' agglomeration and concentration range are important factors affecting the toxicity of these nanoparticles to cells (Akte et al., 2018). Therefore, we have also investigated the effects of PMAA-AgNPs against mammalian cells.

Although in our study a dose-dependent effect of PMAA-AgNPs cytotoxicity can be observed in all the cell type tested, the concentrations required to reduce the mammalian cell viability were considerably higher than those found to inhibit the *C. albicans* growth. Furthermore, none of the tested concentrations were able to reduce the mammalian cells viability by 100%. The treatment with PMAA-AgNPs were able to significantly reduce Vero cells viability only at concentration

superior or equal to $8.0 \mu\text{g ml}^{-1}$. This result is comparable to MFC of most *C. albicans* strains tested. Vero cell line is effective in evaluating the nephrotoxic potential of drug candidate, with a good association between in vivo and in vitro results (Menezes et al., 2013). At subinhibitory concentration ($2.0 \mu\text{g ml}^{-1}$), no significant reduction in the mammalian cell viability was observed. However, the germ tube formation was efficiently inhibited at this same concentration of nanoparticles in combination with fluconazole ($16.0 \mu\text{g ml}^{-1}$).

Hussain et al. (2019) found that spherical AgNPs sizes range between 10 and 90 nm (average size = 80 nm) presented negligible cytotoxicity, suggesting that its use in human at lower concentration is safe, with minimal cell damage to the human cells. It is well known that the cytotoxicity of nanoparticles can be strongly affected by the presence of polymer coatings and surface charge (Akter et al., 2018).

The biocompatibility and bioavailability of nanoparticles are important factors that should be not neglected (Collino et al., 2021). Therefore, the development of novel nanoparticles should be optimized to improve its bioavailability in controlled release formulations. In this regard, PMAA could be a promising drug carrier for the development of smart-controlled release drugs in biomedical applications (Luo et al., 2010).

Synergism between PMAA-AgNPs with fluconazole against resistant *C. albicans* strains indicates that PMAA-AgNPs can overcome fluconazole resistance. Although PMAA-AgNPs presented dose-dependent cytotoxicity on the mammalian cells tested, the inhibitory effect of this nanoparticles alone or in combination was higher in *C. albicans* than in the host cells. This combination can be used carefully as antifungal agent in topical formulations to combat resistant candidiasis. Further in vivo studies are still needed to evaluate the toxicological and pharmaceutical proprieties of PMAA-AgNPs, alone or in combination with fluconazole, aiming to ensure its safety and efficacy in the clinical practice.

ACKNOWLEDGEMENTS

This work was supported by CNPq/FACEPE/CAPES through the National Institute of Photonics (Grant INCT 465763/2014-6). ASLG thanks the financial support from CNPq through grant PQ-1A 307259/2015-3. Support from CAPES to the Graduate Program in Dentistry is also acknowledged (001CAPES-UFPE).

CONFLICT OF INTEREST

No conflict of interest declared.

ORCID

Cecilia Maria Cruz Falcão  <https://orcid.org/0000-0002-8323-0949>

REFERENCES

- Ahmad, A., Wei, Y., Syad, F., Tahir, K., Taj, R., Khan, A.U. et al. (2016) Amphotericin B-conjugated biogenic silver nanoparticles as an innovative strategy for fungal infections. *Microbial Pathogenesis*, 99, 271–281.
- Akter, M., Sikder, M.T., Rahman, M.M., Ullah, A.K.M.A., Hossain, K.F.B., Banik, S. et al. (2018) A systematic review on silver nanoparticles-induced cytotoxicity: physicochemical properties and perspectives. *Journal of Advanced Research*, 9, 1–16.
- Rajput, S., Chand, N. & Mahendra, M. (2013) In situ formation of silver nanoparticles in poly (methacrylic acid) hydrogel for antibacterial applications. *Polymer Engineering & Science*, 53, 1751–1759.
- Carvalho, M.T., de Santana, G.S., de Andrade, A.N., Cabral, A., da Silva Rosa-Leão, N., de Melo-Santos, M.A.V. et al. (2018) Exploiting silver nanoparticles with FMAA against *Aedes aegypti* larvae development: potential larvicidal activity. *Colloidal Nanoparticles for Biomedical Applications XIII*, 10307. <https://doi.org/10.1117/12.2291573>
- CLSI. (2017a) *Performance standards for antifungal susceptibility testing of yeasts*, 1st edition. Wayne, PA: Clinical and Laboratory Standards Institute.
- CLSI. (2017b) *Reference method for broth dilution antifungal susceptibility testing of yeasts*. Wayne, PA: Clinical and Laboratory Standards Institute.
- Collino, C.I., Linao, J.M. & Gutierrez-Millan, C. (2021) Recent advances in functionalized nanomaterials for the diagnosis and treatment of bacterial infections. *Materials Science & Engineering C, Materials for Biological Applications*, 121, 111843.
- Das, B., Tripathy, S., Adhikary, J., Chattopadhyay, S., Mandal, D., Dash, S.K. et al. (2017) Surface modification minimizes the toxicity of silver nanoparticles: an *in vitro* and *in vivo* study. *Journal of Biological Inorganic Chemistry*, 22, 893–918.
- Halboudje, S.D., Ladhav, A.K., Jangid, P.M., Shelar, A.V., Patil, R.H. & Karuppayil, S.M. (2019) Molecular targets of biofabricated silver nanoparticles in *Candida albicans*. *Journal of Antibiotics* (Tokyo), 72, 640–644.
- Hamida, R.S., Ali, M.A., Goda, D.A. & Redhwan, A. (2021) Anticandidal potential of two *Cyanobacteria*-synthesized silver nanoparticles: effects on growth, cell morphology, and key virulence attributes of *Candida albicans*. *Pharmaceutics*, 13, 1688.
- Hampe, I.A.L., Friedman, J., Edgerton, M. & Merschhäuser, J. (2017) An acquired mechanism of antifungal drug resistance simultaneously enables *Candida albicans* to escape from intrinsic host defenses. *PLoS Pathogens*, 13, e1006655.
- Hussain, M.A., Ahmad, D., Anwar, A., Ferveen, S., Ahmed, S., Antis, I. et al. (2019) Combination therapy of clinically approved antifungal drugs is enhanced by conjugation with silver nanoparticles. *International Microbiology*, 22, 239–246.
- Khatoon, N., Sharma, Y., Sarda, M. & Manzoor, N. (2019) Mode of action and anti-*Candida* activity of *Artemisia annua* mediated-synthesized silver nanoparticles. *Journal of Mycology Medicine*, 29, 201–209.
- Lee, B., Lee, M.J., Yun, S.J., Kim, K., Choi, I.H. & Park, S. (2019) Silver nanoparticles induce reactive oxygen species-mediated cell cycle delay and synergistic cytotoxicity with 3-bromopyruvate in *Candida albicans*, but not in *Saccharomyces cerevisiae*. *International Journal of Nanomedicine*, 14, 4801–4816.

- Longhi, C., Santos, J.P., Morey, A.T., Marcatto, P.D., Durán, N., Pinge-Filho, E. et al. (2016) Combination of fluconazole with silver nanoparticles produced by *Fusarium oxysporum* improves antifungal effect against planktonic cells and biofilm of drug-resistant *Candida albicans*. *Medical Mycology*, 54, 428–432.
- Luo, Y.L., Xu, F., Peng, Q.S., Chen, Y.S. & Ma, C. (2010) Preparation and characterization of PMAA/MWCNTs nanohybrid hydrogels with improved mechanical properties. *Journal of Biomedical Materials Research. Part B: Applied Biomaterials*, 92, 243–254.
- Maier, S.A. (2007) *Plasmonics: fundamentals and applications*. New York: Springer Science & Business Media.
- Mutare, T., Nalimmasanga, P., Owunzura, L. & Robertson, V. (2017) Experimental germ tube induction in *Candida albicans*: an evaluation of the effect of sodium bicarbonate on morphogenesis and comparison with pooled human serum. *BioMed Research International*, 2017, 1976273.
- Menezes, C., Valério, E. & Dias, L. (2013) The kidney Vero-E6 cell line: a suitable model to study the toxicity of microcystins. *New Insights into Toxicity and Drug testing*, 2, 29–48.
- Morschhäuser, J. (2016) The development of fluconazole resistance in *Candida albicans* - an example of microevolution of a fungal pathogen. *Journal of Microbiology*, 54, 192–201.
- Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65, 55–63.
- Muñoz, J.L., Roldán, M.V., Rojas, F., de los Angeles Sosa, M., Pellegrini, N. & Giusiano, G. (2019) Antifungal activity of silver nanoparticles in combination with ketoconazole against *Malassezia furfur*. *AMB Express*, 9, 1–9.
- Nadhe, S.R., Singh, R., Wadhvani, S.A. & Chopade, B.A. (2019) *Acinetobacter* sp. mediated synthesis of AgNPs, its optimization, characterization and synergistic antifungal activity against *C. albicans*. *Journal of Applied Microbiology*, 127, 445–458.
- Nguyen, S., Truong, J.Q. & Bruning, J.B. (2020) Targeting unconventional pathways in pursuit of novel antifungals. *Frontiers in Molecular Biosciences*, 7, 621366.
- Nogueira, S.S., de Araujo Nobre, A.R., Mafud, A.C., Guimarães, M.A., Alves, M.M.M., Plácido, A. et al. (2019) Silver nanoparticle stabilized by hydrolyzed collagen and natural polymers: synthesis, characterization and antibacterial-antifungal evaluation. *International Journal of Biological Macromolecules*, 135, 808–814.
- Odds, F.C. (2003) Synergy, antagonism, and what the checkerboard puts between them. *The Journal of Antimicrobial Chemotherapy*, 52, 1.
- Pappas, P.G., Kauffman, C.A., Andes, D.R., Clancy, C.J., Marx, K.A., Ostrosky-Zeichner, L. et al. (2016) Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. *Clinical Infectious Diseases*, 62, e1–e50.
- Pimentel, B.N.A.S., Marin-Dell, F.H., Assis, M., Barbugli, P.A., Longo, F. & Vergani, C.B. (2022) Antifungal activity and biocompatibility of α -AgVO₃, α -Ag₂WO₆, and β -Ag₂MoO₄ using a three-dimensional coculture model of the oral mucosa. *Frontiers in Bioengineering and Biotechnology*, 10, 826123.
- Punjabi, K., Mehta, S., Chavan, R., Chitalla, V., Deogharkar, D. & Deshpande, S. (2018) Efficiency of biosynthesized silver and zinc nanoparticles against multi-drug resistant pathogens. *Frontiers in Microbiology*, 9, 2207.
- Arya, N.R. & Rafiq, N.B. (2020) *Candidians*. Treasure Island (FL): StatPearls Publishing.
- Radhakrishnan, V.S., Dwivedi, S.P., Siddiqui, M.H. & Prasad, T. (2018) In vitro studies on oxidative stress-independent, Ag nanoparticles-induced cell toxicity of. *International Journal of Nanomedicine*, 13, 91–96.
- Radhakrishnan, V.S., Reddy Modam, M.K., Kumar, M., Dwivedi, S.P., Singh, S.P. & Prasad, T. (2018) Silver nanoparticles induced alterations in multiple cellular targets, which are critical for drug susceptibilities and pathogenicity in fungal pathogen. *International Journal of Nanomedicine*, 13, 2647–2663.
- Różalska, R., Sadowska, B., Budzyńska, A., Bernat, P. & Różalska, S. (2018) Biogenic nanosilver synthesized in *Mutarrizium robertsii* waste mycelium extract - as a modulator of *Candida albicans* morphogenesis, membrane lipidome and biofilm. *PLoS One*, 13, e0194254.
- Shah, Z., Hassan, S., Shaheen, K., Khan, S.A., Gul, T., Anwar, Y. et al. (2020) Synthesis of AgNPs coated with secondary metabolites of *Acacia nilotica*: an efficient antimicrobial and detoxification agent for environmental toxic organic pollutants. *Materials Science & Engineering: C. Materials for Biological Applications*, 111, 110829.
- Singh, H.R., Gupta, V.K., Deeba, F., Bajpai, R., Pandey, V., Naqvi, A.H. et al. (2019) Non-toxic and ultra-small biosilver nanoclusters trigger apoptotic cell death in fluconazole-resistant. *Biomolecules*, 9, 47.
- Singh, S., Fatima, Z., Ahmad, K. & Hameed, S. (2018) Fungicidal action of geraniol against *Candida albicans* is potentiated by abrogated CaCdrip drug efflux and fluconazole synergism. *PLoS One*, 13, e0203079.
- Spadaro, D., Barletta, E., Barreca, F., Currò, G. & Neri, F. (2009) PMA capped silver nanoparticles produced by UV-enhanced chemical process. *Applied Surface Science*, 255, 8403–8408.
- Sun, L., Liao, K., Li, Y., Zhao, L., Llang, S., Guo, D. et al. (2016) Synergy between polyvinylpyrrolidone-coated silver nanoparticles and azole antifungal against drug-resistant *Candida albicans*. *Journal of Nanoscience and Nanotechnology*, 16, 2325–2335.
- Zavrel, M. & White, T.C. (2015) Medically important fungi respond to azole drugs: an update. *Future Microbiology*, 10, 1355–1375.

How to cite this article: Falcão, C.M., Andrade, A., Holanda, V.N., de Figueiredo, R.C., Ximenes, E.A. & Gomes, A.S. (2022) Activity of poly(methacrylic acid)-silver nanoparticles on fluconazole-resistant *Candida albicans* strains: Synergistic and cytotoxic effects. *Journal of Applied Microbiology*, 00, 1–10. Available from: <https://doi.org/10.1111/jam.15542>

APÊNDICE C – ARTIGO PUBLICADO NA REVISTA DA ASSOCIAÇÃO MÉDICA BRASILEIRA (FATOR DE IMPACTO: 1.20)

ORIGINAL ARTICLE

Effects of nasal aspiration by the Proetz® method in pediatric patients with sinusitis

Yaskara Amorim Filgueira¹
Vanderlan Nogueira Holanda²
Fernando Luiz Affonso Fonseca³
David Feder³

¹ Departamento de Fisioterapia, Centro Universitário Doutor Leão Sampaio – União Juazeiro do Norte, CE, Brasil

² Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

³ Centro Universitário Saúde ABC, Santo André, SP, Brasil

<https://doi.org/10.1590/1806-9282.66.11.1>

SUMMARY

OBJECTIVE: To characterize the effects of nasal aspiration with Proetz® in peak nasal inspiratory flow (PNIF) in pediatric sinusitis (PS) patients with nasal obstruction.

METHODS: This is a non-randomized descriptive-analytical clinical trial with a quantitative approach. The sample comprised 30 children. Initially, the PNIF was measured and the Visual Analogical Scale (VAS) was used for nasal obstruction, followed by the nasal aspiration procedure. The SNOT-22 questionnaire was applied to the legal guardian of each child, and one week later, it was reapplied for the sake of follow-up.

RESULTS: 16 (53.3%) patients were females and 14 (46.7%) were males, with an average age of 6.4±1.8 years (between 4 and 10 years of age). Analyses of the VAS for obstruction before the intervention revealed that 10 of the participants (33.3%) presented moderate levels, and 20 of them (66.7%) severe levels. However, after the Proetz® method was applied, all the samples (n=30) had mild levels. The PNIF significantly increased after the technique was used, with an improvement of 23.4% in mean values. There was no significant correlation between the VAS and the PNIF.

CONCLUSION: Nasal aspiration with the Proetz® method significantly improved the clinical condition of sinusitis patients with nasal obstruction according to the visual analogical scale, the PNIF, and the SNOT-22 questionnaire. No correlation between the VAS and the PNIF could be found. The study confirms the importance of non-pharmacological interventions in the treatment of sinusitis in children, thus resulting in an improvement in their quality of life.

KEYWORDS: Sinusitis, Respiratory Tract Infections, Child, Nasal Obstruction.

INTRODUCTION

Nasal obstruction is a common symptom of upper-airway disorders in children, and conservative therapy for nasal passage obstruction, whenever indicated, is essential¹. However, to reestablish the drainage pathway by clearing the paranasal sinuses is as important as eliminating the etiologic agent². Some

interference of nasal cleansing and massage over nasal aeration can be observed in children with physiological mouth breathing. Nasal aeration measures showed sensitivity to the cleansing and massage techniques, and measures of nasal geometry confirmed its effect on respiratory physiology³.

DATE OF SUBMISSION: 13-May-2020
DATE OF ACCEPTANCE: 02-Jul-2020
CORRESPONDING AUTHOR: Yaskara Filgueira
Av. Maria Leticia Leite Pereira, s/n, Juazeiro do Norte, CE, Brasil – 63022-145
Tel: +55 88 98832-5774
E-mail: yaskara.filgueira@leao-sampaio.edu.br

In order to relieve the nasal obstruction in the upper airways, some techniques lead to good results, like the nasal instillation of saline solution⁴, therapeutic ultrasound⁵ and nasal aspiration with Proetz®. The method of nasal aspiration with Proetz® was described by and named after Arthur W. Proetz in 1926⁷. It consists of the clearing of the nasal cavity to decongest the upper airways. This technique is performed through the nostrils by means of suction that exerts negative pressure in the nasal cavities. It is based on the air elasticity principle in the paranasal cavities and the gas-compressibility law for the displacement of secretions⁸.

It is known that PA usually causes from 6 to 8 upper airway infectious attacks a year, with high symptomatology of nasal duct obstruction⁹. Therefore, studies on nasal aspiration with the Proetz® method are justified, and if proven effective, it may open doors to the pediatric society to a new therapeutic method based on scientific evidence regarding the efficacy of the technique in the treatment of nasal obstruction.

The guiding question of this study was designed based on the PICO acronym, in which P stands for the Population (pediatric patients - PA), I for Intervention (effects of nasal aspiration with Proetz® method), and CO for Context (nasal obstruction due to sinusitis diagnosis). Therefore, the following question arose: Is the Proetz® method effective for the removal of secretions from the nasal duct in pediatric patients?

The aim of this study was to characterize the effects of nasal aspiration with Proetz® in PNIF in PS patients with nasal obstruction.

METHODS

This is a non-randomized descriptive-analytical clinical trial with a quantitative approach. It was conducted at the Respiratory Physiotherapy Department, pediatric wing, from the Centro Universitário Doutor Leão Sampaio (Unileão), in Juazeiro do Norte, Brazil, in May 2017. The study was approved by the Research Ethics Committee of Unileão under nº 2256330. All the parents or guardians signed a term of consent and patients signed an assent term.

The sample comprised 30 male and female children aged between 4 and 10 years. They were selected by intentional sampling, including sinusitis patients with nasal obstruction whose guardians signed all the terms presented to them. The exclusion criteria were: children with neurologic diseases, with cognitive, renal,

metabolic and/or cardiovascular alterations; those with facial trauma, headache, facial burns, recurrent epistaxis, diagnosis of neoplasia; patients in a mediate or immediate postoperative period; children who could not complete the tests presented by the researchers.

The treatment was carried out in the afternoon, in a climate-controlled environment, at the same temperature proposed in the study by Teixeira et al.¹⁰, namely, between 22 and 24°C. At first, the children were submitted to a clinical examination that included an ectoscopy followed by the measurement of the PNIF and evaluation using the VAS.

The PNIF was measured to analyze how much the nasal obstruction interfered with the intake of air, or if there was no direct influence. The measurement was performed by using an in-check inspiratory flow meter (Clement Clark International) with a padded oronasal mask over the face of the patient. The values of the analysis ranged from 30 to 370 L/min registered on the surface of the cylinder after each inhalation. The result was immediately obtained.

The VAS was carried out to evaluate the level of nasal obstruction according to the child's subjective perception. The scale ranges from 0 to 10, and it is organized by colors that describe the intensity of nasal congestion, which goes from mild to moderate, to severe. This scale has already been used in other studies with good acceptance and promising results for the subjective analysis of pain in children and adolescents, like in the study conducted by Tostes et al.¹¹.

It is important to point out that before the measurement of PNIF was performed, it was clearly and concisely explained to the patient, taking each age into account. The patient was placed in a sitting position, and the mask was properly adjusted to the face so that no air could escape from it. To start the procedure, the command "close your mouth and smell the flower very quickly" was given, which, in a playful way, corresponds to quickly breathing in through the nose. Three measurements were taken from each patient at intervals of 1 minute, and at the end, the highest was selected. If a variation higher than 40 L/min was detected between measurements, a new evaluation was done.

Once the measurements were taken and after a resting period, the SNOT-22 questionnaire was applied. Translated into Portuguese by Kosugi et al.¹², this questionnaire analyzes symptoms during two weeks prior to its application. The questions were adapted so that the legal guardians could answer

them. Although it is self-applicable, the researchers were available to help the respondents, without interfering in their answers, when questions came up.

The SNOT-22 is specific for sino-nasal disorders. It is composed of 22 questions that encompass nasal, paranasal, and psychological symptoms as well as aspects related to the quality of sleep. Each question receives a score from 0 to 5, where 0 is the absence of a condition and 5 is the most severe case of the condition. The questionnaire also evaluates the quality of life of sinusitis patients; therefore, it is also applied whenever interventional processes or therapeutic procedures are to be conducted so that their efficacy and the significant effects on the life of the patient can be analyzed. SNOT-22 is considered the best questionnaire for the evaluation of sinusitis¹⁹.

After all the analyses were carried out, nasal aspiration with the Proetz® method was performed. The patient was in a quiet environment, lying on a stretcher with no head elevation, and the therapist was alongside the head of the stretcher for the procedure. A 10 ml syringe was used for the gradual introduction of 0.9 saline solution to thin the secretions, and then the vacuum aspiration (-150/-180mmHg) technique was applied. The same process was repeated for the second nostril. The whole procedure lasted from 2 to 4 minutes aiming to remove all the secretions from the paranasal sinuses, and the PNIF could then be reassessed.

The statistical analysis was performed using the Shapiro-Wilk test for the analysis of data normality. It was observed that the variable post-inspiratory pressure did not show normal distribution ($p=0.011$), and the variable post-VAS had a probability value close to non-normal distribution (0.071). Hence, the descriptive values mean, standard deviation, median, and 25-75 percentiles were shown. For inferential comparison, the Wilcoxon and Spearman's rank tests were used for the variables from the SNOT-22 questionnaire. The Stata 11 software was used for data analysis.

RESULTS

The sample was composed of 30 children clinically diagnosed with sinusitis. There were 16 females (53.3%) and 14 males (46.7%) at an average age of 6.4 ± 1.8 years, ranging between 4 and 10 years (S^2 3.206 and SE 0.327). The means and medians obtained from the VAS and PNIF before and after the Proetz® method are shown in Table 1.

Initially, it could be observed that the VAS scores significantly improved with the Proetz® method. Before the procedure, 10 (33.30%) of the participants had a moderate score, and 20 (66.70%) had a severe score. However, after the method was used, the entire sample ($n=30$) presented a mild score, indicating that there was a reduction in nasal obstruction according to the patients' perception.

The analysis of the PNIF measurement showed significant improvement after the procedure. Before the application of the technique, the values ranged from 40 to 100 L/min. On the other hand, after the intervention, those values ranged from 50 to 120 L/min in all patients, with an increase of 80% in one of them.

TABLE 1. DESCRIPTIVE VALUES AND TESTING OF HYPOTHESIS FOR THE DIFFERENCES IN THE VISUAL ANALOGICAL SCALE (VAS) AND INSPIRATORY PRESSURE BEFORE AND AFTER THE PROCEDURE.

Indicators	VAS			Inspiratory pressure		
	Before	After	p*	Before	After	p*
Mean (sd)	75 (14)	26 (10)	<0.001	64.0 (20.1)	79.9 (20.5)	<0.001
Median (p25 - p75)	70 (7-8)	2 (2-3)		60 (50-80)	80 (60-100)	

sd = standard deviation; p25 = 25 percentile; p75 = 75 percentile. *Wilcoxon test.

Table 2 shows the Spearman correlation for VAS and PNIF before and after the Proetz® method. No statistical significance could be found in this correlation.

TABLE 2. SPEARMAN CORRELATION BETWEEN VAS BEFORE - INSPIRATORY PRESSURE BEFORE AND VAS AFTER - INSPIRATORY PRESSURE AFTER.

Correlation	Spearman rank correlation	p
VAS before - inspiratory pressure before	0.26	0.168
VAS after - inspiratory pressure after	0.14	0.465

VAS: Visual Analogical Scale

The analyses of the SNOT-22 questionnaire can be found in Table 3.

According to the data supplied by the patients, after the procedure, there was a statistical significance for the variables "need to blow nose", "sneezing", "runny nose", "cough", "post-nasal discharge", "thick nasal discharge", "ear fullness", "ear pain", "facial pain/pressure", "difficulty to fall asleep", "wake up at night", "reduced concentration", "frustrated/restless/irritable", "sad", "decreased sense of smell/taste" and "nasal blockage". The results reveal that there is a direct impact on the patients' quality of life.

TABLE 3. ANALYSIS OF THE VARIABLES FROM THE SNOT-22 QUESTIONNAIRE (N=90)

	BEFORE			AFTER			Wilcoxon ^{***}	
	$\bar{x}$	sd	S ²	$\bar{x}$	sd	S ²	Z	p
Need to blow nose	3.470	0.681	0.464	0.700	0.651	0.424	-4.843	0.001
Sneezing	1.400	1.303	1.697	0.900	0.498	0.248	-3.159	0.002
Runny nose	3.800	0.714	0.510	0.700	0.651	0.424	-4.836	0.001
Cough	0.730	0.786	0.616	0.430	0.504	0.254	-2.066	0.039
Post-nasal discharge	0.930	0.907	0.823	0.500	0.509	0.259	-2.230	0.026
Thick nasal discharge	3.130	1.252	1.565	0.630	0.586	0.309	-4.737	0.001
Ear fullness	0.270	0.450	0.202	0.000	0.000	0.000	-2.828	0.005
Dizziness or vertigo	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000 [†]
Ear pain	0.270	0.450	0.202	0.000	0.000	0.000	-2.828	0.005
Facial pain/pressure	2.570	0.504	0.254	0.630	0.490	0.240	-4.847	0.001
Difficulty to fall asleep	2.270	0.691	0.478	0.670	0.479	0.230	-4.809	0.001
Wake up at night	2.330	0.661	0.437	0.430	0.504	0.254	-4.700	0.001
Lack of a good night's sleep	0.530	0.730	0.533	0.230	0.430	0.185	-1.726	0.084 [*]
Wake up tired	0.430	0.504	0.254	0.230	0.430	0.185	-1.420	0.180 [*]
Fatigue	0.430	0.504	0.254	0.230	0.430	0.185	-1.342	0.180 [*]
Reduced productivity	0.530	0.507	0.257	0.370	0.490	0.240	-1.508	0.132 [*]
Reduced concentration	1.300	0.794	0.631	0.470	0.507	0.257	-3.000	0.001
Frustrated/restless/irritable	2.030	0.689	0.447	0.570	0.504	0.254	-4.932	0.001
Sad	2.000	0.830	0.693	0.130	0.346	0.120	-4.686	0.001
Embarrassed	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000 [†]
Decreased sense of smell/taste	2.030	0.809	0.654	0.470	0.507	0.257	-4.421	0.001
Nasal blockage	3.230	0.898	0.806	0.700	0.651	0.424	-4.390	0.001

$\bar{x}$ (mean); sd (standard deviation); S² (variance); Z (Wilcoxon value); p (significance <0.05); *Statistically non-significant; *** Non-parametric Wilcoxon signed-rank test.

Source: Own authorship.

DISCUSSION

Besides drug therapies and surgical approaches that target the improvement in airway clearance, there are other resources that can bring the breathing function closer to normal. Such resources consist of a clinical procedure called nasal irrigation, which allows for an improvement in bilateral air intake and output through the cleansing of the airways with 0.9 saline and non-invasive aspiration. However, the results obtained from this procedure regarding nasal permeability are usually subjectively evaluated. The quantitative measurements of peak nasal inspiratory and expiratory flow are of utmost importance¹⁴.

A study conducted by Fritz¹⁵ showed the great efficacy of the Proetz® method in children in the treatment of paranasal sinus obstruction, especially the ethmoid sinus. Such results minimize or eliminate the complications brought by sinusitis in this population. Stancic¹⁶ had a sample of 166 children aged between 6 months and 14 years with upper airway obstructions who did not respond to conventional pharmacological treatment. When the Proetz® method was used, it helped in the patients' full recovery from nasal obstruction conditions.

The result obtained from the use of the Proetz® method regarding the improvement in nasal clearance and permeability increases aeration efficiency due to the clearance of the paranasal sinuses. Therefore, the effect of the method on nasal permeability, in structural terms related to the nasal mucosa, is significant, the functional effect on aeration was a fact that can be considered satisfactory for the recovery from nasal obstructive conditions. Tugrul et al.¹⁷ worked with a sample of 90 children suffering from acute rhinosinusitis on whom the nasal obstruction treatment was performed. There was a significance in the values of PNIF after the procedure. Soler et al.¹⁸ conducted a study with individuals between 2 and 21 years old diagnosed with chronic rhinosinusitis. They underwent conservative treatment for 6 months before and after the intervention. According to the results obtained from the SNOT-22 questionnaire, the patients seemed to be confident regarding their overall quality of life with mean values 42.2 ± 19.2 vs. 10.4 ± 9.7 p <0.0001.

Ciprandi et al.¹⁹ studied the correlation of VAS for nasal obstruction and the PNIF. The researchers

confirmed the relevance of the clinical use of the scale, with Spearman's coefficient $\rho=0.879$ ($p<0.001$).

Hence, the application of the method in PA with nasal obstruction is strongly recommended. After the Proetz® method, according to the VAS for nasal obstruction, patients described their pain as mild, and an improvement in nasal obstruction and in nasal aeration could be observed. The insufficient sample size, as well as the lack of comparative literature on the subject (very few similar studies using the same technique in different populations), were factors that posed as limitations to the current study.

CONCLUSION

It could be observed that nasal aspiration with the Proetz® method significantly improved the clinical condition of sinusitis patients with nasal obstruction

according to the visual analogical scale, the PNIF, and the SNOT-22 questionnaire. No correlation between the VAS and the PNIF could be found. The study confirms the importance of the application of non-pharmacological interventions in the treatment of sinusitis in children, thus resulting in an improvement in their quality of life. Additionally, this group of researchers hopes to enrich the national literature with further knowledge on the real effects of Proetz®, a valuable tool for pediatric physiotherapy.

Author's Contribution

Yaskam Amorim Filgueira: Conceptualization, Validation, Investigation, and Writing; Vanderlan Nogueira Holanda: Writing - Review & Editing; Fernando Luiz Affonso Fonseca: Investigation, Supervision, Project Management; David Feder: Investigation, Supervision, Writing - Editing, Project Management.

RESUMO

OBJETIVO: Caracterizar os efeitos da aspiração nasal com Proetz® na pfo. do fluxo inspiratório nasal (Pnif) em pacientes com sinusite pediátrica (SP) com obstrução nasal.

MÉTODOS: Trata-se de um ensaio clínico analítico descritivo, não randomizado, com abordagem quantitativa. A amostra foi composta por 30 crianças. Inicialmente, o Pnif foi medido e a escala visual analógica (EVA) foi utilizada para obstrução nasal, seguida do procedimento de aspiração nasal. O questionário Snot-22 foi aplicado ao responsável legal de cada criança e, uma semana depois, foi reaplicado para fins de acompanhamento.

RESULTADOS: Dezoito (60%) pacientes eram do sexo feminino e 12 (40%) do sexo masculino, com idade média de 6,411,8 anos (entre 4 e 10 anos). A análise da EVA para obstrução antes da intervenção revelou que dez dos participantes (33,3%) apresentaram níveis moderados e 20 deles (66,7%), níveis graves. No entanto, após a aplicação do método Proetz®, todas as amostras (n=30) apresentaram níveis leves. O Pnif aumentou significativamente após a utilização da técnica, com uma melhoria de 23,4% nos valores médios. Não houve correlação significativa entre EVA e Pnif.

CONCLUSÃO: A aspiração nasal com o método Proetz® melhorou significativamente o quadro clínico de pacientes com sinusite com obstrução nasal, de acordo com a escala visual analógica, o Pnif e o questionário Snot-22. Não foi encontrada correlação entre EVA e Pnif. O estudo confirma a importância de intervenções não farmacológicas no tratamento da sinusite em crianças, resultando em melhoria na sua qualidade de vida.

PALAVRAS-CHAVE: Sinusite. Infecções respiratórias. Criança. Obstrução nasal.

REFERENCES

1. Lehtolainen N. Obstruction nasale de l'enfant. *Ann Fr Oto-Rhino-Laryngol Pathol Cervico-Faciale*. 2016;133(3):164-6.
2. Nienkoetter J M, Fernandes M, Schivinski CIS. Fisioterapia e pneumonia em crianças: uma revisão sistemática. *Pediatr Mod*. 2012;48(5).
3. Melo ACC, Gomes ACC, Cunha DA, Lima SJF, Lima WRP, Cunha RR, et al. Muçangas nasais em crianças com respiração oral após a limpeza e massagem nasal. *CoDAS*. 2016;28(6):770-7.
4. Bram TCS. Efeitos da desobstrução rinofaríngea retrograda isolada e associada à instilação de soro fisiológico (0,9% NaCl) sobre as propriedades do muco nasal, a celularidade e as citocinas em lavado nasal e sintomas nasais de otodistas profissionais expostos à poluição da cidade de Belo Horizonte [Tese de doutorado]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2014.
5. Ansari NN, Naghdi S, Fathali M, Bartley J, Rasak MS. A randomized clinical trial comparing pulsed ultrasound and erythromycin phonophoresis in the treatment of patients with chronic rhinosinusitis. *Physiother Theory Pract*. 2015;31(3):166-72.
6. Sonethua E, Sávio ACM, Galdas LS, Gurgel MP. Atuação fisioterapêutica na sinusite crônica. *Rev Bras Fisioter*. 2010;14(supl. 1):436.

7. Gouzy R, Gouzy J. La méthode de déplacement de Proetz. La Lecture d'Oto-rhino-laryngologie et de Chirurgie Cervico-faciale. 1993;247:7-8.
8. Pinto RWNT, Pinto RMM, Tarias UP. Intranasal acute sinus barotrauma. Braz J Otorhinolaryngol. 1975;42(2):162-7.
9. Becker K. Anesthesia in children with a cold. Curr Opin Anaesthesiol. 2012;25(3):333-9.
10. Teixeira RU, Zapodini CE, Alves PS, Costa EA. Peak nasal inspiratory flow evaluation as an objective method of measuring nasal airflow. Braz J Otorhinolaryngol. 2011;77(4):43-80.
11. Tostes MA, Braga JAP, Lins CA, Hilario MOE. Avaliação de dor em crianças e adolescentes portadores de doença falciforme. Rev Clin Méd. 2008;17(3-6):141-7.
12. Kosugi EM, Chen YG, Fonseca VM, Curiano MM, Mendes Neto JA, Gregório LC. Translation, cross-cultural adaptation and validation of SinoNasal Outcome Test (SNOT) - 22 to Brazilian Portuguese. Braz J Otorhinolaryngol. 2011;77(5):663-9.
13. Caminha GP, Melo Junior JT, Hopkins C, Pizzichini E, Pizzichini MM. SNOT-22: psychometric properties and cross-cultural adaptation into the Portuguese Language spoken in Brazil. Braz J Otorhinolaryngol. 2012;78(6):34-9.
14. Silva AML. Rinometria acústica: valores de referência numa população de estudantes universitários [Dissertação de Mestrado]. Lisboa: Faculdade de Ciências Médicas, Universidade Nova de Lisboa; 2012.
15. Fritz K. The adenoids of the child and the irrigation treatment by Proetz in short general anaesthesia (author's transl). Laryngol Rhinol Otol (Stuttgart). 1960;39(1):30-3.
16. Stancic M. Sinobronchial syndrome and obstructive nonspecific pulmonary diseases in children. Srp Arh Celok Lek. 1991;119(11-12):344-8.
17. Tugrul S, Dogan R, Ermi SB, Menn A, Dettua n C. The use of large-volume low pressure nasal saline with fluticasone propionate for the treatment of pediatric acute rhinosinusitis. Int J Pediatr Otorhinolaryngol. 2014;78(6):1393-9.
18. Soler ZM, Rosenbloom JS, Skarada D, Gutman M, Hui M, Nguyen SA. Prospective, multicenter evaluation of balloon sinus dilation for treatment of pediatric chronic rhinosinusitis. Int Forum Allergy Rhinol. 2017;7(3):225-9.
19. Ciprandi G, Mora F, Cassano M, Gallini AM, Mora R. Visual Analog Scale (VAS) and nasal obstruction in persistent allergic rhinitis. Otolaryngology Head Neck Surg. 2009;141(4):527-9.



ANEXO A – CERTIFICADO DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS

CENTRO UNIVERSITÁRIO DR.
LEÃO SAMPAIO - UNILEÃO



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ESTUDO DO EFEITO DE HETEROCÍCLICOS 1,2,3-TRIAZÓLICOS E 1,2,4-OXADIAZÓLICOS SOBRE CÉLULAS MONONUCLEARES DO SANGUE PERIFÉRICO (PBMC) E HEMÁCIAS HUMANAS

Pesquisador: VANDERLAN NOGUEIRA HOLANDA

Área Temática:

Versão: 3

CAAE: 38726620.6.0000.5048

Instituição Proponente: INSTITUTO LEAO SAMPAIO DE ENSINO UNIVERSITARIO LTDA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.323.854

Apresentação do Projeto:

O projeto trata sobre os efeitos dos derivados de 1,2,3-triazol e 1,2,4-oxadiazol sobre células do sangue periférico humano e seus possíveis efeitos adversos em sistemas biológicos, com objetivo de diminuir a quantidade de fármacos com alto potencial tóxico no tratamento de patologias humanas ou até mesmo corrigi-las ainda na fase de experimentação pré-clínica. Dentre as possibilidades de testes in vitro, a avaliação do efeito de candidatos a fármacos em amostras humanas, fornece informações que se aproximam dos testes clínicos, possibilitando traçar de maneira mais fidedigna o perfil toxicológico dessas substâncias.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar o efeito dos derivados de 1,2,3-triazol e 1,2,4-oxadiazol sobre células do sangue periférico humano.

Objetivo Secundário:

Determinar o percentual hemolítico dos derivados de 1,2,3-triazol e 1,2,4-oxadiazol em hemácias de fenótipos A, B, AB e O e o efeito sobre PBMC humano

Avaliação dos Riscos e Benefícios:

Os riscos relacionados a coleta de sangue venoso são:

Endereço: Av. Maria Leticia Leite Pereira, s/n

Bairro: Planalto

CEP: 63.010-970

UF: CE

Município: JUAZEIRO DO NORTE

Telefone: (88)2101-1033

Fax: (88)2101-1033

E-mail: cep.leaoesampaio@leaoesampaio.edu.br

**CENTRO UNIVERSITÁRIO DR.
LEÃO SAMPAIO - UNILEÃO**



Continuação do Parecer: 4.323.854

- a) ocorrência de dor no local da punção;
- b) formação de hematoma;
- c) ardência;

Benefícios:

Os benefícios relacionados a participação na pesquisa são:

- a) identificação do grupo sanguíneo;
- b) contribuição para a elucidação do efeito de candidatos a fármacos contra a leishmaniose sobre células do sangue;

Comentários e Considerações sobre a Pesquisa:

A pesquisa apresenta-se como de grande importância no âmbito dos estudos bioquímicos e farmacológicos. Com o avanço da ciência é possível desenvolver novos compostos que possuam potencial para combater as Leishmanioses e dentre eles pode-se citar heterociclos já conhecidos, como os oxadiazóis e triazóis.

Considerações sobre os Termos de apresentação obrigatória:

O pesquisador apresentou:

1. Projeto na plataforma Brasil;
2. Folha de rosto devidamente assinada;
3. Instrumento para coleta dos dados;
4. Termo de Consentimento Livre Esclarecido;
5. Termo de Consentimento Pós - Esclarecido;
6. Projeto detalhado em Pdf;
7. Cronograma de Execução;
8. Carta de Anuência.

Recomendações:

Recomenda-se que após a realização do projeto seja enviado para este CEP o relatório final da pesquisa.

Conclusões ou Pendências e Lista de Inadequações:

Todas as pendências foram sanadas e elucidadas

Considerações Finais a critério do CEP:

Endereço: Av. Maria Leticia Leite Pereira, s/n
 Bairro: Planalto CEP: 63.010-970
 UF: CE Município: JUAZEIRO DO NORTE
 Telefone: (88)2101-1033 Fax: (88)2101-1033 E-mail: cep.leaosampaio@leaosampaio.edu.br

**CENTRO UNIVERSITÁRIO DR.
LEÃO SAMPAIO - UNILEÃO**



Continuação do Parecer: 4.323.854

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1635465.pdf	06/10/2020 17:02:52		Aceito
Projeto Detalhado / Brochura Investigador	Doc1.pdf	06/10/2020 17:02:39	VANDERLAN NOGUEIRA HOLANDA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Doc2.pdf	06/10/2020 17:02:15	VANDERLAN NOGUEIRA HOLANDA	Aceito
Outros	Doc6.pdf	01/10/2020 19:26:02	VANDERLAN NOGUEIRA HOLANDA	Aceito
Folha de Rosto	DOC.pdf	23/09/2020 15:00:18	VANDERLAN NOGUEIRA HOLANDA	Aceito
Outros	Doc4.pdf	22/09/2020 20:23:06	VANDERLAN NOGUEIRA HOLANDA	Aceito
Outros	Doc3.pdf	22/09/2020 20:11:36	VANDERLAN NOGUEIRA HOLANDA	Aceito
Cronograma	Doc5.pdf	22/09/2020 20:09:03	VANDERLAN NOGUEIRA HOLANDA	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

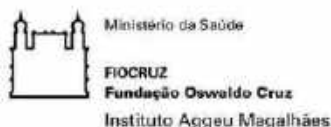
Não

JUAZEIRO DO NORTE, 06 de Outubro de 2020

Assinado por:
JOSE LEANDRO DE ALMEIDA NETO
(Coordenador(a))

Endereço: Av. Maria Leticia Leite Pereira, s/n
Bairro: Planalto CEP: 63.010-970
UF: CE Município: JUAZEIRO DO NORTE
Telefone: (88)2101-1033 Fax: (88)2101-1033 E-mail: cep.leaosampaio@leaosampaio.edu.br

ANEXO B – CERTIFICADO DE APROVAÇÃO DA COMISSÃO ÉTICA NO USO DE ANIMAIS (CEUA)



COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado “**SÍNTESE E AVALIAÇÃO DO POTENCIAL DE NOVOS HETEROCÍCLICOS NITROGENADOS NO DESENVOLVIMENTO DE NOVOS FÁRMACOS CONTRA A LEISHMANIOSE E DOENÇA DE CHAGAS**” protocolado sob nº **146/2019** pela pesquisadora Regina Celia Bressan Queiroz de Figueiredo está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Instituto de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (CEUA/IAM). Na presente versão, este projeto está licenciado e tem validade até 30 de dezembro de 2023 com a finalidade de pesquisa científica. É responsabilidade da coordenadora do projeto notificar à CEUA de quaisquer alterações em relação ao projeto. A coordenadora concorda que nenhuma dessas mudanças serão implementadas antes de serem aprovadas pela CEUA/IAM.

Quantitativo de Animais Aprovados								
Animal	Espécie e linhagem	Quant. (total)	Sexo			Idade	Peso	Origem
			♂	♀	Ambos			
Camundongo	BALB/c	744	708	36		30 dias de idade	± 20g	Biotério IAM
Camundongo	Swiss	184	184			30 dias de idade	± 20g	Biotério IAM
Total		928						

Recife (PE, Brasil), 29 de Abril de 2020

Virginia M. Barros de Lorena

Virginia Maria Barros de Lorena
Coordenadora CEUA/IAM
Mat. SIAPE 1.984.445