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*Ação in vitro do óleo essencial de Lippia alba em culturas de promastigotas de Leishmania
chagasi*

ITUIUTABA

2025

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Ação *in vitro* do óleo essencial de *Lippia alba* em culturas de promastigotas de *Leishmania chagasi*

Trabalho de Conclusão de Curso apresentado ao Instituto de Ciências Exatas e Naturais do Pontal da Universidade Federal de Uberlândia como requisito parcial para obtenção do título de bacharel em Ciências Biológicas.

Área de concentração: Parasitologia

Orientador: Prof^ª Dr^ª Karine Rezende de Oliveira

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À minha mãe e minha avó, que sempre
estiveram ao meu lado e tornaram possível
essa conquista.

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“Se escutar uma voz dentro de você dizendo
‘Você não é um pintor’, então pinte sem parar,
de todos os modos possíveis, e aquela voz será
silenciada.”

(Vincent van Gogh)

RESUMO

As leishmanioses são doenças de caráter zoonótico, causadas por protozoários do gênero *Leishmania* e, com alta incidência mundial. A forma visceral apresenta alta letalidade e os tratamentos existentes se baseiam em fármacos de manipulação endovenosa ou intramuscular, com taxas de reincidência e contraindicações. Por sua vez, a planta *Lippia alba* já foi descrita como agente antiparasitário contra helmintos e protozoários sanguíneos. Assim, este estudo buscou avaliar a ação direta do óleo essencial de *L. alba* sob formas promastigotas de *Leishmania chagasi*. O óleo da planta foi extraído por hidrodestilação e posteriormente quimiotipado por cromatografia gasosa e espectrometria de massas. Em paralelo, foram cultivadas formas promastigotas de *L. chagasi* em cultura axênica, a 28°C. Subsequentemente, os parasitos foram incubados em placa de 96 poços com diferentes diluições do óleo, e em seguida, tratados com uma solução reveladora de resazurina para a leitura no fluorímetro (550nm de excitação e 590nm de emissão). A partir dos valores de densidade óptica obtidos calculou-se a viabilidade parasitária. A quimiotipagem demonstrou que a *L. alba* utilizada neste ensaio pertence ao quimiotipo linalol. Os ensaios *in vitro* revelaram que, na maior concentração testada, 500µg/mL, o óleo demonstrou melhor ação inibitória, eliminando aproximadamente 32% dos parasitos. Portanto, foi constatado que o óleo essencial de *L. alba*, em altas concentrações, exibiu potencial inibitório contra as promastigotas de *L. chagasi in vitro*.

Palavras-chave: Leishmaniose; Óleo essencial; *Lippia alba*; *Leishmania chagasi*; Promastigotas.

ABSTRACT

Leishmaniasis are zoonotic diseases caused by protozoa of the genus *Leishmania* spp., with a high global incidence. The visceral form are often highly lethal, and existing treatments rely on intravenous or intramuscular administration of drugs, which are associated with recurrence and significant contraindications. Notably, the plant *Lippia alba* has been previously described as an antiparasitic agent effective against both blood and tissue helminths (worms) and protozoa. Thus, this study aimed to evaluate the direct effect of *Lippia alba* essential oil on promastigotes of *Leishmania chagasi*. The oil was extracted via hydrodistillation and subsequently underwent chemotyping by gas chromatography and mass spectrometry. Simultaneously, *L. chagasi* promastigotes were maintained in axenic culture at 28°C. The parasites were then incubated with different dilutions of the essential oil and treated with a resazurin-based developer solution for fluorescence measurement using a fluorimeter (550 nm excitation, 590 nm emission). Parasitic viability was determined based on the optical density values obtained. Chemotyping revealed that the *L. alba* sample used in this study belongs to the linalool chemotype. *In vitro* assays demonstrated that the inhibitory effect of the essential oil was most pronounced at the highest concentration tested (500µg/mL), achieving approximately 32% of the parasites. Thus, the essential oil of *L. alba* at high concentrations exhibited potential inhibitory activity against *L. chagasi in vitro*.

Keywords: Leishmaniasis; Essential oil; *Lippia alba*; *Leishmania chagasi*; Promastigotes.

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1 INTRODUÇÃO

A leishmaniose visceral é uma doença de caráter zoonótico e de alta incidência mundial, ocorrendo, principalmente, em países em desenvolvimento (Yamey, 2002), com cerca de 50 a 90 mil casos registrados por ano, de acordo com dados da *World Health Organization* – WHO (2023). A doença se destaca por ser o quadro clínico mais grave, além de ser um problema endêmico de mais de 100 países, entre eles o Brasil, com uma população de risco estimada de 350 milhões de pessoas (Akhoundi *et al.*, 2016). A transmissão se dá por meio da picada da fêmea de flebotomíneos infectada, dos gêneros *Phlebotomus* ou *Lutzomyia*, tidos popularmente como “mosquitos-palha” (WHO, 2023).

Entre os agentes etiológicos responsáveis pelo desenvolvimento da doença estão protozoários pertencentes à família Trypanosomatidae, do gênero *Leishmania*, e pertencente ao complexo “*Leishmania donovani*”, a *Leishmania chagasi* é uma das espécies causadoras da forma visceral da doença (WHO, 2023). Os parasitos deste grupo específico possuem algumas características que os distinguem dos demais, destacando-se o tropismo visceral, condição esta que os possibilita invadir órgãos profundos depois da colonização das células da pele, e que, conforme afirmam Zhang *et al.* (2014), pode estar associada à uma sequência de polimorfismos ou amplificações em alguns genes do parasito.

As opções de tratamento existentes ainda são escassas e baseadas principalmente em antimoniais pentavalentes comercializados a mais de 40 anos, o que propiciou o desenvolvimento de resistência por muitas cepas (Brochu *et al.*, 2003; Brochu, Haimeur, Ouellette, 2004; Croft, Barrett, Urbina, 2005; Choudhury *et al.*, 2008). Além disso, tais fármacos apresentam contraindicações e efeitos colaterais nocivos, como nefrotoxicidade, pancreatite e cardiotoxicidade (Zaghloul & Al-Jasser, 2004; Matoussi *et al.*, 2007; Shahian & Alborzi, 2009).

Com base nessas evidências, é necessário desenvolver terapias alternativas que apresentem menores taxas de danos adversos ao paciente e maior especificidade ao parasito. Nesse sentido, os produtos naturais são uma importante fonte de moléculas ativas com atividade farmacológica, e com grande potencial para o desenvolvimento de novos tratamentos (Raynaud-Le Grandic *et al.*, 2004; Osorio *et al.*, 2007; Albernaz *et al.*, 2010; De Medeiros *et al.*, 2011; Neira *et al.*, 2018; Teles *et al.*, 2019; Marques *et al.*, 2020; Araújo *et al.*, 2022).

Lippia alba (Mill.) N. E. Br. é uma espécie de planta aromática com flor, pertencente à família Verbenaceae, nativa da América Latina (Brasil), que se desenvolve naturalmente em

solos arenosos, nas margens de rios e lagos, em regiões de clima tropical e subtropical (Zoghbi *et al.*, 1998). Tareau, Palisse e Odonne (2017), em um estudo de levantamento etnofarmacológico, destacaram sua ampla utilização pela comunidade em geral, principalmente contra problemas digestivos e respiratórios, também salientando seus efeitos, sedativo e detoxificante. Além disso, estudos também já demonstraram potencial antimicrobiano do óleo essencial da planta contra protozoários sanguíneos da família Trypanosomatidae (Neira *et al.*, 2018; Pagotti *et al.*, 2021).

Com base nisto, e tendo em vista a importância da leishmaniose como problema de Saúde Pública (WHO, 2023), este trabalho teve como principal objetivo investigar a ação do óleo essencial de *L. alba* em culturas de promastigotas de *L. chagasi*, a fim de identificar uma possível terapia alternativa para a Leishmaniose Visceral.

2 DESENVOLVIMENTO

2.1 Revisão Bibliográfica

Os protozoários pertencentes à família Trypanosomatidae, do gênero, *Leishmania*, são os agentes etiológicos responsáveis pelas diferentes manifestações das leishmanioses, doenças ainda muito negligenciadas e conhecidas por sua ocorrência, principalmente, em países em desenvolvimento (Yamey, 2002). Elas podem se apresentar na forma de quatro entidades nosológicas distintas, a depender da espécie envolvida na infecção e da área geográfica, podendo estas ser, cutânea, cutâneo-mucosa, cutânea difusa ou visceral (Calazar) (Rey, 2008).

É característico dos parasitos deste grupo apresentarem, em seus ciclos vitais, uma fase intracelular nos tecidos do hospedeiro, denominada amastigota, e outra, que se desenvolve no tubo digestivo dos vetores invertebrados, chamada promastigota. Tais patógenos são transmitidos aos hospedeiros vertebrados através da picada da fêmea de um flebotomíneo infectada, dos gêneros *Phlebotomus* ou *Lutzomyia*, tidos popularmente como “mosquitos-palha”. Até o momento, já foram identificadas cerca de 50 espécies do parasito, das quais 20 ocasionam as variadas síndromes da doença em mamíferos (Rey, 2008; Santarém; Cardoso; Cordeiro-da-Silva, 2023).

Entre estas, e pertencente ao complexo “*Leishmania donovani*”, a *Leishmania chagasi* é uma das espécies causadoras da forma visceral da patologia. Este grupo específico possui algumas características que os distinguem dos demais, entre as quais destaca-se o tropismo visceral. Esta condição é o que a possibilita viver em temperaturas em torno dos 37°C, sendo uma adaptação crucial para a invasão de órgãos profundos depois da colonização das células da pele (Rey, 2008).

Por outro lado, Campos-Ponce *et al.*, (2005) citado no trabalho de Laurenti *et al.*, (2021), aponta para uma provável relação existente entre as condições do sistema imunológico do paciente e a capacidade da patologia avançar para os órgãos internos. Em indivíduos imunocompetentes, o parasito pode ser eliminado naturalmente pela ativação dos fagócitos em células efectoras, que acontece através da secreção de citocinas, como o interferon gama (IFN- γ), pelas células T e *natural killers* (Kolodziej & Kiderlen, 2005).

Com base nisso, e em razão de uma resposta imunológica inata imatura, associados a quadros de desnutrição, observa-se que, crianças com menos de 10 anos de idade são muito mais suscetíveis a desenvolver casos mais graves de leishmaniose visceral (Kimberlin *et al.*,

2021). Scarpini *et al.* (2022), em seu trabalho de revisão, indica que tal fenômeno se segue após a infecção dos macrófagos da epiderme, uma vez que alguns desses, não tendo sido estimulados a extinguir o microrganismo, podem migrar para os órgãos linfoides e, de lá, alcançar regiões mais profundas.

Em consequência disso, o indivíduo acometido pode apresentar quadros sintomatológicos que incluem febre, perda de peso, esplenomegalia, hepatomegalia variável, pancitopenia (com maior frequência anemia e trombocitopenia), aumento no nível de enzimas hepáticas, e hipoalbuminemia com hipergammaglobulinemia (Bern *et al.*, 2005; Aronson *et al.*, 2016; Kimberlin *et al.*, 2021).

Cabe ressaltar que, *L. chagasi* se mantém disponível na natureza graças a reservatórios vertebrados, principalmente cães, mas também em outros animais silvestres, tais como canídeos, gambás e roedores sinantrópicos, apresentando assim um caráter zoonótico (Bern; Maguire; Alvar, 2008; Molina *et al.*, 2012; Pace, 2014; Eshaghi; Hajihassani, 2021; Mohebbi, 2013 *apud* Safavi, 2021).

O calazar é o quadro clínico mais grave dentre as possíveis manifestações da leishmaniose, além de ser um problema endêmico de mais de 100 países, entre eles o Brasil, com uma população de risco estimada de 350 milhões de pessoas (Santarém; Cardoso; Cordeiro-da-Silva, 2023). Também destaca-se o fato de que, de acordo com a *Pan American Organization* associada a Organização Mundial da Saúde (OMS), em 2022 foram registrados em oito países da América Latina um total de 1.834 novos casos da doença, sendo 92% notificados no Brasil.

Entre as formas de tratamento, um levantamento bibliográfico realizado por Teixeira *et al.* (2019), reforçou a existência de escassas opções de quimioterápicos, comercializadas a mais de 40 anos, o que propiciou o desenvolvimento de resistência por muitas cepas. Entre esses fármacos, destacam-se, principalmente, o antimoniato de meglumina (Glucantime®) e a Anfotericina B, que apresentam efeitos colaterais nocivos, como nefrotoxicidade, anorexia, dor abdominal, letargia e cardiotoxicidade (Croft; Oliaro, 2011; De Menezes *et al.*, 2015 *apud* Teixeira *et al.*, 2019).

Com base nesse dilema, evidencia-se a necessidade de terapias alternativas que apresentem menores taxas de danos adversos ao paciente e maior especificidade ao parasito. A partir daí, alguns estudos, como os realizados por Kolodziej & Kiderlen (2005), demonstraram que determinados produtos de origem natural e extratos de plantas podem apresentar atividades imunomodulatórias em macrófagos, tornando-os células efectoras. Os autores também salientaram para a importância de a droga “apresentar efeito leishmanicida

direto e seletivo, bem como conseguir estimular devidamente o sistema imunológico do paciente”, sem o comprometimento de células próprias, como é o caso das medicações ainda utilizadas.

Em um de seus trabalhos onde foram testados 67 tipos de taninos e compostos estruturalmente relacionados em culturas de *L. donovani* e *L. major*, Kolodziej & Kiderlen (2005), concluíram que nenhuma das substâncias, com exceção de derivados do ácido cafeico, apresentou atividade leishmanicida contra as formas promastigotas. No entanto, os autores observaram o contrário ao realizar os testes nas formas amastigotas, sugerindo que a célula fagocítica foi estimulada a secretar óxido nítrico (NO) contra os patógenos.

Em adendo, Marques *et al.* (2020) demonstraram, em seu estudo patenteado com o cafeato de isopentila (ICaf), molécula derivada do ácido cafeico, resultados promissores contra *Leishmania*, bem como a possibilidade de melhorar sua solubilidade ao associá-la à β -ciclodextrina, tornando-a biodisponível no organismo. Tal iniciativa vem de encontro a uma possível solução para uma das dificuldades consideradas na revisão realizada por Souza *et al.* (2022), para a utilização de óleos essenciais (OEs) como terapêutica, tendo em vista que são substâncias “altamente voláteis, com baixa estabilidade oxidativa, hidrofobicidade e, dependendo do tipo e concentração, citotoxicidade ao macrófago”.

Como forma de solucionar tais dificuldades, trabalhos já têm demonstrado que a hidrofobicidade pode ser superada através da formulação do óleo em nanococleatos ou lipossomas (Machín *et al.*, 2019), assim como a citotoxicidade aos macrófagos pode ser reduzida pela nanoencapsulação do composto (Galvão *et al.*, 2020).

Souza *et al.* (2022), em seu trabalho de revisão, também reuniram uma série de estudos com OEs contra diversas espécies de *Leishmania*, onde a grande maioria demonstrou grande potencial antiparasitário. Os autores ainda defendem que os OEs possuem ação direta no patógeno através da indução da morte celular programada, como foi identificado por Islamuddin *et al.* (2014) ao visualizar a externalização da fosfatidilserina para o folheto externo da membrana mitocondrial, e fragmentação do DNA nuclear. Porém, conforme afirmam Carvalho *et al.* (2017), as formas amastigotas tendem a ser mais sensíveis, uma vez que os macrófagos também podem ser estimulados a aumentar a liberação NO, o que favorece a eliminação do parasito.

Além disso, Bouyahya *et al.* (2017) identificou que o mesmo OE pode apresentar diferentes valores de Concentração Inibitória Máxima (IC₅₀) em diferentes espécies de *Leishmania*, o que sugere um potencial específico dos compostos aos seus alvos.

Ao considerar a estrutura química dos OEs, Tasdemir *et al.* (2019) e Silva *et al.* (2017), sugerem que a presença de anéis aromáticos e grupos hidroxilas nas formulações é essencial para a atuação eficiente e direta no patógeno. Hamdi *et al.* (2018), contribui pontuando que a quiralidade dos componentes isolados também aparenta ser um fator importante na atividade antileishmanicida.

Reforçando a temática, Rottini (2011), verificou a atividade dos óleos de *Citrus aurantium*, *Citrus limon*, *Eucalyptus globulus*, *Endlicheria bracteolata* e o composto isolado da *Matricaria chamomilla*, o α -bisabolol, em culturas de promastigotas e amastigotas intracelulares de *L. amazonensis*. Em decorrência dos experimentos, a autora constatou que todos os compostos apresentaram algum efeito leishmanicida contra as formas promastigotas, dos quais o OE de *E. bracteolata* e o princípio ativo α -bisabolol demonstraram excelente ação antiparasitária em amastigotas intracelulares.

Em outra investigação executada por Oliveira *et al.* (2009), OEs de três vegetais, sendo um deles nativos do Cerrado Nordeste, a *Lippia sidoides* Cham, popularmente conhecida por alecrim-pimenta, foram testados em promastigotas de *L. chagasi*. A planta nordestina, cujo quimiotipo predominante era p-Cimeno e Timol, demonstrou efeito ultraestrutural no parasito, sendo possível observar “células disformes, algumas delas com processos mitóticos anormais, e aumento no número e na densidade eletrônica de gotículas de lipídios” (Oliveira *et al.*, 2009). Outra alteração observada foi a “vacuolização citoplasmática, inchaço do corpo celular, do bolso flagelar, das mitocôndrias e das acidocalcisomas” (Oliveira *et al.*, 2009).

A partir desses achados, de Medeiros *et al.* (2011) desenvolveram outro estudo com a mesma planta, a fim de verificar sua ação em promastigotas e amastigotas de *L. amazonensis*. Assim como o grupo anterior, as mesmas alterações ultraestruturais foram observadas, bem como a inibição do crescimento celular do parasito, principalmente das amastigotas intracelulares (IC₅₀ de 34,4 μ g/mL).

Estes estudos abrem portas para investigações quanto ao potencial leishmanicida de outros espécimes pertencentes ao gênero *Lippia*, como por exemplo, a *Lippia alba*, popularmente conhecida por melissa ou erva-cidreira.

Lippia alba (Mill.) N. E. Br. é uma espécie de planta aromática com flor, pertencente à família Verbenaceae, nativa da América Latina (Brasil), que se desenvolve naturalmente em solos arenosos, nas margens de rios e lagos, em regiões de clima tropical e subtropical (Atti-Serafini, 2002 *apud* Silva *et al.*, 2006).

De maneira geral, pode apresentar-se desde formas herbáceas rasteiras, a arbustos e até árvores de pequeno porte (Silva *et al.*, 2006). Tareau, Palisse e Odonne (2017), em um estudo de levantamento etnofarmacológico, destacaram sua ampla utilização pela comunidade em geral, principalmente contra problemas digestivos e respiratórios, também salientando seus efeitos, sedativo e detoxificante.

Em um trabalho de revisão realizado por Hennebelle *et al.* (2008), foram reunidos diversos estudos de aplicações da planta, em diferentes formatos (extratos alcoólicos e óleo essencial), e a partir deles, verificaram-se atividades antimicrobiana (bactérias e fungos, e em alguns casos vírus e protozoários), neuro sedativa, anti-inflamatória, analgésica e antioxidante.

No contexto do potencial antimicrobiano da planta, com foco nos protozoários sanguíneos, Pagotti *et al.* (2021) verificou que o óleo essencial de *L. alba*, do quimiotipo linalol, foi ativo contra formas tripomastigotas e amastigotas de *Trypanosoma cruzi*, o agente etiológico causador da Doença de Chagas. De encontro a este apontamento, Neira *et al.* (2018) identificou que o óleo essencial de *L. alba*, do quimiotipo citral, apresentou atividade contra formas promastigotas e amastigotas de *Leishmania braziliensis*, sendo 2 a 3 vezes mais específico ao parasito do que aos macrófagos.

Tais achados têm sua fundamentação na constituição química dos metabólitos secundários produzidos pela planta para sua defesa ou atração de polinizadores, podendo estes ser das classes dos compostos fenólicos, terpenos ou alcalóides (Cid; Teixeira, 2017). No caso da espécie em questão, inúmeras pesquisas já identificaram mais de um quimiotipo predominante, uma vez que eles podem variar conforme a localidade, o clima, a época do ano em que é realizada a colheita, e a presença ou ausência de predadores (Mattos, 1996 *apud* Silva *et al.*, 2006; Gomes *et al.*, 2019).

3 METODOLOGIA

3.1 Material Botânico

A coleta da espécie da *L. alba* utilizada neste trabalho foi realizada entre os meses de agosto e novembro de 2023, na UFU, *Campus* Pontal, em Ituiutaba. A exsicata da planta está depositada no Herbarium Uberlandense da UFU (HUFU), com número de registro 71.842, na cidade de Uberlândia - MG (Reflora, 2024).

3.2 Extração e purificação do óleo essencial

O óleo essencial foi extraído no Laboratório de Botânica e Ecologia no Domínio Cerrado (LABEC), no Instituto de Ciências Exatas e Naturais do Pontal - UFU, por hidrodestilação, em aparelho do tipo Clevenger, durante 2h, como está descrito na VI edição da Farmacopéia Brasileira, nos meses de março e maio de 2024.

O procedimento se deu de forma seriada devido à capacidade limitada do balão volumétrico, sendo, portanto, executadas 6 extrações, de 35g de material vegetal desidratado com 750mL de água destilada, a fim de se obter ao final, aproximadamente, 1,5 mL de óleo.

O material foi devidamente purificado para a remoção de resíduos de água que estivessem diluídos a partir da solubilização da amostra em diclorometano e posterior adição de sulfato de sódio anidro. A mistura foi filtrada para remoção do precipitado, e o sobrenadante foi coletado em um recipiente coberto por papel alumínio que foi deixado por um período de 24 horas no fluxo laminar até a total evaporação do reagente. O óleo foi armazenado em vidro âmbar e condicionado a -20°C até utilização experimental.

3.3 Quimiotipagem do óleo essencial

O óleo essencial foi quimiotipado no Laboratório de Nanobiotecnologia Prof. Dr. Luiz Ricardo Goulart, da UFU, *Campus* Umuarama, através de cromatografia gasosa e espectrometria de massa (CG/MS). As análises foram realizadas em instrumento da *Agilent Technologies* modelo 7890B GC System/5977B GC/MSD, utilizando coluna cromatográfica capilar Agilent 19091S-433 HP-5ms (30m x 250µm x 0,25µm). A temperatura do injetor foi de 210°C, tendo hélio de alta pureza como gás de arraste, a uma taxa de fluxo constante de 0,9 mL min⁻¹. Foram feitas injeções de 2µL utilizando o modo Split 10:1. O modo Scan (MS)

foi configurado para m/z 35–550, com uma contagem de 150 limiar e atraso de solvente de 3 min. O quadrupolo foi mantido a 150°C com a fonte de íons a 280°C. A temperatura inicial do forno foi programada de 60°C aumentando a uma taxa de 3°C min⁻¹ até 246°C, mantendo essa temperatura por 30 minutos.

Os constituintes químicos do óleo foram identificados através da análise do cromatograma no programa computacional, *Agilent MassHunter Unknowns Analysis*, onde foi possível realizar a comparação dos espectros de massa com base no banco de dados da biblioteca NIST17.L.

3.4 Cultivo dos Parasitos

Para este estudo foram utilizadas promastigotas de *Leishmania chagasi* (MCAN/BR/2002/BH401), cordialmente cedidas pela Dr^a Iasmin Aparecida Cunha Araújo, do Laboratório de Imunopatologia da UFU, *Campus Umuarama*.

Os parasitos foram cultivados em meio α -MEM (10,1g/L), suplementado com 10% (v/v) de Soro Fetal Bovino (SFB) inativado, 100U/mL de penicilina e 100 μ g/mL de estreptomicina, 2mM de L-Glutamina, 20mM HEPES, pH 7,2, a 24 $\pm$ 1°C, em incubadora sob demanda biológica de oxigênio (BOD).

3.5 Ensaios in vitro de viabilidade das promastigotas de *Leishmania chagasi* frente diferentes concentrações do óleo essencial

Os valores de densidade óptica obtidos foram avaliados através do ensaio fluorimétrico por resazurina (Araújo *et al.*, 2022). Os parasitos foram diluídos em meio α -MEM completo, e semeados em placa de 96 poços, de forma a se obter 2,5x10⁵ parasitos por poço. O óleo essencial foi diluído de forma seriada em meio α -MEM completo, em sete concentrações (500-7,8 μ g/mL), segundo Araújo *et al.* (2022), e incubado junto aos parasitos por 48h, a 26°C em estufa. Em sequência, adicionou-se 20 μ L de uma solução de resazurina (0,15 mg/ml) em cada poço, 24h antes da leitura no equipamento. Cada concentração foi testada em triplicata. Como controle positivo, diferentes concentrações de Anfotericina B foram utilizadas (500-7,8ng/mL), e para controle negativo, apenas parasitos diluídos em meio completo. Ambos os controles também foram testados em triplicata.

3.6 Análises estatísticas

Os resultados foram avaliados no programa *GraphPad Prism* 10 com determinação dos gráficos de dose-resposta. O cálculo da inibição parasitária foi realizado a partir dos valores de densidade óptica obtidos dos ensaios com as concentrações de óleo essencial, comparados aos controles positivo e negativo.

4 RESULTADOS E DISCUSSÃO

4.1 Obtenção e quimiotipagem do óleo essencial de *Lippia alba*

Ao final do processo de hidrodestilação, obteve-se aproximadamente 1,5 mL de óleo essencial de *L. alba*, apresentando um rendimento médio de 0,97%, considerando as seis extrações seriadas ao todo.

Após a quimiotipagem, foram identificados um total de 63 compostos significativos (Tabela 1), dos quais 10 apresentaram maior abundância e contribuíram majoritariamente para a definição do quimiotipo da planta (Tabela 2). Tais dados estão, em sua grande maioria, em conformidade com a literatura consultada (Adams, 2007), e podem ser confirmados no cromatograma exposto na

Figura 1.

Destacam-se particularmente entre os componentes identificados, o linalol (51,2%), o eucaliptol (7,67%) e o óxido de cariofileno (5,5%), que contribuíram especialmente para a definição do quimiotipo da *L. alba* utilizada neste ensaio. Vale ressaltar que na estrutura molecular de tais compostos (Figura 2) estão presentes funções orgânicas importantes para a ação direta e mais específica ao parasito, entre elas, grupos hidroxilas e anéis aromáticos, bem como carbonos quirais (Silva *et al.*, 2017; Hamdi *et al.*, 2018; Tasdemir *et al.*, 2019).

Tabela 1 – Composição qualitativa e quantitativa do óleo essencial de *L. alba*, determinado por GC/MS – 63 constituintes significativos.

TR (min)*	Composto	Match factor**	Abundância relativa (%)***
7,4419	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)- ou Sabineno	94,73	1,468
7,9448	β -Myrcene	97,2	0,326
9,4535	Eucalyptol ou 1,8-cineol	96,3	7,671
9,9908	β -Ocimene	98,5	0,663
10,8148	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1 α ,2 α ,5 α)- ou Hidrato de sabineno	96,4	0,221
11,0194	<i>trans</i> -Linalool oxide (furanoid)	97,7	0,248
11,6252	α -Terpineol	88,3	0,199

12,6425	Linalool	89,3	51,201
13,0882	<i>p</i> -Mentha-1,5,8-triene	96,0	0,208
13,4311	<i>p</i> -Mentha-1,5,8-triene	96,8	0,367
13,8998	Propanoic acid, 2-methyl-, 3-hexenyl ester, (E)-	92,0	0,407
14,0712	(+)- <i>cis</i> -Verbenol, acetate	92,0	0,12
14,2198	<i>trans</i> -2-Hexenyl isobutyrate	91,4	0,219
14,7913	Pinocarvone	94	0,177
14,9742	α -Terpineol	89,1	0,209
16,0257	L- α -Terpineol	98,2	0,763
16,2314	(1R)-(-)-Myrtenal	97,9	0,109
16,46	(+)- <i>cis</i> -Verbenol, acetate	93,3	1,94
16,8487	Carveol	91,4	3,535
18,1974	Neral	96,4	0,301
19,5118	Citral	98,1	0,339
22,735	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-, acetate	94	0,329
24,3352	Copaene	98,2	0,147
25,0324	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1 α ,2 β ,4 β)]- ou β -Elemeno	97,9	0,696
26,2668	Caryophyllene	99,6	2,3
26,8155	γ -Elemene	96,7	0,101
27,707	Humulene	97,4	0,599
28,0156	Aromandendrene	97,6	0,196
28,6785	γ -Muurolene	94,5	0,198
28,8957	Germacrene D	96,4	2,529
29,4443	Cubebol	96,5	0,309
29,6615	α -Muurolene	97,8	0,166
29,8901	β -Elemeno	97,5	0,348
30,313	Cubebol	95,9	0,931
30,6102	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S- <i>cis</i>)-	96,9	0,249
31,216	Ledene oxide-(II)	90,8	0,265
31,8332	Ledene oxide-(II)	93,8	0,336

32,0503	Germacrene B	99,3	1,948
32,2332	Nerolidol	96,7	0,798
33,1476	Caryophyllene oxide	95,4	5,557
33,4905	Salvial-4(14)-en-1-one	91,1	0,173
33,9363	β -costol	90,6	0,129
34,1306	Humulenol-II	92,6	0,881
34,3363	4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S,4R,4aS,8aR)-	96,2	0,23
34,5649	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-, [1S-(1 α ,4 α ,4a β ,8a β)]-	91,8	0,179
34,8621	Isospathulenol	94,8	0,239
35,1821	10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5 β -ol	94,4	0,135
35,5365	Aromadendrene oxide-(2)	91	0,369
35,6965	Ylangenol	90,5	0,269
35,8336	(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	88,6	0,414
36,1651	Ylangenal	98,1	1,015
36,348	Cadala-1(10),3,8-triene	83,2	0,108
36,5308	(-)-Spathulenol	95,3	0,488
36,8623	(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	81,9	0,131
37,0795	(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	93,1	0,324
37,9253	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	90,6	0,243
38,2682	(-)-Spathulenol	94,1	0,369
38,4396	Isoaromadendrene epoxide	92,6	0,273
39,7998	β -Copaen-4 α -ol	93	0,693
40,0627	(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	92,5	0,208
40,177	β -Oplopenone	84,8	0,233
62,3509	Heptacosane	94,7	0,297

76,1581	Heptacosane	94,5	0,247
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*TR: Tempo de Retenção dos compostos determinado em minutos.

****Comparação dos espectros de massas obtidos a partir da análise pelo programa *Unknowns* Agilent com os dados da biblioteca NIST17.L.**

***Abundância relativa de cada composto na amostra considerando a área do cromatograma correspondente a cada pico.

Fonte: A autora, 2025.

Tabela 2 – Os 10 constituintes com maior abundância na composição química do óleo essencial de *L. alba*, como determinado por GC/MS.

TR*	Composto	Match	Abundância relativa
(min)		factor**	(%)***
12,6425	Linalool	89,3	51,201
9,4535	Eucalyptol ou 1,8-cineol	96,3	7,671
33,1476	Caryophyllene oxide	95,4	5,557
16,8487	Carveol	91,4	3,535
28,8957	Germacrene D	96,4	2,529
26,2668	Caryophyllene	99,6	2,3
32,0503	Germacrene B	99,3	1,948
16,46	(+)- <i>cis</i> -Verbenol, acetate	93,3	1,94
7,4419	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)- ou Sabineno	94,73	1,468
36,1651	Ylangenal	98,1	1,015

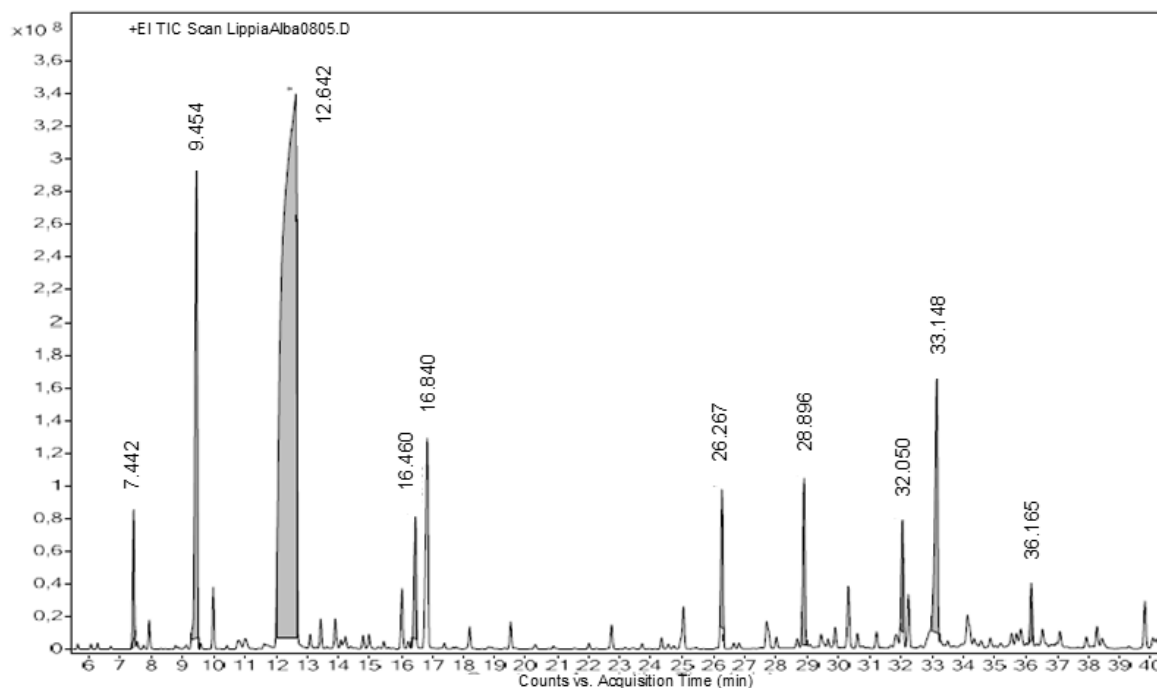
*TR: Tempo de Retenção dos compostos determinado em minutos.

****Comparação dos espectros de massas obtidos a partir da análise pelo programa *Unknowns* Agilent com os dados da biblioteca NIST17.L.**

***Abundância relativa de cada composto na amostra considerando a área do cromatograma correspondente a cada pico.

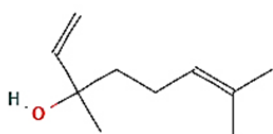
Fonte: A autora, 2025.

Figura 1 – Cromatograma de Íons Totais do óleo essencial de *L. alba*, com amplificação de 6 a 40 minutos.



Fonte: A autora, 2025.

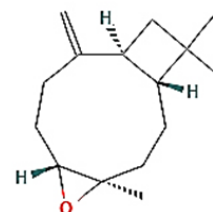
Figura 2 – Estrutura química das substâncias de maior abundância na composição do óleo essencial. (a) Linalool; (b) Eucalyptol; (c) Caryophyllene oxide.



a) Linalool



b) Eucalyptol



c) Caryophyllene oxide

Fonte: National Center for Biotechnology Information (2024).

4.2 Testes *in vitro* do óleo essencial de *L. alba* nas culturas de promastigotas de *L. chagasi*.

Os resultados obtidos neste estudo demonstraram que, em altas concentrações (500 µg/mL), o óleo essencial de *L. alba* apresentou ação contra cerca de 32% das promastigotas de *L. chagasi* em comparação com o controle negativo (

Figura 3). Por esse motivo, o índice de Concentração Inibitória necessária para eliminar 50% dos parasitos (IC₅₀) não foi demonstrado.

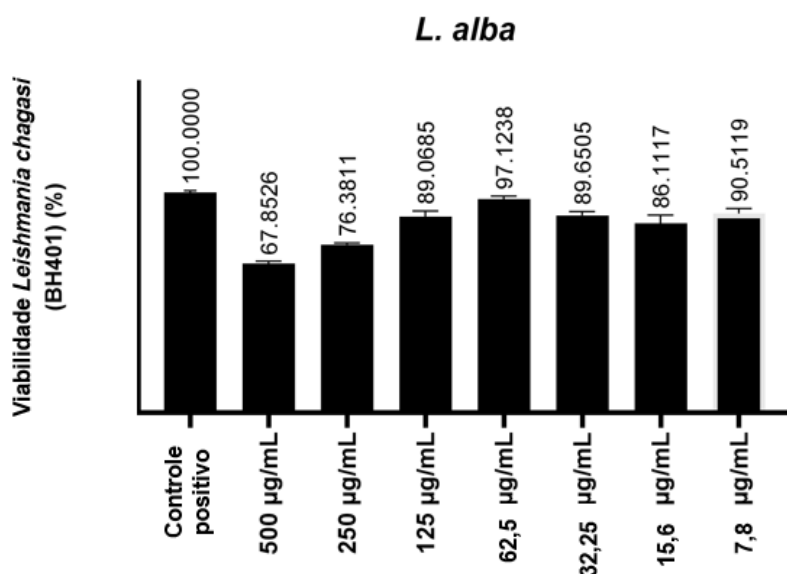
Na Tabela 3 estão descritas as porcentagens de inibição parasitária para cada concentração de óleo testada.

Tabela 3 – Índices de inibição parasitária mediante a aplicação de diferentes concentrações do óleo essencial de *L. alba* (500-7,8µg/mL), nas culturas *in vitro* de *L. chagasi*.

Concentração de óleo	Inibição parasitária (%)
500 µg/mL	32,1474
250 µg/mL	23,6189
125 µg/mL	10,9316
62,5 µg/mL	2,8762
31,25 µg/mL	10,3495
15,6 µg/mL	13,8883
7,8 µg/mL	9,4881

Fonte: A autora, 2025.

Figura 3 – Testes de viabilidade parasitária mediante diferentes concentrações do óleo essencial de *L. alba* (500-7,8µg/mL).



Fonte: A autora, 2025.

Tais resultados sugerem a necessidade de mais estudos serem realizados para detalhar quais dos compostos com maior representatividade no óleo poderiam ter tido participação principal na atividade contra os parasitos, mesmo que mínimo. Para isto, ensaios com esses componentes isolados ou até mesmo nanoencapsulados, podem demonstrar com mais precisão os mecanismos de ação, e propor melhor assimilação da droga pelo parasito (Assolini *et al.*, 2020).

5 CONCLUSÃO

Neste trabalho demonstrou-se que o óleo essencial de *L. alba*, quimiotipo linalol, na concentração de 500µg/mL, apresentou ação contra cerca de 32% das promastigotas de *L. chagasi in vitro*, tornando-se necessário mais estudos complementares a fim de validar esta ação antiparasitária.

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APÊNDICE A – ARTIGO CIENTÍFICO

***In vitro* action of *Lippia alba* essential oil in *Leishmania chagasi* promastigote cultures**

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Abstract

Leishmaniasis are zoonotic diseases caused by protozoa of the genus *Leishmania* spp., with a high global incidence. The visceral form is often highly lethal, and existing treatments rely on intravenous or intramuscular administration of drugs, which are associated with recurrence and significant contraindications. Notably, the plant *Lippia alba* has been previously described as an antiparasitic agent effective against both blood and tissue worms and protozoa. Thus, this study aimed to evaluate the direct effect of *L. alba* essential oil on promastigotes of *Leishmania chagasi*. The oil was extracted via hydrodistillation and subsequently underwent chemotyping by gas chromatography and mass spectrometry. Simultaneously, *L. chagasi* promastigotes were maintained in axenic culture with a suitable medium at 28°C. The parasites were then incubated with different dilutions of the essential oil and treated with a resazurin-based developer solution for fluorescence measurement using a fluorimeter (550 nm excitation, 590 nm emission). Parasitic viability was determined based on the optical density values obtained. Chemotyping revealed that the *L. alba* sample used in this study belongs to the linalool chemotype. *In vitro* assays demonstrated that the inhibitory effect of the essential oil was most pronounced at the highest concentration tested (500µg/mL), achieving approximately 32% of the parasites. Despite this limitation, the

essential oil of *L. alba* at high concentrations exhibited potential inhibitory activity against *L. chagasi* *in vitro*.

Keywords: Leishmaniasis; Essential oil; *Lippia Alba*; *Leishmania chagasi*; Promastigotes.

Introduction

Leishmaniasis are zoonotic diseases with a high worldwide prevalence, occurring mainly in developing countries [1]. According to the World Health Organization (2023) [2], about 50.000 to 90.000 cases of the most severe form of leishmaniasis are reported per year. The disease can manifest in the cutaneous (tegumentary) and/or visceral forms, the latter also known as kala-azar [2]. In turn, the latter stands out as the most severe clinical condition. In addition, it is an endemic problem in more than 100 countries, including Brazil, with an estimated risk population of 350 million people [3].

One of the etiological agents responsible for the disease's development is the protozoa that belongs to the Trypanosomatidae family of the genus *Leishmania* and the “*Leishmania donovani*” complex; *Leishmania chagasi* is one of the species that causes the visceral form of the disease [2]. The parasites of this specific group have some characteristics that distinguish them from the others, especially visceral tropism which is their ability to invade deep organs after colonizing skin cells. This ability, according to Zhang and colleagues [4], may be determined by a sequence of polymorphisms or amplifications in some genes of the parasite. In addition, the parasite is transmitted through the bite of an infected female sandfly of the genera *Phlebotomus* or *Lutzomyia* [2].

Existing treatment options are still scarce and based mainly on pentavalent antimonials marketed for more than 40 years, which has led many strains of *Leishmania* spp. to develop resistance against it [5-8]. In addition, such drugs have contraindications and harmful side effects, such as nephrotoxicity, pancreatitis, and cardiotoxicity [9-11]. Based on this evidence, it is necessary to develop alternative therapies that have few adverse effects to the patient and higher selectivity to the parasite. Thus, natural products are an essential source of active molecules with pharmacological activity and high potential for developing new treatments [12-16].

Lippia alba (Mill.) N. E. Br. is a species of aromatic flowering plant that belongs to the Verbenaceae family, native to Latin America (Brazil), which develops naturally in sandy soils, on the banks of rivers and lakes, in tropical and subtropical climate regions [17]. Considering the plant's antimicrobial potential, Pagotti et al. [18] found that the essential oil

(EO) of *L. alba*, of the linalool chemotype, was also active against trypomastigotes and amastigotes of *Trypanosoma cruzi*, a blood protozoan that also belongs to the Trypanosomatidae family, and which causes the Chagas disease.

Among the possible mechanisms of action of EOs in parasites, studies point that the presence of chirality, aromatic rings, and hydroxyl groups in the formulations of the chemical components might be essential for the action on the pathogen to be efficient and direct [19-21]. Thus, based on this and given the importance of leishmaniasis as a public health problem [2], this study aimed to investigate the action of *L. alba* EO on *L. chagasi* promastigotes in order to identify a possible alternative therapy for treating visceral form.

Materials and Methods

Botanical material

The *L. alba* plant used in this study was collected between August and November 2023 at the Federal University of Uberlândia, Pontal *Campus*, in Ituiutaba (Minas Gerais). The plant's exsiccate is deposited at the Herbarium Uberlandense of Federal University of Uberlândia (HUFU), under the registration number 71.842, in Uberlândia-MG.

Oil extraction

The essential oil was extracted by hydrodistillation in a cleverger apparatus for 2 hours, as described in the 7th edition of the Brazilian Pharmacopoeia [22]. The extraction occurred at the Laboratory of Botany and Ecology in the Cerrado Domain (LABEC in Portuguese) at the Institute of Exact and Natural Sciences of Pontal-UFU.

Due to the volumetric flask's limited capacity, the procedure took place in a serial manner. Six extractions of 35g of dehydrated plant material with 750 mL of distilled water were performed to obtain approximately 1.5 mL of oil at the end.

The material was properly purified from the solubilization of the sample in dichloromethane and the subsequent addition of anhydrous sodium sulfate. The mixture was filtered to remove the precipitate, and the supernatant was collected in a container covered with aluminum foil that was left in the laminar flow for 24 hours until the reagent had totally evaporated. The oil was stored in amber glass and taken to the freezer until the subsequent tests.

Chemotyping

The essential oil was characterized at the Nanobiotechnology Laboratory Prof. Dr. Luiz Ricardo Goulart at UFU, Umuarama *Campus*, using gas chromatography coupled with mass spectrometry (GC-MS).

The analyses were conducted on an Agilent Technologies 7890B GC System coupled with a 5977B GC/MSD detector, equipped with an Agilent 19091S-433 HP-5ms capillary chromatographic column (30m x 250 μ m x 0.25 μ m). The injector temperature was set to 210°C, with high-purity helium as the carrier gas at a constant flow rate of 0.9 mL^{min}⁻¹. Injections of 2 μ L were performed in Split mode 10:1. The mass spectrometry (MS) was configured for an m/z range of 35-550, with a threshold count of 150- and 3-minutes solvent delay. The quadrupole temperature was maintained at 150°C, and the ion source was set to 280°C. The oven temperature was programmed to start at 60°C, increasing at a rate of 3°C min⁻¹ until reaching 246°C, where it was held for 30 minutes.

The chromatographic data was analyzed using the Agilent MassHunter Unknowns Analysis software, which identified the constituents of the essential oil. The mass spectra of the components were compared with NIST17 library database for compound identification.

Parasites Cultures

This study used promastigotes of *Leishmania chagasi* (MCAN/BR /2002/BH401), which were cordially provided by Dr. Iasmin Aparecida Cunha Araújo from the Immunopathology Laboratory of the UFU, Umuarama *Campus*.

Parasites were grown in inactivated α -MEM medium (Sigma Aldrich, San Luis, Missouri, USA) (10.1g/L) supplemented with 10% (v/v) Fetal Bovine Serum (Sigma Aldrich, San Luis, Missouri, USA), 100U/mL penicillin and 100 μ g/mL streptomycin, 2mM L-glutamine, 20mM (Gibco™), HEPES, pH 7.2, at 24 $\pm$ 1°C, in a BOD incubator.

***In vitro* viability assays of *Leishmania chagasi* promastigotes against different concentrations of *L. alba* essential oil**

The optical density values obtained were evaluated using a resazurin colorimetric assay (Sigma Aldrich, San Luis, Missouri, USA) [16]. Parasites were diluted in supplemented α -MEM medium and maintained in a 96-well plate, with a final concentration of 2.5x10⁵ parasites per well. After, the essential oil was serially diluted in complete α -MEM medium at seven concentrations (500-7.8 μ g/mL) and incubated with the parasites for 48 hours at 26°C in an oven. Next, 20 μ L of a resazurin solution (0.15 mg/ml) was added to each well 24 hours

before reading the fluorimeter (Spectramax M2, Molecular Devices LLC, USA) at 550 nm excitation and 590 nm emission.

Each concentration was tested in triplicate. As a positive control, different concentrations of Amphotericin B (Sigma Aldrich, San Luis, Missouri, USA) were used (500-7.8ng/mL). For negative control, only parasites diluted in a complete medium were used. Both controls were also tested in triplicate.

Statistical analyses

Results were evaluated in the GraphPad Prism 10 program using dose-response plots. The parasitic inhibition was calculated from the optical density values obtained from the tests with the concentrations of essential oil, which were compared with the positive and negative controls.

Results and Discussions

Obtaining and chemotyping the essential oil of *Lippia alba*.

At the end of the hydrodistillation process, approximately 1.5 mL of oil was obtained, with an average yield of 0.97%, considering the six serial extractions performed.

After chemotyping, a total of 63 significant compounds were identified, of which ten were more abundant and contributed mainly to defining the plant chemotype (Table 1). Such data is mostly in accordance with the literature consulted [23] and can be confirmed in the chromatogram (Fig. 1) shows. Particularly noteworthy among the identified components are linalool (51.2%), eucalyptol (7.67%), and caryophyllene oxide (5.5%), which contributed mainly to the definition of the *L. alba* chemotype used in this assay. Notably, the molecular structure of such compounds contains organic functions that are important for direct and more parasite-specific action, including hydroxyl groups, aromatic rings, and chiral carbons [19-21].

Table 1 The ten constituents with the highest abundance in the chemical composition of *Lippia alba essential* oil, as GC/MS determines.

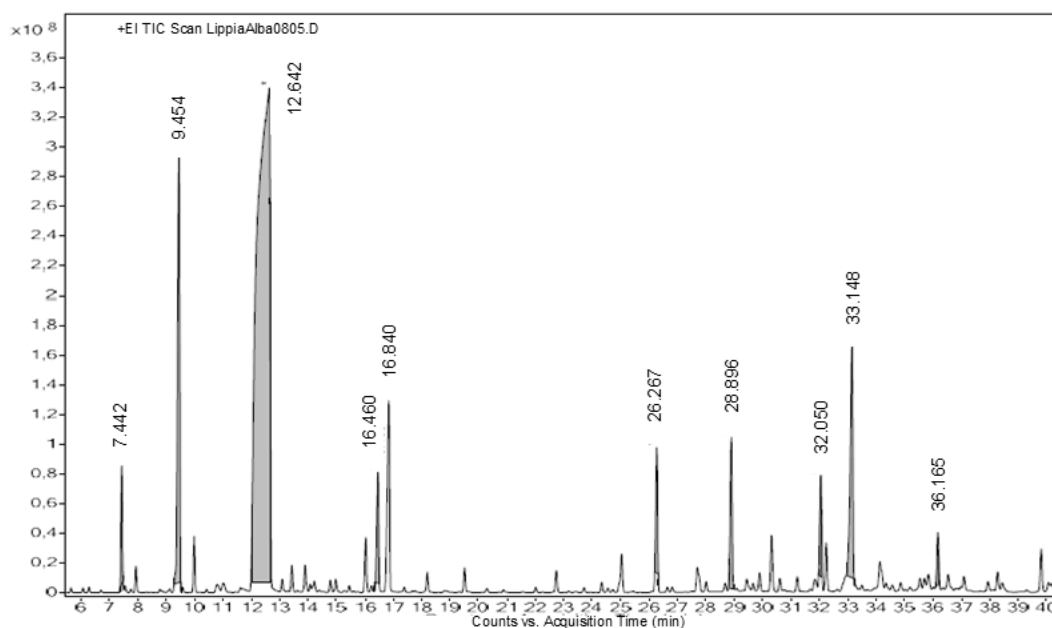
TR* (min)	Compound	Match factor**	Relative Abundance (%)***
12,6425	Linalool	89,3	51,201
9,4535	Eucalyptol ou 1,8-cineol	96,3	7,671
33,1476	Caryophyllene oxide	95,4	5,557
16,8487	Carveol	91,4	3,535
28,8957	Germacrene D	96,4	2,529
26,2668	Caryophyllene	99,6	2,3
32,0503	Germacrene B	99,3	1,948
16,46	(+)- <i>cis</i> -Verbenol, acetate	93,3	1,94
7,4419	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)- ou Sabineno	94,73	1,468
36,1651	Ylangenal	98,1	1,015

*Retention time of compounds determined in minutes.

**Comparison of the mass spectra obtained from the analysis by the Unknowns Agilent program with the data from the NIST17 library. L.

*** Relative abundance of each compound in the sample considering the area of the chromatogram corresponding to each peak.

Fig. 1 Total Ion Chromatogram of *L. alba* essential oil, with amplification from 6 to 40 minutes, highlighting the ten main components identified in the essential oil.



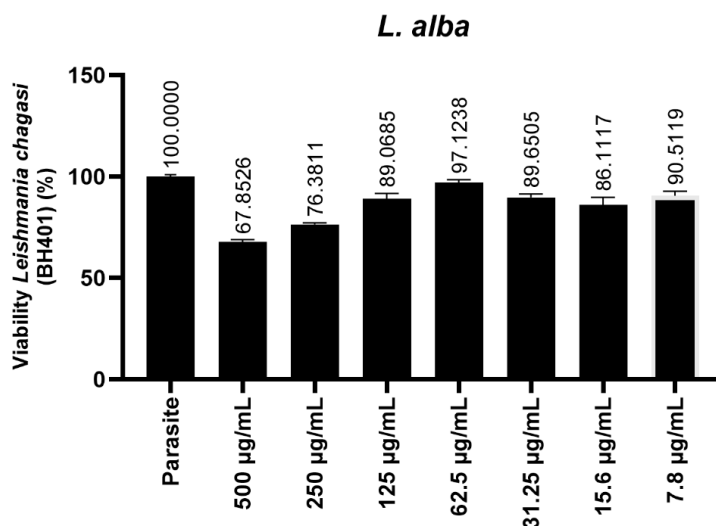
***In vitro* tests of *L. alba* essential oil in *L. chagasi* promastigotes cultures.**

The results of this study demonstrated that, at high concentrations (500 $\mu\text{g/mL}$), *L. alba* essential oil showed action against about 32% of *L. chagasi* promastigotes when compared to the negative control (Fig. 2). For this reason, the Inhibitory Concentration index required to eliminate 50% of the parasites (IC_{50}) could not be calculated. Table 2 describes the percentages of parasitic inhibition for each oil concentration tested.

Table 2 Indexes of parasitic inhibition by applying different concentrations of *L. alba* essential oil (500-7.8 $\mu\text{g/mL}$) in *in vitro* cultures of *L. chagasi*.

Oil concentration	Parasitic inhibition (%)
500 $\mu\text{g/mL}$	32.1474
250 $\mu\text{g/mL}$	23.6189
125 $\mu\text{g/mL}$	10.9316
62.5 $\mu\text{g/mL}$	2.8762
31.25 $\mu\text{g/mL}$	10.3495
15.6 $\mu\text{g/mL}$	13.8883
7.8 $\mu\text{g/mL}$	9.4881

Fig. 2 Parasitic viability tests using different concentrations of *L. alba* essential oil (500-7.8µg/mL). Bar graph showing the percentage of live promastigotes after *in vitro* assays.



These

results suggest that additional studies are necessary to detail which essential oil compounds could participate in the direct action on parasites. For this, assays with these isolated components [16], or even nanoencapsulated, can explain the mechanisms of action and propose ways for the parasite to better assimilate the drug [24].

Conclusion

This work demonstrated the inhibitory action potential of *Lippia alba* essential oil, linalool chemotype, against *Leishmania chagasi* promastigotes *in vitro*. However, further studies are necessary to identify the isolated action of this compound.

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University of Uberlândia, Umuarama *Campus*, for her assistance and guidance regarding chromatographic analyses and oil purification processes.

Authors' Contributions. All authors contributed to the project's execution. Vitória Helena Alecrim Rocha Medeiros collected and dried the botanical material, extracted and purified the essential oil of *L. alba*, analyzed the chromatogram obtained in the material's chemotyping, maintained the cultures of *L. chagasi*, and wrote the paper. Iasmin Aparecida Cunha Araújo performed the *in vitro* assays of the essential oil in the cultures of *L. chagasi* promastigotes, as well as the statistical analyses. Arlene Bispo dos Santos Nossol, Mário Machado Martins, and Luciana Machado Bastos carried out the oil chemotyping process. Juliana Aparecida Povh aided in and guided the essential oil extraction process. Karine Rezende de Oliveira guided and aided in all stages of execution, especially the maintenance of *L. chagasi* cultures, *in vitro* tests, and the paper writing. All authors read and approved the final manuscript.

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Data availability: All data generated during this study is included in this published paper.

Conflict of Interest Declaration: The authors declare no conflicts of interest.

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ANEXO A – NORMAS DE SUBMISSÃO DA REVISTA CURRENT MICROBIOLOGY

Normas disponíveis no link: <https://link.springer.com/journal/284/submission-guidelines>

Instructions for Authors

Editorial procedure

Single-blind peer review

This journal follows a single-blind reviewing procedure.

This journal also publishes special/guest-edited issues. The peer review process for these articles is the same as the peer review process of the journal in general.

Additionally, if a guest editor authors an article in their issue/collection, they will not handle the peer review process

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink “Submit manuscript” and upload all of your manuscript files following the instructions given on the screen.

Source Files

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

- The name(s) of the author(s)
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- A clear indication and an active e-mail address of the corresponding author
- If available, the 16-digit [ORCID](#) of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Large Language Models (LLMs), such as [ChatGPT](#), do not currently satisfy our [authorship criteria](#). Notably an attribution of authorship carries with it accountability for the work, which cannot be effectively applied to LLMs. Use of an LLM should be properly documented in the Methods section (and if a Methods section is not available, in a suitable alternative part) of the manuscript. The use of an LLM (or other AI-tool) for "AI assisted copy editing" purposes does not need to be declared. In this context, we define the term "AI assisted copy editing" as AI-assisted improvements to human-generated texts for readability and style, and to ensure that the texts are free of errors in grammar, spelling, punctuation and tone. These AI-assisted improvements may include wording and formatting changes to the texts, but do not include generative editorial work and autonomous content creation. In all cases, there must be human accountability for the final version of the text and agreement from the authors that the edits reflect their original work.

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

For life science journals only (when applicable)

- Trial registration number and date of registration for prospectively registered trials
- Trial registration number and date of registration, followed by “retrospectively registered”, for retrospectively registered trials
-

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Statements and Declarations

The following statements should be included under the heading "Statements and Declarations" for inclusion in the published paper. Please note that submissions that do not include relevant declarations will be returned as incomplete.

- **Competing Interests:** Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Please refer to “Competing Interests and Funding” below for more information on how to complete this section.

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

Text

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using [Springer Nature’s LaTeX template](#).

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data).

Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

The entries in the list should be numbered consecutively.

If available, please always include DOIs as full DOI links in your reference list (e.g. “<https://doi.org/abc>”).

- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. *Eur J Appl Physiol* 105:731-738. <https://doi.org/10.1007/s00421-008-0955-8>

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. *N Engl J Med* 341:325–329

- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. *J Mol Med*. <https://doi.org/10.1007/s001090000086>

- Book

South J, Blass B (2001) *The future of modern genomics*. Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) *The rise of modern genomics*, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. <http://physicsweb.org/articles/news/11/6/16/1>. Accessed 26 June 2007

- Dissertation

Trent JW (1975) *Experimental acute renal failure*. Dissertation, University of California

Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations, see

[ISSN.org LTWA](http://www.issn.org/LTWA)

If you are unsure, please use the full journal title.

Authors preparing their manuscript in LaTeX can use the bibliography style file `sn-basic.bst` which is included in the [Springer Nature Article Template](#).

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.

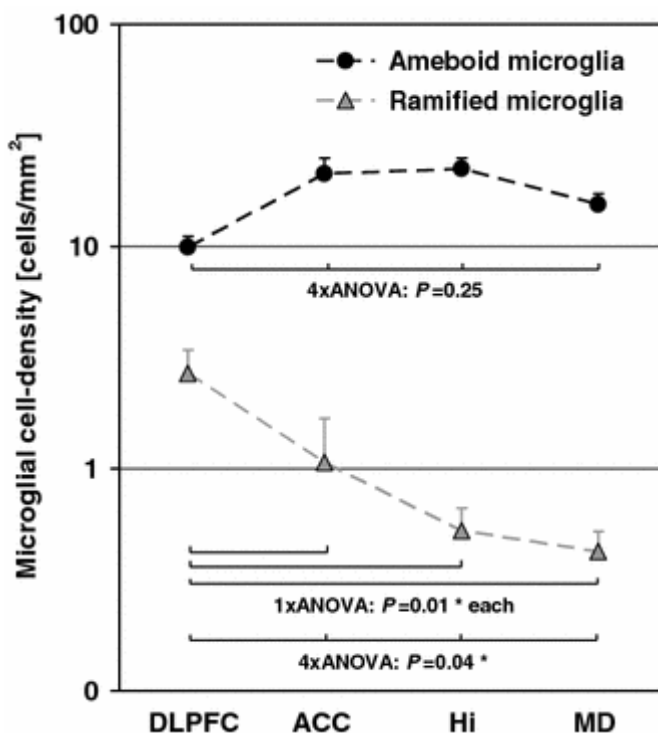
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and Illustrations Guidelines

Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

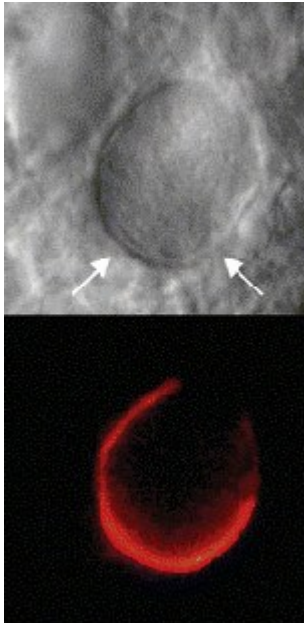
Line Art



- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.

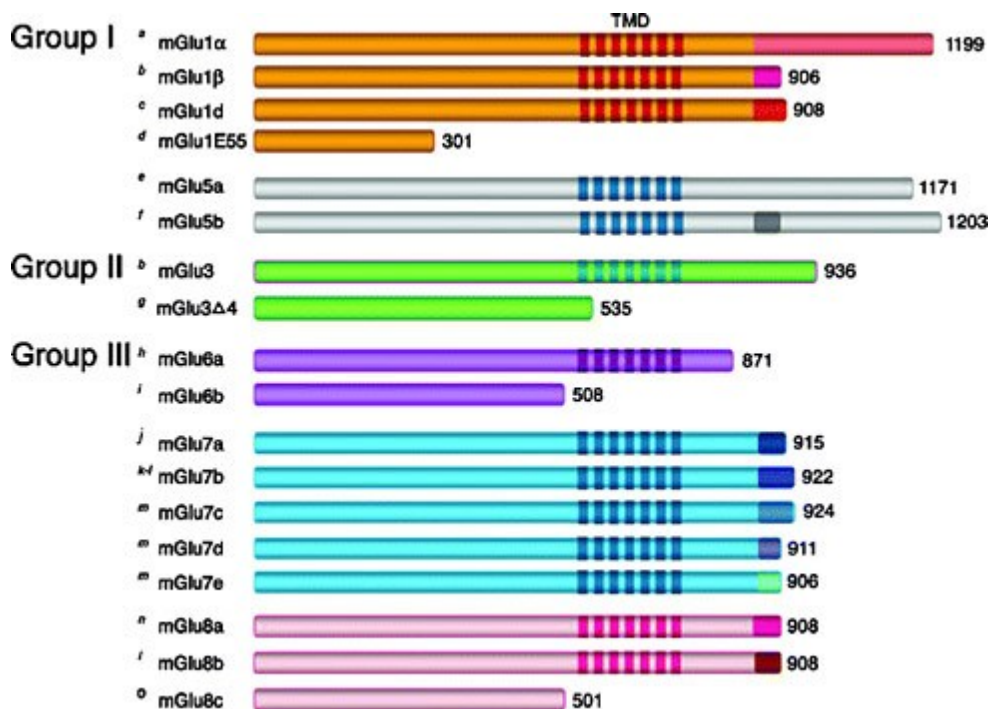
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

- Figures should be submitted within the body of the text. Only if the file size of the manuscript causes problems in uploading it, the large figures should be submitted separately from the text.
- When preparing your figures, size figures to fit in the column width.
- For large-sized journals the figures should be 84 mm (for double-column text areas), or 174 mm (for single-column text areas) wide and not higher than 234 mm.
- For small-sized journals, the figures should be 119 mm wide and not higher than 195 mm.

Permissions

If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

- All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)

- Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements)
- Any figure lettering has a contrast ratio of at least 4.5:1

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Supplementary Information (SI)

Springer accepts electronic multimedia files (animations, movies, audio, etc.) and other supplementary files to be published online along with an article or a book chapter. This feature can add dimension to the author's article, as certain information cannot be printed or is more convenient in electronic form.

Before submitting research datasets as Supplementary Information, authors should read the journal's Research data policy. We encourage research data to be archived in data repositories wherever possible.

Submission

- Supply all supplementary material in standard file formats.
- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author.
- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.

Audio, Video, and Animations

- Aspect ratio: 16:9 or 4:3
- Maximum file size: 2 GB
- Minimum video duration: 1 sec
- Supported file formats: avi, wmv, mp4, mov, m2p, mp2, mpg, mpeg, flv, mxf, mts, m4v, 3gp

Text and Presentations

- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

Spreadsheets

- Spreadsheets should be submitted as .csv or .xlsx files (MS Excel).

Specialized Formats

- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

Collecting Multiple Files

- It is possible to collect multiple files in a .zip or .gz file.

Numbering

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
- Refer to the supplementary files as “Online Resource”, e.g., "... as shown in the animation (Online Resource 3)", "... additional data are given in Online Resource 4”.
- Name the files consecutively, e.g. “ESM_3.mpg”, “ESM_4.pdf”.

Captions

- For each supplementary material, please supply a concise caption describing the content of the file.

Processing of supplementary files

- Supplementary Information (SI) will be published as received from the author without any conversion, editing, or reformatting.

Accessibility

In order to give people of all abilities and disabilities access to the content of your supplementary files, please make sure that

- The manuscript contains a descriptive caption for each supplementary material

- Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

Generative AI Images

Please check [Springer's policy on generative AI images](#) and make sure your work adheres to the principles described therein.

Article Types

Requirements for Article Types

Microorganism Identification (applies to all article types);

New Microbial isolates

For any attempt to affiliate an isolate to a validly described prokaryotic species the stretch of the 16S rRNA gene sequence should be at least 1000 nt long. Shorter sequences result in an uncertain affiliation. The 16S rRNA gene sequence must be compared to those of type strains when assigning a name to an isolate, e.g. when using BLAST-NCBI tick the option 'Limit to sequences from type material' or, in the case of EzBioCloud restrict the search only to type strains and denote the type strain with a superscript T after the strain number.

In those cases in which the 16S rRNA gene sequence similarity is higher than 98.7% to a group of highly related species, additional evidence must be provided before a name is given to an isolate, such as generated by wet-lab DNA hybridization or genome based dDDH, or ANI analysis between an isolate and type strains of the most highly related species, to test the genomic uniqueness of a novel isolate. Also, MALDI-TOF or the phylogenetic assessment by one or more housekeeping gene sequences are helpful to confirm the membership of an isolate to a validly named species. In the absence of any of these data authors should be prudent to actually name an isolate but either use the name of a species group or the Genus spp category.

As ITS regions alone are insufficient to unambiguously name a new fungal isolate in a given genus or genus complex the literature must be searched for more discrimination barcoding regions to support the naming. In the absence of such data the authors must be refrain of applying an explicit species name to an isolate.

Names given for OTU- defined 'species' at a 97% cut-off value of short 16S rRNA gene sequence stretches (e.g. V3, V3/V4 regions) are highly speculative, not re-assessed by different approaches but once published, taken for granted. As species name-attached sequences enter databases the likelihood for a future even higher erroneous identification is

predictable and author should be refrain from naming species on the basis of such shaky scientific grounds.

Please, see [this Editorial](#) for further information about affiliations of new isolates.

New archaeal and bacterial Species

It is expected that descriptions of novel Archaeal and Bacterial species will include data from genome sequence analyses (see specific guidance on Descriptions and Style Guide of Novel Microbial Taxa link for further details).

for fungi see: <https://doi.org/10.1186/s43008-021-00063-1>

for prokaryotes see: <https://doi.org/10.1099/ijsem.0.000778>

Research Articles

The text should be clear and concise and should not exceed 6,000 words (excluding references). The maximum number of figures is 5 (up to 4 panels each).

The maximum number of references is 50. Exceptions may be considered should there be a compelling reason to exceed these parameters.

Structure: Introduction, Materials and Methods, Results, Discussion, Conclusion & References.

Abstract: Structured abstract, maximum of 250 words. The abstract should provide the context or background for the study and should state the study's purpose, basic procedures, main findings and principal conclusions. It should emphasize new and important aspects of the study or observations, note important limitations, and not overinterpret findings.

The abstract should not contain any undefined abbreviations or unspecified references.

Keywords: Include 4-6 keywords following the Abstract.

Body of Manuscript: The text should be divided into the following sections:

Introduction, Materials and Methods, Results, Discussion, Conclusion.

Introduction: Provide a context or background for the study (that is, the nature of the problem and its significance). State the specific purpose or research objective of, or hypothesis tested by, the study or observation. Cite only directly pertinent references and do not include data or conclusions from the work being reported.

Materials and Methods: The guiding principle of this section should be clarity about how and why a study was done in a particular way. Materials and Methods section should aim to be sufficiently detailed such that others with access to the data would be able to reproduce the results. A statement indicating that the research was approved by an independent local, regional or national review body should be included.

Results: Present your results in logical sequence in the text, tables, and figures, giving the main or most important findings first. Do not repeat all the data in the tables or figures in the text; emphasize or summarize only the most important observations. Provide data on all primary and secondary outcomes identified in the Materials and Methods section. Give numeric results not only as derivatives (e.g. percentages) but also as the absolute numbers from which the derivatives were calculated, and specify the statistical significance attached to them, if any.

Discussion: Begin the discussion by briefly summarizing the main findings and explore possible mechanisms or explanations for these findings. Emphasize the new and important aspects of your study and put your findings in the context of the totality of the relevant evidence. State the limitations of your study and explore the implications of your findings for future research and or policy.

Conclusion: Link the conclusions with the goals of the study but avoid unqualified statements and conclusions not adequately supported by the data.

Short Communications

Submissions should provide a high-quality original research presented in a short format.

Short Communications should be of broad interest to the Microbiology community.

Abstract: 250 words maximum

Keywords: Include 4-6 keywords following the Abstract.

Word Count: 3,000 words (applies to the body of the manuscript only) and up to 30 references.

The maximum number of figures is 5 (up to 4 panels each).

Structure: Introduction, Materials and Methods, Results and Discussion, Conclusion & References

(mini)-Reviews

Review articles should address a focused topic, issue or question.

They should be balanced and up-to-date. They should not simply restate the literature but rather interpret and integrate the findings to provide a framework for understanding the literature on a particular topic.

The length of a Review Article will depend on the topic covered but should not exceed 8,000 words (excluding references) or 5,000 words in case of a mini- Review.

The maximum number of references is 100.

Structure: Abstract, Keywords, Introduction, Discussion, Conclusion & References

The Discussion section of the review should be organized into sub-sections headed by informative subtitles.

We welcome topical review articles on the above topics but the content should be discussed with the Editors prior to submission indicating how this manuscript would be different from recent review articles in the field.

Letters to the Editor

Letters to the Editor should focus on news or on an article that has been published in a journal within the previous six months.

Letters should focus on some aspect(s) of the paper that is, in the author's opinion, in some way worthy of comment.

All Letters to the Editor must address a scientific issue in an objective fashion, should have fewer than 1,000 words (main body text), up to 6 references.

New Isolates

The microorganisms used in the study and in particular new isolates must be deposited in two publicly accessible culture collects located in two different countries. The collections must be listed in the WDCM (see the below Links for a complete list of the WDCM culture collections which are all suitable).

The authors must refer to the collection and the strain number in the text to ensure that the strains are available to other scientists.

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